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Phosphorus fertilisation management modifies the biodiversity of AM fungi in a tropical savanna forage system

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ABSTRACT

In the present study we investigated how the community of arbuscular mycorrhizal fungi (AMF) in roots of *Centrosema macrocarpum* responded to different doses and sources of phosphorus (40 kg ha^{-1} of P as rock phosphate, 150 kg ha^{-1} of P as rock phosphate and 75 kg ha^{-1} of P as diammonium phosphate together with 75 kg ha^{-1} of P as rock phosphate) in a Venezuelan savanna ecosystem. We also related AMF diversity to soil parameters (total N, total P, available P, extractable K, total Ca, total Mg, total Fe, total Cu, total Zn, total Mn, glomalin-related soil protein, microbial biomass C, dehydrogenase, urease and acid phosphatase activities, water-soluble carbon and carbohydrates and aggregate stability) at different doses of P. The AM fungal small-subunit (SSU) rRNA genes were subjected to PCR, cloning, SSCP, sequencing and phylogenetic analyses. Nine fungal types were identified: six belonged to the genus *Glomus* and three to *Acaulospora*. The majority of fungal types showed high similarity to sequences of known glomalean isolates: Aca 1 to *Acaulospora mellea*, Aca 2 to *Acaulospora rugosa*, Aca 3 to *Acaulospora spinosa*, Glo 1 to *Glomus intraradices* and Glo 3 to *Glomus fasciculatum*. The control treatment was dominated by species belonging to the genus *Acaulospora*. However, when the soil was fertilised with low doses of P, the colonisation of roots increased and there was a change in the AMF diversity, the genus *Glomus* dominating. The AM development and the abundance of AM fungal types in roots were decreased dramatically by the fertilisation with high doses of P, without differences between the sources of P used. The available P in soil was negatively correlated with the AMF diversity. In conclusion, the application of low doses of P as rock phosphate stimulated mycorrhization and enhanced the soil quality parameters except water-soluble carbohydrates, helping to offset a loss of fertility in P-poor tropical savanna soils.

1. Introduction

Savanna soils occupy an important part of the Venezuelan territory and they are characterised by very low fertility, acidity and low productivity; therefore, the quality of forage for animals is low (Jordan, 1984). This is due to the fact that savanna ecosystems show a strong rain-dry seasonality. Under these conditions, erosion of the soil increases and its content of plant nutrients, principally of phosphorus (P), is reduced (Tiessen et al., 1984).

Centrosema macrocarpum Benth is a leguminous forage planted as a cover crop and used in silvopastoral systems in the Venezuelan savanna ecosystems. Leguminous covers are used as protein and energy sources for animals in the drought season, the most critical season for the production of forage in these ecosystems. However, their establishment is frequently limited by the low levels of available phosphorus in acid tropical soils. In such soils, an adequate phosphorus application is necessary for optimum growth and crop yields.

The direct use of rock phosphate as a fertiliser is a practice used widely under different soil and culture conditions. It has been shown in numerous experiments that rock phosphate has excellent properties and its use in P-poor soils shows promise (Gighuru and Sánchez, 1988; Vela, 1991; Arévalo et al., 2003; Alguacil et al., 2003).

The arbuscular mycorrhizal fungi (AMF) are one of the main components of the soil microbiota in most agroecosystems. They are obligate root symbionts that form complex communities in soils and contribute to nutrient cycling, especially of P, and plant growth and health (Smith and Read, 1997). The composition and diversity of AMF communities can be affected both qualitatively and quantitatively by agricultural management practices (Miller et al., 1995; Mathimaran et al., 2007). Previous studies have shown that agricultural practices such as tillage, P fertilisation and crop cultivation

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have a negative impact on the AMF association in temperate and tropical agroecosystems (Jansa et al., 2002; Gosling et al., 2006; Preger et al., 2007; Alguacil et al., 2008). It has been suggested that the AM fungal diversity affects the plant biodiversity and the ecosystem productivity and stability (Van der Heijden et al., 1998), so that the loss of fungal biodiversity through human activities may have a negative impact on ecosystem functioning.

In general, P fertiliser application depresses infection by AMF (Schubert and Hayman, 1986; Ryan and Graham, 2002) although it may not have any influence (Salinas et al., 1985; Mathimaran et al., 2007). Arias et al. (1991), in their study on acid soils of the savannas in Colombia, found that P fertilisation with high levels of triple super-phosphate inhibited mycorrhizal infection in *Stylosanthes capitata* plants inoculated with *Glomus manihotis* but not in two other legumes studied (*C. macrocarpum* and *Pueraria phaseoloides*). A basic requisite, for a more effective management and preservation of AMF diversity in agricultural management ecosystems, is to know the species composition and thus to be able to understand the mycorrhizal function. However, to the best of our knowledge, no data are available concerning the effect of different doses and sources of P fertiliser application to soil on AMF diversity in savanna agroecosystems. Thanks to the advances in molecular biology techniques in recent years, it is possible to identify the diversity of the AMF colonising plant roots. The single-stranded conformation polymorphism (SSCP) approach is a very sensitive and reproducible technique that has been applied successfully in studies in order to analyse the sequence diversity of AM fungi within roots (Kjøller and Rosendahl, 2000; Alguacil et al., 2009a,b).

Therefore, the objectives of this work were: 1. To test whether different amounts and forms of phosphorus fertiliser applied to soil influence the AMF diversity associated with *C. macrocarpum* roots in a tropical savanna ecosystem. 2. To check whether the changes in soil parameters mediated by the different phosphorus fertilisation procedures can be related with the AMF diversity.

