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ORIGINAL ARTICLE

Colonization and molecular diversity of arbuscular mycorrhizal fungi associated with the rhizosphere of cowpea (*Vigna unguiculata* (L.) Walp.) in Benin (West Africa): an exploratory study

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Abstract Arbuscular mycorrhizal symbiosis is an important plant root–fungal partnership/interaction that affects the growth response of crops. We have investigated the molecular diversity of arbuscular mycorrhizal fungi (AMF) colonizing cowpea roots and the associated rhizosphere soil to test the hypothesis that community diversity in rhizosphere soil is similar to that in cowpea (*Vigna unguiculata*) roots. Cowpea plants were grown in farmers' fields located in seven agro-ecological zones of Benin, and soil and root samples were collected. The molecular diversity of the AMF in these samples was assessed after amplification of the large ribosomal subunit of DNA extracted from the soil and the root samples. At fruition, the frequency of mycorrhizal infection was unaffected by the agro-ecological zone, but there were significant differences in the intensity of AMF colonization among the zones. Multiple regression analysis showed that the main factor affecting mycorrhizal frequency at flowering was available phosphorus. Phylogenetic analysis revealed 25 operational taxonomic units belonging to two fungal families (Glomeraceae and Gigasporaceae). The diversity of AMF colonizing roots of cowpea in Benin was high and fairly similar to that in the rhizosphere soil but with a prevalence of the Glomeraceae. Despite the absence of strict host specificity in mycorrhizal symbiosis, there was a preferential association between some AMF species and cowpea cultivar IT96D-610.

Keywords AMF diversity · Arbuscular mycorrhizas · Operational taxonomic unit · Rhizosphere · *Vigna unguiculata*

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Introduction

The symbiosis between arbuscular mycorrhizal fungi (AMF) and terrestrial plants is one of the most widespread (Parniske 2008; Smith and Read 2008) and ancient (Redecker 2000) plant–microbe interactions. Establishment of an active mycorrhizal symbiosis is an important beneficial factor in terms of improved plant nutrient uptake, such as phosphate (Harrison 1999), and plant health, by buffering biotic (Harrier and Watson 2004; Hao et al. 2012) and/or abiotic stresses (Rivera-Becerril et al. 2005; Smith and Read 2008). The development of extraradical arbuscular mycorrhizal mycelium also improves soil stability (Rillig et al. 2002) and water retention (Augé 2004; Cho et al. 2006; Bedini et al. 2009). Consequently, AMF are perceived as an important component of the plant–soil system, creating symbiotic associations with most land and cultivated plants (Wang and Qiu 2006) and

improving crucial ecosystem processes (Harrier and Watson 2004; van der Heijden et al. 2008; Hao et al. 2009; Gianinazzi et al. 2010) and plant productivity (van der Heijden et al. 1998; Smith and Read 2008).

Although the benefits of AMF symbiosis are well-known, studies on the Glomeromycota fungi in sub-Saharan Africa (SSA) and the application of these fungi in agriculture are still rudimentary. Previous works have mainly focused on the identification of AMF in tropical forests (Hawley et al. 2004; Houngnandan et al. 2009) or have studied the impact of land use intensity on AMF communities in different climatic zones (Franke et al. 2006; Mathimaran et al. 2007; Tchabi et al. 2008). Cowpea [*Vigna unguiculata* (L.) Walp.] is cultivated in 45 countries around the world, with SSA accounting for 84 % of the total world production (Abate et al. 2011). In this region, cowpea is a major food legume, but few studies have focused on its relation with AMF (Tawaraya 2003). One recent study has helped to identify the diversity of AMF associated with this crop in different agro-ecosystems of Benin based on spore morphology (Johnson et al. 2013). However, one of the limitations of studies based on this traditional method is that some species of AMF do not sporulate (Wubet et al. 2004), possibly leading to an incomplete image of the diversity of functionally active AMF colonizing roots (Helgason et al. 1999). The results of a number of other studies have provided evidence for preferential associations between plants and AMF species (Bever et al. 2001; Pivato et al. 2007; Cesaro et al. 2008).

Over the past two decades, molecular approaches have been widely used to characterize AMF communities in grasslands (Gollotte et al. 2004; Oehl et al. 2004a; Hijri et al. 2006; Gai et al. 2009; Binet et al. 2013) and agricultural ecosystems (Jansa et al. 2002; Stukenbrock and Rosendahl 2005; Castillo et al. 2006; Brito et al. 2012). However, to our knowledge, no research on the genetic diversity of AMF colonizing cowpea roots in sub-Saharan fields has been reported. A better understanding of the community structure of AMF in soils under cultivation and in the roots of crops is a fundamental prerequisite for their eventual valorization as bio-fertilizers. Hence, the aim of our study was to investigate AMF diversity associated with *Vigna unguiculata* cv. IT96D-610, by analysis of the large subunit (LSU) ribosomal DNA genes (van Tuinen et al. 1998) in roots and rhizosphere soil collected from fields of Benin. The following questions were addressed: (1) Do agro-ecosystems or the phenology of cowpea affect mycorrhizal colonization of roots? (2) What is the main abiotic factor affecting the root colonization of cowpea? (3) Is the AMF community diversity in rhizosphere soil similar to that in cowpea roots? (4) Are there preferential associations between IT96D-610 cowpea cultivar and AMF?

Materials and methods

Study sites

The survey was carried out between July and November 2008 in the major agro-ecological zones in Benin where cowpea is produced (geographical position: 6°–12°50 N; 1°–3°40 E) (see Johnson et al. 2013). In each agro-ecological zone, four cowpea farmers were asked to participate in the study, and in each farmer's field the same cultivar of cowpea, IT96D-610, provided by the International Institute of Tropical Agriculture (IITA–Ibadan station) was sown. IT96D-610 is an erect cowpea plant and an early-maturing cultivar (maturity period 65–70 days). Overall, 28 farmers' fields located in seven agro-ecological zones were sampled (see Johnson et al. 2013). Average annual precipitation data for longer than a 5-year period were obtained for each site from the pan-African meteorological service (ASECNA).