2. Materials and methods

2.1. Study site

The experimental area was located in the Experimental Station "La Iguana", Guárico State, in North-eastern of Venezuela (8°25′N and 65°25′W, 120 m above sea level). The climate is markedly tropical isothermic, with an annual average rainfall of 1369 mm (concentrated in a rainy season between Jun and October), and a mean annual temperature of 27.9 °C. The soil in the experimental area is classified as Typic Plinthustults (Ultisol) in the USDA soil classification system (SSS, 2006).

2.2. Experimental design

Legume forage centrosema (*C. macrocarpum* Benth) was planted as a cover crop in experimental plots in January 2004. The design of the experiment was a full randomized design with five replication plots (15 m × 60 m each) in an experimental area of 18,000 m^2. One year later (2005), were applied the treatments. The treatments consisted of four fertiliser management, with variations in the phosphorous dose and source applied to soil once a year. The same management system was applied for three consecutive years (2005–2007).

Control: Fertilisation without added P (150 kg ha^{-1} urea–0 P–100 kg ha^{-1} K_2O).
PR: Fertilisation with low dose of P as rock phosphate (150 kg ha^{-1} urea–40 kg ha^{-1} P–100 kg ha^{-1} K_2O).
APR: Fertilisation with high dose of P as rock phosphate (150 kg ha^{-1} urea–150 kg ha^{-1} P–100 kg ha^{-1} K_2O).
PR + DP: Fertilisation with high dose of P, 50% as diammonium phosphate and 50% as rock phosphate (150 kg ha^{-1} urea–75 + 75 kg ha^{-1} P–100 kg ha^{-1} K_2O).

The rock phosphate used in this experiment come from Venezuela (Riecito Rock) and is composed by 30.2% P_2O_5, 38.6% CaO, 29.0% SiO_2, 0.2% MgO, 0.1% Fe_2O_3, 0.3% Al_2O_3, and 1.6% CO_2, with 53% of solubility in citric acid. The amount of P to be added to each plot was weighed individually and incorporated by thoroughly mixing with the soil.

2.3. Sampling

All samples were collected in August 2008 (rainy season), almost 5 years after *C. macrocarpum* was established. Soil cores (5 cm diameter) were collected from the 0–15 cm layer of all 5 replicates plots for each fertilizing treatment. Four cores were collected at 15 m intervals along the south-western to north-eastern transect of each replicate plots. Subsequently, the four soil cores from each plot were composited, the field moist soil was gently broken apart, air-dried, passed through and 2 mm sieved, and stored at room temperature until further analysis.

At the same time, five plants per treatment, each plant taken from a different plot, were sampled and taken to the laboratory (a total of 20 plants). Plants, including root system, were collected and placed in polyethylene bags for transport to the laboratory, where fine roots were separated from the soil. Roots were then briefly rinsed, quickly dried on paper and used partly for morphological and partly for molecular analysis.

2.4. Mycorrhizal determinations

The percentage of mycorrhizal root colonisation was estimated by visual observation of fungal colonisation after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), according to Phillips and Hayman (1970). The extent of mycorrhizal colonisation was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980).

2.5. Soil biological, biochemical and physical analyses

Soil microbial biomass C was determined using a fumigation–extraction method (Vance et al., 1987). Ten grams of soil at 60% of its field capacity are fumigated in a 125-ml Erlenmeyer flask with purified $CHCl_3$ (Panreac) for 24 h placed in a glass desiccator. After removal of residual $CHCl_3$, 40 ml of 0.5 M K_2SO_4 (Panreac) solution is added and the sample is shaken for 1 h before filtration of the mixture. The K_2SO_4-extracted C was determined with an automatic carbon analyser for liquid samples (Shimadzu TOC) and microbial biomass C is calculated as the difference between fumigated and non-fumigated samples.

Acid phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (PNPP, 0.115 M) (Fluka) as substrate. Two ml of 0.5 M sodium acetate (Panreac) buffer at pH 5.5 using acetic acid (Naseby and Lynch, 1997) and 0.5 ml of substrate were added to 0.5 g of soil and incubated at 37 °C for 90 min by shaking. The reaction was stopped by cooling at 2 °C for 15 min. Then, 0.5 ml of 0.5 M $CaCl_2$ (Panreac) and 2 ml of 0.5 M NaOH (Panreac) were added, and the mixture was centrifuged at 4000 rpm for 5 min. The *p*-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm (Tabatabai and Bremner, 1969). Controls were made in the same way, although the substrate was added before the $CaCl_2$ and NaOH.

Dehydrogenase activity was determined according to García et al. (1997) and Trevors (1984). For this, 1 g of soil at 60% of its field capacity was exposed to 0.2 ml of 0.4% INT (2-piodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride) (Sigma) in distilled water for 20 h at 22 °C in the dark. The INTF (iodo-nitrotetrazolium formazan) formed was extracted with 10 ml of methanol (Panreac) by shaking vigorously for 1 min and filtering through Whatman no. 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

Urease and N-(-benzoyl-L-argininamide) (BAA) hydrolyzing protease activities were determined in 0.1 M phosphate (Panreac) buffer at pH 7; 1 M urea (Panreac) and 0.03 M BAA (MP Biomedicals) were used as substrates, respectively. Two millilitres of buffer and 0.5 ml of substrate were added to 0.5 g of soil sample, which was incubated at 30 °C (for urease) or 39 °C (for protease) for 90 min. Both activities were determined as the NH_4 + released in the hydrolysis reaction (Nannipieri et al., 1980).

Soil pH was measured in a 1:5 (w/v) aqueous solution. In soil aqueous extracts, water-soluble carbon was determined in a Shimadzu TOC-5050A analyser of C for liquid samples. Water-soluble carbohydrates were determined by the method of Brink et al. (1960).

The percentage of stable aggregates was determined by the method described by Lax et al. (1994). A 4 g aliquot of sieved (0.2–4 mm) soil was placed on a small 0.250 mm sieve and wetted by spray. After 15 min the soil was subjected to an artificial rainfall of 150 ml with energy of 270 J m^{-2}. The remaining soil on the sieve was placed in a previously weighed capsule (T), dried at 105 °C and weighed (P1). Then, the soil was soaked in distilled water and, after 2 h, passed through the same 0.250 mm sieve with the assistance of a small stick to break the remaining aggregates. The residue remaining on the sieve, which was made up of plant debris and sand particles, was dried at 105 °C and weighed (P2). The percentage of stable aggregates with regard to the total aggregates was calculated by $(P1 - P2) \times 100/(4 - P2 + T)$.

Glomalin-related soil protein (GRSP) was determined in the easily extractable glomalin form according to Wright and Anderson (2000). It was extracted from soil samples sieved between 0.2 and 4 mm with 20 mM sodium citrate (Panreac) (pH 7.0) at a rate of 250 mg of aggregates in 2 ml of buffer and autoclaving at 121 °C for 30 min. The supernatant was removed and two additional sequential 1-h extractions were performed. All supernatants from a sample were combined, the volume was measured, an aliquot was centrifuged at 10,000 × g for 15 min to remove soil particles and Bradford-reactive (Bio-Rad) total protein was measured.

The total N was determined with the Kjeldahl method, consisting of titration after distillation and sample digestion. Available P, extracted with 0.5 M $NaHCO_3$ (Panreac) was determined by colorimetry according to Murphy and Riley (1962). Extractable (with ammonium acetate) K was measured by atomic adsorption spectroscopy on a UNICAM 969 AA-Spectrometer.

Total P, total Ca, total Mg, total Fe, total Cu, total Zn and total Mn contents were determined by nitric–perchloric digestion: 1 g of crushed sample was placed in a Kjeldahl flask, and 10 ml of concentrated HNO_3 (Panreac) plus 10 ml of concentrated $HClO_4$ (Panreac) were added. The mixture was heated at 210 °C for 90 min. When cool, the content of the tubes was filtered through an Albert® 145 ashless filter paper, and the volume completed at 50 ml by washing the Kjeldahl flasks with HCl 0.5 N several times and filtering. All elements were quantified using with an ICP-MS (Thermo electron corporation Mod. IRIS intrepid II XDL).

2.6. DNA extraction and PCR

For each sample, total DNA was extracted from root material (representing approx. 5–8 cm root length) using a DNeasy plant mini Kit following the manufacturer's recommendations (Qiagen). The roots samples were placed into a 2-ml screw-cap propylene tube and the DNA extracts were obtained by disrupting roots with a sterile disposable micro-pestle in liquid nitrogen. The DNA was resuspended in 20 μl of water. Several dilutions of extracted DNA (1/10, 1/50, 1/100) were prepared. Partial ribosomal small-subunit (SSU) DNA fragments were amplified using the universal eukaryotic primer NS31 (Simon et al., 1992) as forward primer and a mixture of equal amounts of the AM1 (Helgason et al., 1998), AM2 and AM3 (Santos-González et al., 2007) primers as the reverse primer combination. PCR reactions were carried out in a final volume of 25 μl using the "ready to go" PCR beads (Amershan Pharmacia Biotech), 0.2 μM dNTPs and 0.5 μM of each primer (PCR conditions: 95 °C for 3 min, then 30 cycles at 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and 30 s; and 72 °C for 8 min). As a template, 2 μl of extracted DNA was used in all reactions. Positive and negative controls using PCR positive products and sterile water respectively were also included in all amplifications. DNA extracts were stored at −20 °C.

All the PCR reactions were run on a Perkin Elmer Cetus DNA Thermal Cycler. Reactions yields were estimated by using a 1.2% agarose gel containing ethidium bromide.

2.7. Cloning, generation of SSCPs and sequencing

The PCR products were purified using a Gel extraction Kit (Qiagen) cloned into pGEM-T Easy (Promega) and transformed into *Escherichia coli* (Xl1 blue). Thirty two putative positive transformants were screened in each resulting SSU rRNA gene library, using 0.7 unit of RedTaq DNA polymerase (Sigma) and a re-amplification with NS31/AM1, AM2, AM3 primers with the same conditions described above.

Positive clones from each sample were analysed by Single-Stranded Conformational Polymorphism (SSCP). Samples for SSCP were prepared by mixing 5 μl of the clone PCR product with 5 μl loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 0.5M EDTA). The samples then were denatured at 95 °C for 5 min, placed on ice to stabilise single strands and then loaded on a 20 × 20 cm × 0.75 mm gel containing 0.6× MDE gel (BMA, Rockland, ME, USA), 10% ammonium persulfate (APS), 10× TBE buffer, and 1% N-Tetramethylethylenediamine (TEMED). Electrophoresis was performed in 0.6× TBE buffer and run at 20 °C at constant 150 V for 16 h in a D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Bands were visualised by silver staining using a DNA Silver Staining Kit provided by Bio-Rad.

The SSCP banding patterns obtained from different root samples were compared by eye, and the bands were grouped according to similar mobility. Examples of each SSCP pattern were chosen for sequencing. They were grown in liquid culture and the plasmid extracted using the QIAprep Spin Miniprep Kit (Qiagen). The sequencing was done by Molecular Biology Laboratory (SACE – Murcia University, Spain) using the universal primers SP6 and T7. Sequence editing was done using the program Sequencher version 4.1.4 (Gene Codes Corporation). Representative sequences of the clones generated in this study for each fungal type have been deposited at the National Centre for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov) under the accession numbers FN430571–FN430588.

Sequences were screened for possible chimeric origin using the Chimera Detection Function of the Ribosomal Database Project (RDP) internet site (http://rdp8.cme.msu.edu/html/analyses.html).