Soil and root sampling

Samples were collected at two different stages of cowpea development (flowering and fruition). At the flowering stage, cowpea roots of ten randomly selected plants within each field were harvested. At the same time, soil samples (100 g) were collected around these randomly selected plants at a depth of 0–15 cm. At fruition (or green-mature stage: peas are fully developed and the majority of the pods are mature), in addition to soil sampling, roots were also collected on another ten cowpea plants in each of the 28 fields.

Soil samples were air-dried, sieved (2 mm), homogenized, placed in closed plastic bags and kept at room temperature. Sampled cowpea roots were washed, dried with tissue paper and divided into two parts. One part was maintained in plastic bags at 4 °C pending arbuscular mycorrhizal (AM) colonization assessment; the remaining part of each root system was cut into small pieces and dried overnight at 50 °C (Farmer et al. 2007; Branco et al. 2013). Dried samples were stored at room temperature until further (molecular) analysis.

At the time of sowing, we also carried out a short survey on previous agricultural practices carried out by each farmer in his field.

Soil physical and chemical analyses and characterization of land use

From each soil sample (cowpea field × phenology stage), we used one subsample for determination of the physical and chemical properties, including soil texture, pH, total carbon (C), total N, total P, available P, exchangeable K, exchangeable Mg, exchangeable Ca and cation exchange capacity (CEC). The analyses of the soil samples were as previously described (Johnson et al. 2013). To characterize the land use

system in the farmers' fields, Joosteen's roving coefficient (R) (Vine 1968) was calculated according to the following equation:

$$R = \frac{U}{V} \times 100$$

where U is the land use period (year), and V is the sum of land use period and fallow period (year). R is a roving coefficient which takes into account for calculation the length of the land use period and the length of the fallow period. In shifting cultivation, a low R value means that the field is cultivated for a very short period with a long fallow period, while a high R value means the field is intensively and regularly cultivated.

Assessment of root colonization by AMF

Samples (1–5 g) of fine roots were cleared and bleached in 10 % KOH for 60 min at 90 °C, rinsed in water, immersed in 10 % H_2O_2 for 45 min and stained in 0.05 % trypan blue in lactophenol (Phillips and Hayman 1970) for 72 h to visualize all fungal structures. Stained roots were cut into 1- or 2-cm-long fragments. Per root sample, 30 root fragments were mounted on glass slides. Each fragment was observed under a microscope (100× magnification) to estimate the extent of AMF colonization. The mycorrhizal frequency (F), which corresponds to the ratio of colonized versus non-colonized root fragments, and the intensity of colonization of the root cortex (M), which corresponds to the percentage cover of AMF colonization in each root fragment, were evaluated microscopically using the notation scale described by Trouvelot et al. (1986).

DNA extraction from rhizosphere soils and roots

Only cowpea roots and soil samples collected in farmers' fields at fruition were analyzed for molecular diversity. For this assay, approximately 100 mg of roots was collected by randomly taking fragments from the root samples obtained from each field and grinding these to a homogenous powder in liquid nitrogen. For the rhizosphere soil sampled in each field around the ten selected plants at fruition, an aliquot (100–200 mg) of homogenized soil was used for genomic DNA extraction. DNA from soil and roots was extracted using the NucleoSpin®Plant II kit (Macherey-Nagel GmbH & Co., Germany) following the manufacturer's protocol for the isolation of DNA from soil samples and plant tissues.

PCR amplification of a partial LSU rDNA region

In order to increase the amount of DNA available for cloning, each DNA extract was submitted to nested PCR reactions to strengthen the efficiency of the amplification. The primer pair LR1 and NDL22, previously designed by van Tuinen et al. (1998) from alignments of the 5′ end of the large ribosomal subunit, which targets eukaryotes was used for the first nested PCR amplification, and the primer pair FLR3 and FLR4 (Gollotte et al. 2004) which targets Glomeromycota fungi was used for the second.

Reactions were performed in a final volume of 20 μl containing 2 μl of 10× PCR buffer (10 mM Tris–HCl, pH 9.5, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 0.2 mg/ml bovine serum albumin) (Q-BIOgene, France), 200 mM dNTPs, 500 nM of each primer, 0.8 U of Taq DNA polymerase (Q-BIOgene) and 5 μl of diluted or undiluted root or soil genomic DNA extract. Each reaction was performed in a thermal cycler (T3000 thermocycler; Biometra, Germany) programmed as follows: initial denaturation cycle at 95 °C (3 min), annealing at 58 °C or 60 °C (1 min), extension at 72 °C (1 min), followed by 34 cycles of denaturation at 93 °C (1 min), annealing at 58 or 60 °C (1 min) and extension at 72 °C (1 min). The program was concluded with a final extension phase at 72 °C for 10 min. The first PCR amplicons were diluted 1/100, and 5 μl of this dilution was used as a template for the second reaction targeting AMF, at the same amplification conditions as described above.

PCR products were separated by gel electrophoresis on 1.2 % agarose gel in TAE buffer (40 mM Tris, pH 7.8, 20 mM acetic acid and 2 mM EDTA), and DNA was visualized under UV light after staining with 0.1 % ethidium bromide (Sambrook et al. 1989).

Cloning, sequencing and construction of LSU rDNA libraries

The PCR products generated from rhizosphere soil and from the cowpea roots using FLR3 and FLR4 were pooled before cloning as previously suggested (Renker et al. 2006; Higo et al. 2014). PCR products generated from rhizosphere soil and root tissues using primers FLR3 and FLR4 were cloned into pCR®2.1-TOPO® using the TOPO TA Cloning® kit according to the manufacturer's protocol (Invitrogen SARL, France) and transformed into competent *Escherichia coli* One shot® TOP10 (Invitrogen SARL). Plasmid clones were identified based on blue–white screening. The presence of cloned inserts was checked by PCR using the primer pair 18.1 (5'-GTCACGTTGTAAAACG-3′) and 18.2 (5′-AGCT ATGACCATGATTAC-3′) directly on the bacterial colonies diluted in sterile distilled water and lysed at 90 °C for 5 min.

Two AMF libraries of LSU rDNA genes from rhizosphere soil and mycorrhizal cowpea roots were established. Inserts from 50 randomly selected clones in each LSU rDNA library were sent for dye-terminator sequencing at MWG Biotech (Germany).