2.8. Phylogenetical analysis

Sequence similarities were determined using the Basic Local Alignment Search Tool (BLASTn) sequence similarity search tool

(Altschul et al., 1997) provided by GenBank. Phylogenetic analysis was carried out on the sequences obtained in this study and those corresponding to the closest matches from GenBank. Sequences were aligned with other published glomeralean sequences using the program ClustalX (Thompson et al., 1997) and the alignment was adjusted manually in GeneDoc (Nicholas and Nicholas, 1997) Neighbour-Joining (NJ) phylogenetic analyses (Saitou and Nei, 1987) were performed with the program PAUP4.08b (Swofford, 2002) and using the default parameters. *Paraglomus brasilianum*, ancient taxa of the phylum Glomeromycota, was used as the out-group.

2.9. Diversity of AM fungal community

The Shannon–Weaver (H') index was calculated as an additional measure of diversity, as it combines two components of diversity, i.e., species richness and evenness. It is calculated from the equation $H' = -\sum pi(\ln pi)$, where pi is the proportion of individuals found in the ith species (in a sample, the true value of pi is unknown but is estimated as ni/N [here and throughout, ni is the number of individuals in the ith species]).

2.10. Statistical analysis

Treatment effects on measured variables (colonisation and soil properties) were tested by analysis of variance, and comparisons among treatment means were made using a Least-Significant Difference (LSD) test calculated at $P < 0.05$. The effect of fertilisation treatment on the abundance of different AMF phylotypes was assessed by univariate ANOVA tests. Correlation analysis between all the soil parameters measured, colonized root length and the AM fungal types was carried out using Pearson's rank correlation coefficients. Statistical procedures were carried out with the software package SPSS 17.0 for Windows (Ferrán Aranaz, 1996).

In order to investigate the influence of different fertilisation treatments on the distribution of AMF phylotypes and to correlate the AM fungal community composition with the soil quality parameters, ordination analyses were conducted in CANOCO for Windows v. 4.5 (Ter Braak and Smilauer, 2004) using the presence or absence data for each root sample. Initial detrended correspondence analysis suggested a unimodal character of the data response to the sample origin (the lengths of gradients were >4); therefore, the canonical-correspondence analysis (CCA) was used.

The presence or absence of AMF phylotypes in each root sample was used to construct the sampling effort curves (with 95% confidence intervals) using the software EstimateS 8.00 (Colwell, 2005). The sample order was randomized by 100 replications.

3. Results

3.1. Degree of mycorrhization and biomass production of C. macrocarpum

The highest percentage colonisation (75.3%) was found in the treatment with low doses of P as rock phosphate (Table 1). The centrosema roots of the control treatment showed significantly higher colonisation rates than when grown with treatments involving high doses of P (ARP and RP + DP). The lowest degree of root colonisation was observed in the treatment where different sources of P were added.

The *C. macrocarpum* crop biomass yield under the different fertiliser treatments applied was as follow: C = 21.33 Mg ha^{-1}; RP = 23.34 Mg ha^{-1}; ARP = 24.41 Mg ha^{-1}; RP + DP = 27.99 Mg ha^{-1}. There were no significant differences among treatments.

3.2. Physical, biological and chemical analyses of the soil

The percentage of stable aggregates, the glomalin-related soil protein and the total N were significantly higher in the treatment with the lowest P dose than in the control treatment; however, no significant differences with respect to treatments APR and RP + DP were found (Tables 1 and 2).

The soils from treatments C and RP (control and low dose of P) showed significantly higher phosphatase and dehydrogenase activities and water-soluble C than soils that received high doses of P (ARP and RP + DP) (Tables 1 and 2).

The concentration of available P increased proportionally with the P dose applied to the soil; thus, the values for the APR and PR + DP treatments were significantly higher and the lowest value occurred for the C treatment (Table 2).

All three treatments that added P to soil gave significantly higher urease activity than the control. When the high dose of P was added as rock phosphate, the concentrations of microbial biomass C and extractable K were the lowest (Tables 1 and 2).

The treatment with different sources of P (RP + DP) produced a significantly higher content of water-soluble carbohydrates (Table 2).

There were no differences among the four treatments with respect to pH, total P, total Ca, total Mg, total Cu, total Zn and total Mn (Tables 1 and 2).

3.3. Molecular analysis

All the PCR experiments were run using DNA preparations consisting of pooled roots of individual plants. The occurrence of AMF was monitored in 20 root samples (five root samples for each treatment). Template DNA was amplified successfully with the combination of primers AM1, AM2 and AM3/NS31. All samples

Table 1
Physical and biological properties of rhizosphere soil, degree of AM fungal colonisation and number of AMF phylotypes in roots of *C. macrocarpum* in response to different fertilisation treatments ($n = 5$).

Treatments	AS (%)	pH (H_2O)	AMF	GRSP (mg g^{-1} soil)	MBC (mg kg^{-1})	DHS (μg INTF g^{-1} soil)	UR (μmol NH_3 g^{-1} h^{-1})	PHP (μmol PNP g^{-1} h^{-1})	CRL (%)
C	18.9a	5.1a	4.8a	397a	59a	11.9b	1.36a	0.58b	61.0c
RP	24.9b	4.8a	5.0a	532b	66b	14.04b	2.47b	0.66b	75.3d
ARP	20.4ab	4.7a	1.8b	461ab	54a	7.5a	2.62b	0.41a	35.3b
RP + DP	17.9a	4.6a	1.8b	456ab	63ab	10.7a	2.61b	0.42a	14.4a

AS: Aggregate stability; AMF: number of arbuscular mycorrhizal fungi phylotypes; GRSP: glomalin-related soil protein; MBC: microbial biomass C; DHS: Dehydrogenase; UR: Urease; PHP: Phosphatase; CRL: Colonized root length. Values in row followed by the same letter do not differ significantly ($P < 0.05$) as determined by the LSD test. C = control; RP = fertilisation with 25% P as rock phosphate; ARP = fertilisation with 100% P as rock phosphate; RP + DP = fertilisation with 50% P as rock phosphate and 50% P as diammonium phosphate.