Sequences analysis and phylogenetic inference

The sequences of the cloned PCR products were submitted to the BLASTN algorithm (Altschul et al. 1997) in order to verify the glomeromycotan origin of the sequences, to check the presence of chimerical sequences, to exclude potential contaminant sequences (e.g., bacteria, unspecific amplifications of other genome regions) and to identify similar sequences from the database. Subsequently, they were deposited in GenBank (http://www.ncbi.nlm.nih.gov) and assigned accession numbers from HG515402 to HG515486 inclusive.

Multiple alignments over 377 bp were performed on DNA sequences, including the variable domains D1 and D2, of the two LSU rDNA clone libraries and on known sequences from public databases having the highest degree of similarity, using the MAFFT (http://mafft.cbrc.jp/alignment/server/) algorithm. The alignments were manually optimized with Se-Al 2.0 software (University of Oxford, UK). Finally, a phylogenetic inference was carried out by distance analysis using the neighbor-joining (NJ) algorithm (Saitou and Nei 1987) with the Kimura 2-parameter model and using *Mortierella chlamydospora* as an outgroup. The reliability of the clades of the phylogenetic tree was assessed using the bootstrap method with 1000 replications. A phylogram was drawn and visualized using a NJ plot (http://biom3.univ-lyonl.fr, University of Lyon, France).

Definition of the operational taxonomic unit and nucleotide sequence accession numbers

As single morphospecies and even individual spores of Glomeromycota may contain multiple slight variants of rDNA, AMF cannot be identified on the basis of a single ribosomal sequence. For this reason, operational taxonomic units (OTUs) or ribotypes were determined as distinct monophyletic groups in the phylogenetic trees. Only clades that were supported by bootstrap values of≥95 % were used (Holland et al. 2014). Splitting of the OTU was avoided unless there was positive evidence for doing so, such as homologies with known AMF species. AMF genera or morphospecies associated to each OTU were attributed on the basis of the position of already known sequences from databases.

Diversity analysis

Rarefaction curves were constructed with the Analytical Rarefaction program version 1.3 (http://www.uga.edu/~strata/software/) in order to determine whether the number of clones sequenced sufficiently represented Glomeromycota diversity in the cowpea roots and in the rhizosphere soil. The results were fitted to the Michaelis–Menten equation $\left(y = \frac{ax}{b+x}\right)$ which is known to accurately fit rarefaction curves. This model was optimized using a Levenberg–Marquardt nonlinear fitting algorithm and was used for extrapolating these curves to estimate biodiversity (Colwell and Coddington 1994).

The overall AMF OTU richness in cowpea roots and rhizosphere soil was estimated using the Chao method (Chao 1987). The richness estimator $\hat{S}_{\text{Chao1}}$ was computed using EstimateS software version 9.0.0 (Colwell 2013).

In order to analyze AMF diversity in cowpea roots and in rhizosphere soil, two of the most commonly used indices of species diversity that combine information on species richness and relative abundance in different ways were computed: the Shannon–Wiener's diversity index H' (Shannon 1948) and Simpson's diversity index D (Simpson 1949). The indices were computed using the following formulas:

$$H' = -\sum_{i=1}^{S} p_i ln p_i$$

where p_i is the proportion of sequences belonging to each OTU relative to the total number of sequences and S is the number of OTUs (OTU richness).

$$D = \frac{1}{\sum_{i=1}^{S} \frac{n_i(n_i-1)}{N(N-1)}}$$

where n_i is the number of sequences of each OTU and N is the total number of sequences. In both formulas, i represents each OTU.

The Pielou's index E (Pielou 1966) was used to assess evenness of the OTU distribution within cowpea roots and rhizosphere soil. It was calculated as indicated below:

$$E = \frac{H'}{H'_{\max}} \; with \; H'_{\max} = ln\, S$$

Where H' is the Shannon–Wiener's diversity index.

In order to determine whether the community composition of AMF OTUs associated with rhizosphere soil was similar to the one within cowpea roots, the classic Jaccard index of similarity, (J_{clas}) (Jaccard 1912) was calculated. This index uses OTU presence/absence information for the two ecological systems A and B to measure the proportion of OTUs present in both ecological systems based on the total number of OTUs present in at least one of the ecological systems. The index is defined as:

$$J_{clas} = \frac{c}{a+b+c}$$

where a is the number of AMF OTUs occurring only in ecological system A (rhizosphere soil of cowpea), b is the number of OTUs occurring only in ecological system B (cowpea

roots) and c is the number of species in both ecological systems. However, the classic Jaccard index of compositional similarity (J_{clas}) is strongly affected by the size of sample and undersampling, especially for assemblages with numerous rare species, and it also induces severe negative bias. Therefore, to compare more rigorously the AMF compositional similarity within rhizosphere soil and cowpea root samples, Chao's estimator for Chao's Abundance-based Jaccard similarity index ($\widehat{J}_{abd}$), which has been proved to be strikingly resistant to undersampling (Chao et al. 2005), was computed using EstimateS software version 9.0.0 (Colwell 2013) following the formula below:

$$\widehat{J}_{abd} = \frac{\widehat{U}\widehat{V}}{\widehat{U} + \widehat{V} - \widehat{U}\widehat{V}}$$

where $\widehat{U}$ and $\widehat{V}$ are the estimators of the total relative abundances of OTU belonging to the ecological systems A (rhizosphere soil) and B (cowpea roots), respectively. $\widehat{U}$ and $\widehat{V}$ are calculated as suggested by Chao et al. (2005).

Analytical and statistical methods

One-way analysis of variance (ANOVA) was performed to assess the effects of agro-ecological zone and phenological stage on the mycorrhizal colonization parameters (F and M) and diversity indices based on OTUs. This ANOVA was performed with nontransformed data after ensuring conformity of the data with ANOVA assumptions (normality and homogeneity of variance), with the exception of the percentage values of the frequency and intensity of mycorrhizal colonization, which were transformed using an arcsine function. For data which did not match ANOVA assumptions even after transformation, nonparametric variance analysis (Kruskall–Wallis test) or Wilcoxon paired test were used.