Table 2
Chemical properties of rhizosphere soil in response to different fertilisation treatments ($n = 5$).

Treatments	WSCH (μg g^{-1})	WSC (μg g^{-1})	N (g kg^{-1})	P (μg g^{-1})	TP (μg g^{-1})	K (μg g^{-1})	Ca (μg g^{-1})	Mg (μg g^{-1})	Fe (μg g^{-1})	Cu (μg g^{-1})	Zn (μg g^{-1})	Mn (μg g^{-1})
C	30a	102b	0.34a	3.8a	230a	8.76c	13.35a	5.97a	21.16a	3.19a	4.19a	2.17a
RP	28a	124b	0.51b	5.3b	243a	9.05c	13.88a	6.75a	20.95a	3.00a	3.95a	1.87a
ARP	28a	93a	0.48ab	16.6c	258a	3.31a	12.40a	7.35a	22.41a	2.95a	4.39a	1.75a
RP + DP	46b	92a	0.44ab	15.9c	252a	6.04b	11.44a	7.00a	22.47a	3.05a	3.69a	1.76a

WSCH: Water-soluble carbohydrates; WSC: Water-soluble C; N: Total N; P: P available; TP: Total P; K: Extractable K; Ca: Total Ca; Mg: Total Mg; Fe: Total Fe; Cu: Total Cu; Zn: Total Zn; Mn: Total Mn. Values in row followed by the same letter do not differ significantly ($P < 0.05$) as determined by the LSD test. C = control; RP = fertilisation with 25% P as rock phosphate; ARP = fertilisation with 100% P as rock phosphate; RP + DP = fertilisation with 50% P as rock phosphate and 50% P as diammonium phosphate.

generated PCR products of the expected band of 550 bps, which were used for cloning and creating a clone library. From the 20 clone libraries, a total of 640 clones were screened by PCR (32 clones per sample); out of these, 503 contained the SSU rRNA gene fragment. All of these 503 clones were subjected to SSCP analysis. Representative clones for each pattern were sequenced, for a total of 66 sequences, while the remaining 437 clones were classified by SSCP pattern. After preliminary BLAST searches, a total of 354 clones had a high degree of similarity to sequences from taxa belonging to the phylum Glomeromycota (Table 3) while the remaining clones were identified as non-AMF since they showed high similarity to sequences from the Ascomycota. Sequences belonging to the Ascomycota were detected in all clone libraries. The band patterns that produced sequences of non-glomalean origin in the SSCP gels were distinguished easily, as they migrated further in the gel and showed migration patterns different to those of glomalean bands. Since the aim of this study was to study the diversity of the AMF, we did not consider the presence of non-AMF for this purpose.

3.4. Identification of AM fungal groups

All sequences had high similarity (98–100% identity) to AM fungi and belonged to members of the phylum Glomeromycota (Fig. 1). The constructed alignment included 43 sequences that were downloaded from GenBank and the 18 different glomalean sequences that were recovered in this study. Some clones produced the same sequence and are represented just once in the alignment. The alignment produced a tree that revealed that the sequences belong to the Glomerales and Diversisporales groups. The sequences obtained in this study were clustered in nine discrete groupings or fungal types with support in the NJ bootstrap analysis of ≥80%. The nine sequence groups were mainly consistent with SSCP patterns and were grouped in two families: the Glomeraceae and the Acaulosporaceae. Six of these sequence groups belong to the genus *Glomus* and three fungal types belong to the genus *Acaulospora*.

The majority of AM fungal types found in this study clustered closely with sequences from AM fungi in culture: Aca 1, Aca 2 and Aca 3 include sequences with 98%, 98% and 97% identity to the sequences of *Acaulospora mellea*, *Acaulospora rugosa* and *Acaulospora spinosa*, respectively. Glo 1 and Glo 3 clustered together with *Glomus intraradices* BEG123 and *Glomus fasciculatum* BEG53, respectively. Glo 2 clustered together with the taxonomically-undescribed *Glomus* sp. isolate AY129631. Glo 4 was related to root-derived sequences in GenBank belonging to uncultured glomalean species and therefore not characterised taxonomically. Glo 5 and Glo 6 did not seem to be related to any sequences of AM fungi in culture or found previously in the database; thus, they could be species unknown until now.

Table 3
Number of clones detected for each fungal type according to SSCP pattern in the roots of *C. macrocarpum* in response to different fertilisation treatments ($n = 5$).

	Fertilisation treatments				Total	
	C	RP	ARP	RP + DP	*n*	%
Glo 6	–	44	29	41	114	32.20
Glo 5	–	52	31	30	113	31.92
Glo 1	–	27	–	–	27	7.63
Aca 1	9	17	–	–	26	7.35
Glo 3	–	21	–	–	21	5.93
Glo 4	18	–	–	–	18	5.08
Aca 3	14	–	–	–	14	3.96
Glo 2	11	–	–	–	11	3.11
Aca 2	10	–	–	–	10	2.82
Total	62	161	60	71	354	100

Abbreviations are: C = control; RP = fertilisation with 25% P as rock phosphate; ARP = fertilisation with 100% P as rock phosphate; RP + DP = fertilisation with 50% P as rock phosphate and 50% P as diammonium phosphate.

3.5. AM fungal community distribution

The observed absolute numbers of AMF phylotypes per root sample for each treatment were compared using analysis of variance (Table 1). The treatment harbouring the highest mean number of AMF phylotypes was RP (5), which differed significantly from the ARP (2) and RP + DP (2) treatments according to the least-significant difference test. There were no significant differences between the mean numbers of AMF phylotypes found with the C and RP treatments.

The control and the RP treatment, which received a low dose of P, showed the most-diverse communities of AMF, each one hosting five of the nine fungal types found in this study, although the community compositions of the AMF differed (Table 3). These treatments showed similar Shannon–Weaver diversity indices ($H' = 1.58$ for C and $H' = 1.52$ for RP). Aca 2, Aca 3, Glo 2 and Glo 4 seemed to be specific to the control treatment. Glo 1 and Glo 3 were found exclusively in the RP treatment (fertilisation with a low dose of P). The two fertilisation treatments where a high dose of P was added (rock phosphate alone or with diammonium phosphate) gave the lowest AM fungal species diversity. In both treatments the same two fungal types were found and the diversity index was similar ($H' \approx 0.7$). Glo 5 and Glo 6 were found exclusively in the three treatments which had received P fertilisation and accounted for 64.12% of the AM fungal clones analysed. Aca 1 seemed to be sensitive to the higher dose of P applied since it was found only in the control and RP treatments (Table 3).

The sampling effort curve (Fig. 2) showed that the number of root samples analysed was sufficient to detect the majority of fungal types present in the roots; in fact, the curve reached saturation.

The biplot diagram of this CCA (Fig. 3) confirms the results described previously. Thus, the diagram shows that the C and RP treatments were distant from each other and also from the RP + DP and ARP treatments, demonstrating that they harboured distinct AM fungal communities. However, the AM fungal communities found in these two treatments (RP + DP and ARP) were shared, so the RP + DP treatment appears close to the ARP treatment. Fig. 3 also shows clearly the AMF phylotypes occurring in each of these

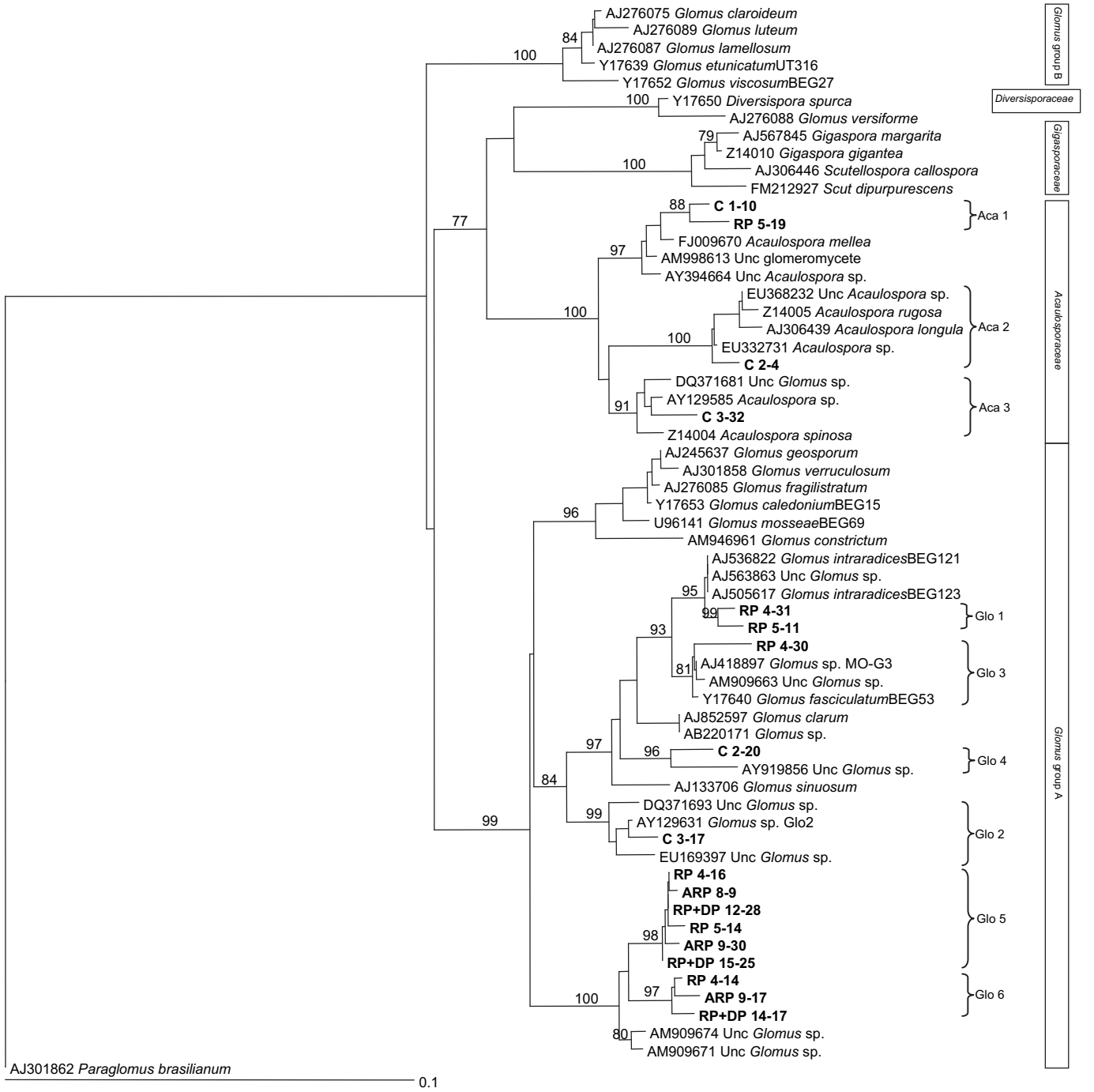

Fig. 1. Neighbour-Joining (NJ) phylogenetic tree showing examples of the AM fungal sequences types isolated from roots of *C. macrocarpum* in the four different P fertiliser treatments and reference sequences from GeneBank. All bootstrap values >80% are shown (100 replicates). Sequences obtained in the present study are shown in bold type. They are labelled with the number of treatment from which they were obtained (C = control, RP = low doses of P as rock phosphate, ARP = high doses of P as rock phosphate, RP + DP = high doses of P 50% as rock phosphate and 50% as diammonium phosphate) and clone identity number. Group identifiers (for example Aca 1) are AM fungal sequences types found in our study. *Paraglomus brasilianum* was used as out-group.

four fertilisation treatments; for example, Aca 2, Aca 3, Glo 2 and Glo 4 were found exclusively in treatment C and Glo 1 and Glo 3 appeared only in the RP treatment.