Pearson's correlation analysis was used to determine relationships between mycorrhizal colonization parameters of *V. unguiculata* and edapho-climatic variables (percentage of sand, silt, and clay, pH, total C, total N, total P, available P, exchangeable K, exchangeable Mg, exchangeable Ca, CEC, Joosteen's roving coefficient R and average annual precipitation) in the 28 cowpea fields. In a next step, stepwise regression analysis was realized to derive the best-fit regression model with mycorrhizal colonization parameters and abiotic variables.

The diversity t test described by Magurran (1988) was used to compare Shannon's diversity index of AMF OTUs in rhizosphere soil and in cowpea roots. In order to determine whether the distribution of the AM fungal OTUs in the rhizosphere soil and in the cowpea roots was independent, a Fisher's exact test was inferred. This test enabled us to evaluate whether a dependence between OTUs and ecological systems (rhizosphere soil or cowpea roots) did or did not exist.

All statistical analyses were carried out using SAS software version 9.2 (SAS Institute, Cary, NC, USA), except for the diversity t test which was implemented in the PAST software version 2.01 (Hammer et al. 2001).

Results

Cowpea roots colonization by AMF

Roots of *V. unguiculata* from all the fields were colonized to a certain extent by AM fungi. At the flowering stage, mycorrhizal frequency (F) and intensity (M) varied from 13.6 to 70 % and from 3.6 to 15 %, respectively. The frequency of mycorrhizal infection (F) and the level of mycorrhizal colonization (M) were affected by agro-ecological zone at a p value of 0.028 and 0.0001, respectively (Fig. 1). At fruition (Fig. 2), F and M varied between 48 and 82 % and between 5 and 43 %, respectively. At this stage, the frequency of mycorrhizal colonization (F) was unaffected by agro-ecological zone (p=0.46), but there were significant differences in intensity of arbuscular mycorrhizal colonization (M) between the zones (p<0.015). Likewise, a comparative analysis of the frequencies (F) and the intensities of mycorrhization in each agro-ecosystem showed that, in general, F and M were significantly higher at fruition than at flowering (Fig. 3).

Relationship between mycorrhizal colonization and edaphic conditions

At flowering, the frequency (F) and intensity (M) of mycorrhizal colonization were negatively correlated with available P and the Joosteen's roving coefficient (R) (Table 1). Stepwise regression analysis on all measured edapho-climatic factors identified soil available P content (negative influence) as a significant explanatory variable affecting mycorrhizal colonization frequency at flowering. At flowering, 78 % of the variation in F was explained by soil available P content (Fig. 4).

OTUs detected in the rhizosphere and diversity analysis

Partial LSU sequences of AMF colonizing cowpea roots and associated rhizosphere soils from different agro-ecosystems in Benin were amplified by the primer pair FLR3 and FLR4. One hundred amplicons of the expected size ranging from 309 to 377 bp in length were sequenced. BLAST searches in the GenBank database showed that all sequences obtained in this study had a high similarity (70–100 %) to AMF and

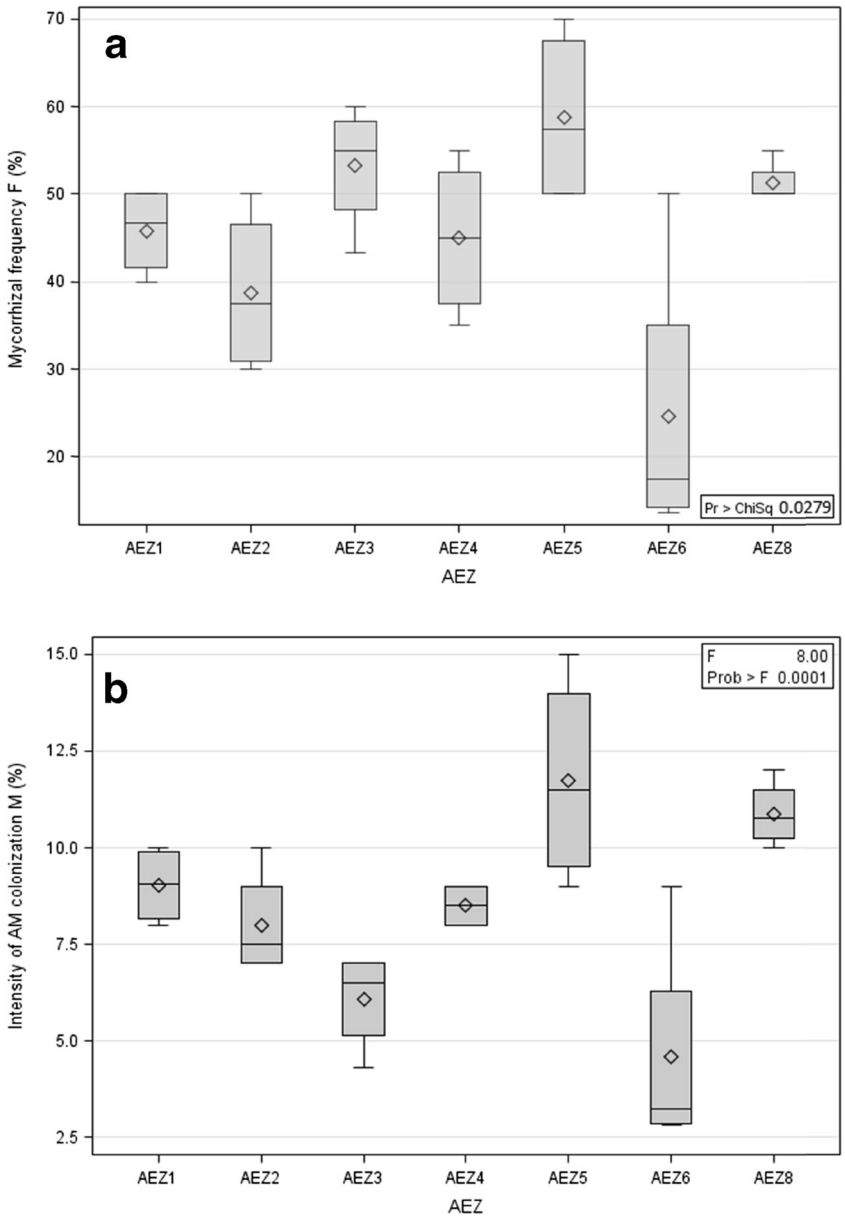


Fig. 1 Box plots showing the frequency (*F*) and intensity (*M*) of arbuscular mycorrhizal (AM) colonization in cowpea (*Vigna unguiculata*) roots from different agro-ecological zones (*AEZs*) at flowering. *Length of box* Interquartile range, *symbol in box interior* group mean, *horizontal line in box* group median, *whiskers* group minimum and maximum values. **a** The Kruskall–Wallis test shows that frequency of mycorrhizal infection (F) was affected by agro-ecological zone. **b** The analysis of variance (ANOVA) demonstrates that there were significant differences in the level of mycorrhizal colonization (M) between zones