3.6. Relationship between the biodiversity of AMF and the soil quality properties

The biodiversity of the AMF, measured as the Shannon–Weaver index, was correlated negatively ($r = -0.999$; $P < 0.05$) with the available P in the soil. No other significant correlation was found between this diversity index and the other soil properties. Highly-positive, significant correlations were observed between the urease activity and the Glo 5 and Glo 6 fungal types ($r = 0.994$; $P < 0.01$) as well as between the water-soluble carbon and the Glo 1 and Glo 3 phylotypes ($r = 0.953$; $P < 0.05$). However, there was a significant, negative correlation ($r = -0.994$; $P < 0.01$) between the urease activity and the fungal types found exclusively in the control treatment (Aca 2, Aca 3, Glo 2 and Glo 4), as shown by the high

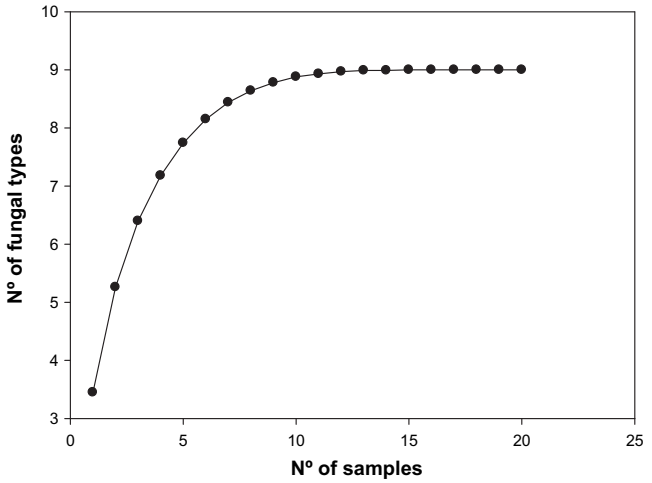

Fig. 2. Sampling effort curve for the AM fungal types richness observed in this study. The sample order was randomized by 100 replications in EstimateS, version 8.0 (Colwell, 2005).

separation or distance between them in the diagram (Fig. 3). The diagram also shows that the Aca 1 fungal type was correlated positively with the acid phosphatase activity ($r = 0.963$; $P < 0.05$) and negatively with the available P ($r = -0.995$; $P < 0.01$).

4. Discussion

This study shows that application of different doses and sources of P fertiliser to soil in a tropical savanna affected the AM fungal diversity and composition in *C. macrocarpum* roots. We found that the control treatment was dominated by five different AM fungal types from nine fungal types detected in the whole study. However, when a low dose of P fertiliser was added to the soil (RP treatment), the community composition of the AMF changed and it was dominated by *Glomus* spp., four new fungal types appearing: Glo 6, Glo 5, Glo 1 and Glo 3. From these fungal types, only Glo 5 and Glo 6 remained in the soil when high doses of P were applied (ARP and RP + DP). Therefore,

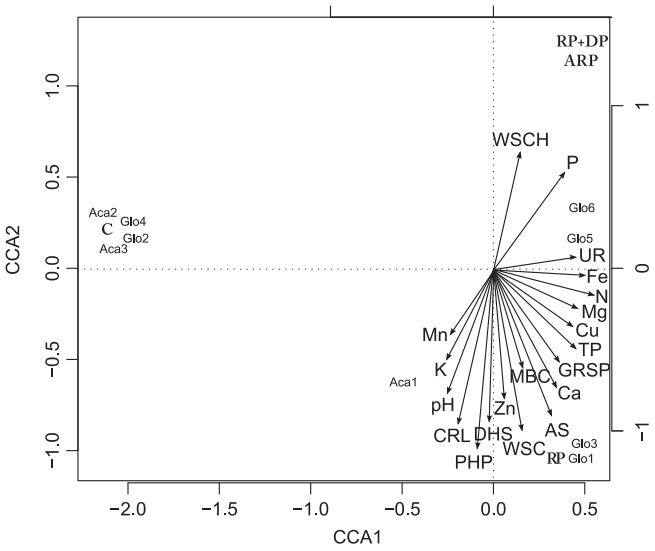

Fig. 3. Canonical-correspondence analysis (CCA) of the AMF communities found and soil properties at the four different fertilisation treatments. The eigenvalues of the first and second axes in the two-dimensional ordination diagrams are as follows: CCA1: 0.49 and CCA2: 0.40.

these results suggest that P fertilisation could induce a shift in the community composition of AMF. In accordance with our results, it has been reported that suitable additions of NPK fertiliser could improve the diversity of AMF in some soils, but increasing P fertilisation markedly reduced the species diversity of AMF and altered the species composition (Johnson, 1993; Kahiluoto et al., 2001; Wang et al., 2009). This indicates that the species composition of the AMF appears to vary in response to nutrient additions in field studies. In our case only the available P was modified significantly in the soil after the fertilisation treatments, while other nutrients such as total P, total Fe, total Ca, total Mg, total Cu, total Zn and total Mn were not altered significantly. Although the mechanism determining the influence of P fertilisation on the community structure of AMF is mostly unknown, the response of AMF to fertilisation may be related partly to the changes in soil quality and therefore to different requirements of the fungi for C, N or P (Treseder and Allen, 2002).

The fertilisation treatments used in our study affected differently the soil properties measured and therefore the soil quality. Thus, following the low dose of P as rock phosphate (RP) there was an increase in biomass C, water-soluble carbon and the dehydrogenase, urease and phosphatase activities with respect to the control treatment. These parameters have been used frequently as indicators of soil microbial activity (De Luca and Keeney, 1993; García et al., 1997). The application of low-to-moderate amounts of P fertiliser to P-deficient soils may reactivate microbial populations and consequently the AMF (Picone, 2002), since AMF are key components of the soil microbiota and interact with other microorganisms in the rhizosphere, affecting the soil microbiological properties (Jeffries et al., 2003). Thus, formation of AMF might have altered root biomass and exudation, which influence the quantity and quality of C delivered to the soil via fungal hyphae (Marschner et al., 1997). Therefore, the fact that the water-soluble carbon content was higher with the RP treatment than with the other treatments (C, ARP and RP + DP) could be due to higher rhizodeposition of soluble-C fractions, as a consequence of the higher percentage of root colonisation under this treatment. Also, the higher level of stable aggregates resulting from the RP treatment can be attributed to the proliferation of fungal hyphae (Roldán et al., 1994; Jeffries and Barea, 2000). In this regard, a significant, positive correlation between the Glo 1 and Glo 3 fungal types and the WSC ($P < 0.05$) was found. The stimulating effect in the soil of low doses of P might have selected exclusive morphotypes able to function better with the sources of P available in the soil, helping to improve some properties of the soil and therefore its quality.

The Aca 2, Aca 3, Glo 2 and Glo 4 fungal types were detected only in the control treatment, suggesting that these AM fungi are highly sensitive to any change in their habitat due to P addition. By contrast, Glo 5 and Glo 6, which were the most abundant in our study, were found in all three treatments where P was added and their abundance was correlated positively with urease activity ($P < 0.01$), suggesting that the soil conditions where urease activity significantly increased are suitable for the proliferation of these species. The significant increases in urease activity in the P-fertilised soils (RP, ARP and RP + DP) may have been due to enhanced root exudation, that can stimulate microbial activity and, in turn, intracellular enzyme activity. Root exudates can be a source of easily-degradable N compounds, such as amino acids and small peptides, which are able to induce urease synthesis (García-Gil et al., 2000). Higher urease activity implies higher availability of N in soil. This has been confirmed by several experiments (Treseder and Allen, 2002; Wang et al., 2009) which showed that, besides P content, high nitrogen content in soil can also affect the species composition of AMF and the percentage of root colonisation (Blanke et al., 2005).

With high doses of P (rock phosphate with or without diammonium phosphate), the content of available P was increased and the values of some parameters related to soil quality (DHS, PHP and WSC) were diminished by both treatments (APR and RP + DP), without differences between the two P sources used in spite of the higher solubility of diammonium phosphate. Also, the percentage of root colonisation and the diversity of AMF were lowest with these two fertiliser treatments. The fungal type Aca 1 was not detected following these treatments involving the highest amounts of added P, so we may conclude that this fungal sequence type possesses a higher degree of sensitivity to high levels of available P in soil, as shown by the negative correlation ($P < 0.01$). In general, we found a significant, negative correlation between the available P in the soil and the biodiversity of AMF, in agreement with the findings of several earlier experiments (Ryan and Graham, 2002; Treseder and Allen, 2002) that showed that the role of AMF diminished in agricultural soils with high P availability. It is important to note that in the treatments which included high doses of P (APR and RP + DP) the highest numbers of Ascomycetes, also called dark septate endophytes (DSE), were found. The Ascomycetes might displace the glomalean fungi when mineral nutrient demands are low and the AMF cease to obtain carbohydrates from their host (Alguacil et al., 2008). Therefore, the lower diversity of AMF in soils fertilised with high doses of P also could be due to competition for nutrients with other fungal symbionts.

We found that AMF communities in P-poor, savanna tropical soils were dominated by *Glomus* and *Acaulospora* spp. In a morphological study carried out in a similar ecosystem, Cuenca et al. (1998) reported the dominance of the same AMF species as we found here; although it is difficult to compare directly the results of molecular studies of the diversity of AMF in roots with spore morphology in the soil, these data suggest that these two AMF genera occur typically in P-poor, tropical soils.

In the treatments which gave a low level of available P in the soil (C and RP), an acceptable plant production was maintained compared with the soils that received high doses of P (Table 2). This could be attributable to a very effective association with AMF; in fact, centrosema roots showed a high percentage of mycorrhizal infection (an average of 68%) and also higher diversity of AMF compared with the treatments involving high doses of P (APR and PR + DP). Huang et al. (2005) and Alguacil et al. (2005) showed that plant productivity in a given system can be dependent on the diversity of symbionts. The role of AMF in increasing the uptake of P by their host plants is well known (Smith and Read, 1997). The AMF can contribute to the plant uptake of mineral nutrient P, by producing extracellular phosphatases and accessing distant P sources which are otherwise unavailable to the host plant (Joner and Johansen, 2000). Another mycorrhizal contribution to plant P uptake could be related to AM hyphae exploring a greater soil volume and inducing physiological changes to favour the establishment of P-solubilising and mineralising microorganisms in the mycorrhizosphere (Linderman, 1988; Singh and Kapoor, 1998).

5. Conclusions

This study shows that the application of low doses of P to soil as rock phosphate maintained the previous levels of AM fungal diversity, although it modified the AM fungal community composition, increased the percentage of root colonisation and improved properties related to soil quality. However, the application of high doses of P decreased markedly the AM fungal diversity, independent of the P source. Therefore, the application of low doses of P as rock phosphate can stimulate mycorrhization and help to offset the loss of biological fertility in P-poor, tropical savanna soils.

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