belonged to the Glomeromycota phylum, thereby confirming the specificity of the FLR3–FLR4 primer pair for the detection of Glomeromycota. The phylogenetic analysis of sequences allowed the determination of 25 monophyletic OTUs, supported by high bootstrap values (>95 %). Of these, 23 belonged to the Glomeraceae family and two belonged to the Gigasporaceae family (Fig. 5). A high proportion of the sequences obtained (90 %), originating from both cowpea root samples and rhizosphere soil, clustered with uncultured Glomeromycota or were new sequences. Only a small proportion of these sequences (10 %) clustered with previously identified AMF sequences. These were related to the cultured AMF species: Glo2 for *Rhizophagus irregularis*, Glo06 for *R. clarum*, Glo07 for *Sclerocystis sinuosum*, Glo11 for *Funneliformis mosseae*, Glo12 for *Septoglomus viscosum* (syn. *G. viscosum*) and Gig01 for *Gigaspora margarita* (Fig. 5). Glomeraceae species were predominant in rhizosphere soil (96 %) and in cowpea roots (92 %). The OTU Glo01, which had a high similarity (93 % identity) with *R. irregularis*, was the most abundant OTU (22 %) in cowpea roots.

In order to determine whether the number of clones was sufficient to represent Glomeromycota diversity in the subsamples of rhizosphere soil and cowpea roots, we constructed rarefaction curves (Fig. 6). The data indicated that the number of sequences analyzed per ecological system (rhizosphere soil or cowpea roots) did

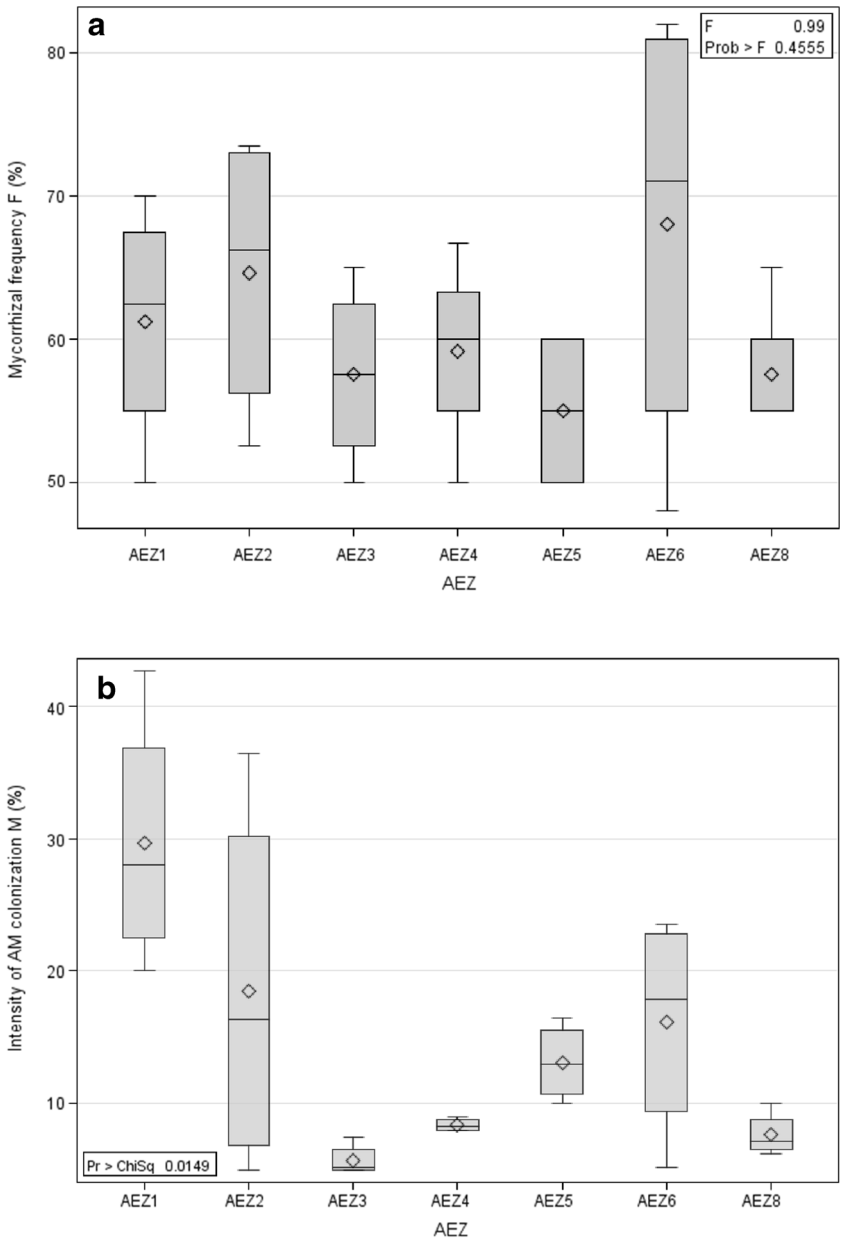


Fig. 2 Box plots showing the frequency (*F*) and intensity (*M*) of AM colonization in cowpea roots from different AEZs at fruition. **a** The results of the ANOVA demonstrate that there was no significant difference ($F=0.99$; $Prob=0.4555,>0.05$) in mycorrhizal frequency (F) between the zones. **b** The results of the Kruskall–Wallis test show that intensity of mycorrhizal colonization (M) between zones was affected by agro-ecological zone ($Pr=0.0149$). See caption to Fig. 1 for explanation of box plot

not provide full coverage of AMF diversity. Fitted equations and extrapolation of the rarefaction curves (Fig. 7) suggested that the diversity plateau would be at 36 and 23 Glomeromycota species in the rhizosphere soil and in cowpea roots, respectively, showing that with only 50 sequences per sample, only 50 and 70 % of the expected diversity, respectively, was described.

The Shannon–Wiener diversity index (*H'*) calculated on the basis of relative abundance of OTUs in rhizosphere soil did not significantly differ from that in mycorrhizal cowpea roots. The Simpson's diversity index (*D*) and AMF OTU richness were in the same range in rhizosphere soil and in cowpea roots (Table 2). Similarly, the estimated AMF OTU richness in the rhizosphere soil ($\hat{S}_{\mathrm{Chao1}}=32\pm10$) overlapped that in cowpea roots ($\hat{S}_{\mathrm{Chao1}}=23\pm7$) (Table 2). Moreover, Pielou's evenness index (*E*) was nearly the same for both ecological systems.

Distribution of AM fungal OTU in rhizosphere soils and roots

The classic Jaccard index of similarity (J_{clas}) and Chao's estimator for Chao's Abundance-based Jaccard's similarity index ($\widehat{J}_{abd}$) between AMF community composition

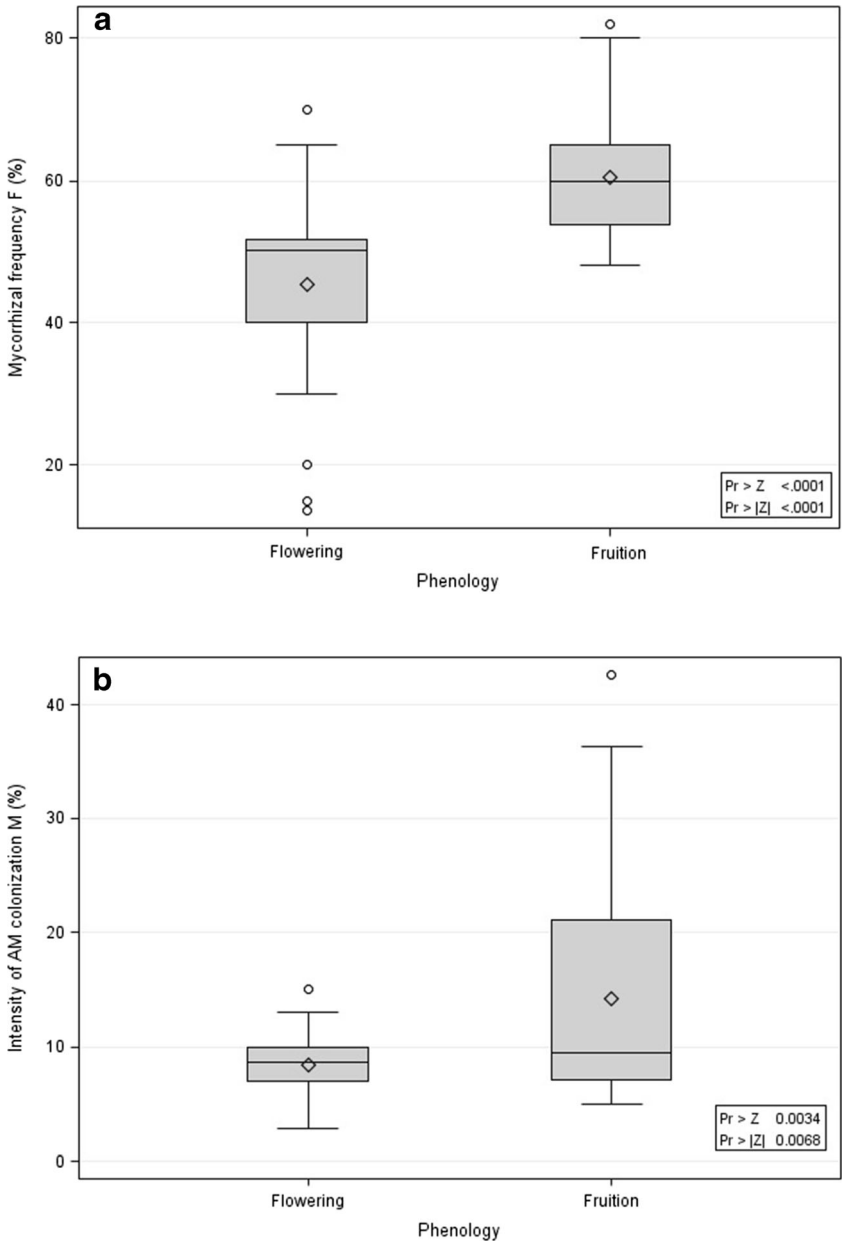


Fig. 3 Box plots showing frequency (*F*) and intensity (*M*) of AM colonization in cowpea roots at flowering and at fruition. The results of the Wilcoxon test show that F (**a**) and M (**b**) were significantly higher at fruition than at flowering. See caption to Fig. 1 for explanation of box plot

Table 1 Relationships between arbuscular mycorrhizal colonization frequency (F) and intensity (M) and two edaphic parameters from 28 cowpea fields in different agro-ecological zones of Benin

Edaphic parameter[a]	Flowering		Fruition	
	F	M	F	M
R	−0.51*	−0.53*	0.41*	0.36 ns
Available P	−0.88***	−0.63**	−0.09 ns	−0.09 ns

Significant at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; *ns* not significant

Values represent Pearson's correlation coefficient(*r*) (n=28 cowpea fields)

[a] R, Joosteen's roving coefficient; available P, soil available phosphorus content

in rhizosphere soil and in cowpea roots were moderate (0.40 and 0.46, respectively). In addition, the Fisher's exact test performed in order to determine whether the distribution of the AMF OTUs in the rhizosphere soil of cowpea plants or in the roots was independent revealed the existence of a highly significant dependency (df=24; p=3.54E-08) between AMF OTUs and ecological systems (rhizosphere soil or cowpea roots). Therefore, these analyses show that Glomeromycota species tend not to be randomly distributed; rather, they choose preferentially their ecological system. The studied cowpea cultivar (IT96D-610) may establish a preferential symbiosis with some AMF species.

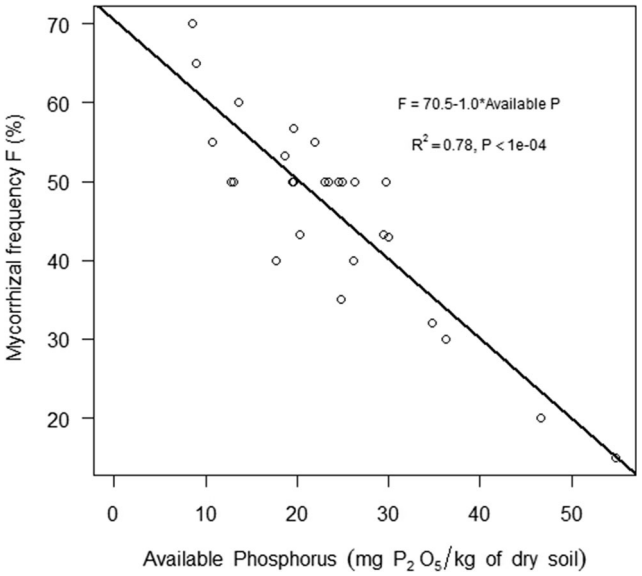


Fig. 4 Relationship between AM colonization frequency (*F*) and soil available phosphorus (*P*) content at flowering

Discussion

Mycorrhizal colonization of roots

Cowpea has a good mycotrophic status, and arbuscules and vesicles are found in its roots. A comparative analysis of the frequencies and the intensities of mycorrhization in each of our agro-ecosystems showed that, in general, F and M were significantly higher at fruition than at flowering, indicating that phenology affected the mycorrhizal colonization of roots. This result corroborates previous findings (McArthur and Knowles 1993) which showed that physiological conditions can affect the mycorrhizal colonization of roots. Indeed, McArthur and Knowles (1993) observed that potato roots colonized by *Glomus fasciculatum* declined during the period of rapid tuber growth. In the case of cowpea, mycorrhizal colonization could have been affected not only by the plant phenology but also, at fruition, by the longer time of permanence of the roots in the soil. This hypothesis may be supported by the findings of Muthukumar and Udaiyan (2000) who reported that the annual and biennial species investigated in their study had significantly lower colonization levels than the perennial species.

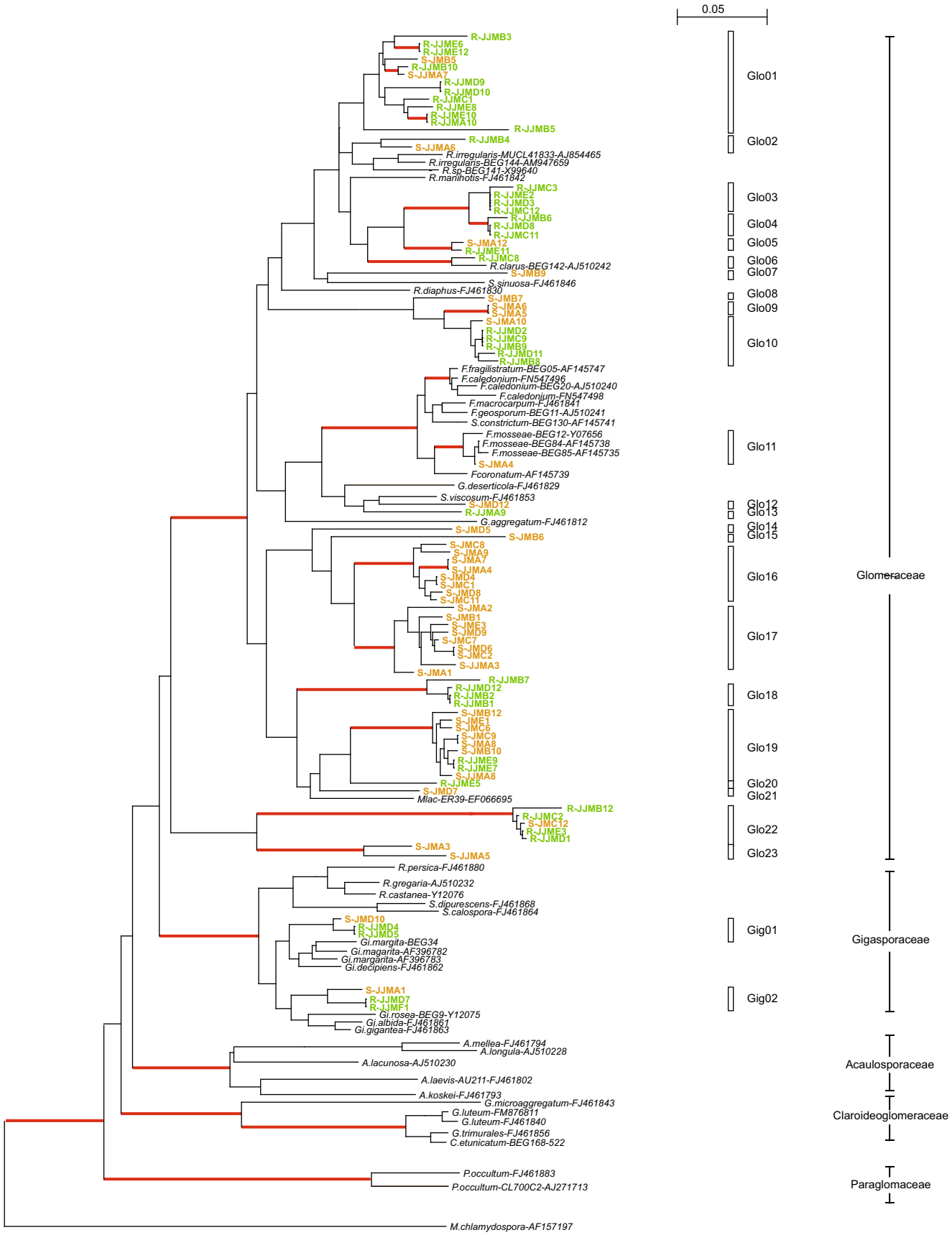
Although the presence of arbuscule is a significant indication of mutualism between plants and AM fungi (Smith and Smith 1989), we did not assess the degree of arbuscular presence in this study as we had no specific research question related to this mycorrhizal colonization parameter. We highly recommend that future studies provide more detailed data on AM colonization assessment.

The results of our study show that the main factor affecting F at flowering was available P. It has already been reported (Michel-Rosales and Valdes 1996; Uhlmann et al. 2006) that biotic factors, such as spore density, are not the main factor(s) affecting mycorrhization but that other, more decisive abiotic factors may exist. Other propagules, such as viable pieces of hyphae and colonized root pieces with vesicles of AM fungi, could be factors affecting mycorrhization (Douds et al. 2011). Factors known to influence mycorrhization include P levels (Michel-Rosales and Valdes 1996), soil pH and total or extractable K (Chen et al. 2008). Nevertheless, it is well known that the response of mycorrhizal colonization to available P is variable (Jasper et al. 1989; Treseder 2013). Indeed, some studies have found a positive relationship between AMF colonization level and available P (Sanginga et al. 1999; Chen et al. 2008), while others showed a negative one (Cuenca and Meneses 1996; Sanginga et al. 1999). Therefore, it would appear that available P can influence mycorrhizal colonization either positively or negatively. The negative correlation can be explained by the well-known depressive effect of P on AM colonization (Bruce et al. 1994). On the other hand, in soils which were severely P-deficient, a positive relationship is generally observed and can be explained by the stimulating effect of P. Thus, the negative effect observed in our survey may be explained simply by the fact that the sampled cowpea fields were not severely P-deficient (Johnson et al. 2013).

Diversity of AMF

Molecular biology has provided new techniques for the analysis of fungal populations forming mycorrhizas (Robinson-Boyer et al. 2009). We successfully performed nested PCR amplification of the same region with the primer pair FLR3–FLR4. The FLR4 primer has a perfect match only with glomalean fungal LSU rDNA sequences, and although the FLR3 primer is able to recognize DNA from some Basidiomycetes as well as Glomeromycetes, this primer pair only amplified Glomeromycota sequences (Gollotte et al. 2004; Mummey and Rillig 2007). This might explain why overall sequenced FLR3–FLR4 PCR products in this study mostly had a high level of similarity (70–100 %) with Glomeromycota LSU rDNA sequences. The size of the FLR3–FLR4 amplicons obtained by the nested PCR ranged from 309 to 377 bp, a variation which is in agreement with those of AMF genera, ranging from 300 to 350 bp for *Gigaspora*, *Scutellospora*, *Acaulospora* and *Entrophospora* to about 380 bp in *Glomus* (van Tuinen et al. 1998; Mummey and Rillig 2007).

Phylogenetic analysis revealed that our sequences could be clustered in 25 AMF monophyletic OTUs or ribotypes, among which only 10 % of sequences were assigned or close to the known species, including *Rhizophagus irregularis*, *R. clarus*, *Sclerocystis sinuosa*, *Funneliformis mosseae*, *Glomus viscosum*, *Gigaspora margarita* and *G. decipiens*. However, it is uncertain whether each arbuscular mycorrhiza fungal OTU represents a single morphospecies, or whether


0.05
R-JJMB3
R-JJME6
R-JJME12
S-JMB5
R-JJMB10
S-JJMA7
R-JJMD9
R-JJMD10
R-JJMC1
R-JJME8
R-JJME10
R-JJMA10
R-JJMB5
R-JJMB4
S-JJMA6
R.irregularis-MUCL41833-AJ854465
R.irregularis-BEG144-AM947659
R.sp-BEG141-Y99640
R.manihotis-FJ461842
R-JJMC3
R-JJME2
R-JJMD3
R-JJMC12
R-JJMB6
R-JJMD8
R-JJMC11
S-JMA12
R-JJME11
R-JJMC8
R.clarus-BEG142-AJ510242
S-JMB9
S.sinuosa-FJ461846
R.diaphus-FJ461830
S-JMB7
S-JMA6
S-JMA5
S-JMA10
R-JJMD2
R-JJMC9
R-JJMB9
R-JJMD11
R-JJMB8
F.fragilistratum-BEG05-AF145747
F.caledonium-FN547496
F.caledonium-BEG20-AJ510240
F.caledonium-FN547498
F.macrocarpum-FJ461841
F.geosporum-BEG11-AJ510241
S.constrictum-BEG130-AF145741
F.mosseae-BEG12-Y07656
F.mosseae-BEG84-AF145738
F.mosseae-BEG85-AF145735
S-JMA4
Fcoronatum-AF145739
G.deserticola-FJ461829
S.viscosum-FJ461853
S-JMD12
R-JJMA9
G.aggregatum-FJ461812
S-JMD5
S-JMB6
S-JMC8
S-JMA9
S-JMA7
S-JJMA4
S-JMD4
S-JMC1
S-JMD8
S-JMC11
S-JMA2
S-JMB1
S-JME3
S-JMD9
S-JMC7
S-JMD6
S-JMC2
S-JJMA3
S-JMA1
R-JJMB7
R-JJMD12
R-JJMB2
R-JJMB1
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R-JJME7
S-JJMA8
R-JJME5
S-JMD7
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R-JJMB12
R-JJMC2
S-JMC12
R-JJME3
R-JJMD1
S-JMA3
S-JJMA5
R.persica-FJ461880
R.gregaria-AJ510232
R.castanea-Y12076
S.dipurescens-FJ461868
S.calospora-FJ461864
S-JMD10
R-JJMD4
R-JJMD5
Gi.margita-BEG34
Gi.magarita-AF396782
Gi.margarita-AF396783
Gi.decipiens-FJ461862
S-JJMA1
R-JJMD7
R-JJMF1
Gi.rosea-BEG9-Y12075
Gi.albida-FJ461861
Gi.gigantea-FJ461863
A.mellea-FJ461794
A.longula-AJ510228
A.lacunosa-AJ510230
A.laevis-AU211-FJ461802
A.koskei-FJ461793
G.microaggregatum-FJ461843
G.luteum-FM876811
G.luteum-FJ461840
G.trimurales-FJ461856
C.etunicatum-BEG168-522
P.occultum-FJ461883
P.occultum-CL700C2-AJ271713
M.chlamydospora-AF157197
Glo01
Glo02
Glo03
Glo04
Glo05
Glo06
Glo07
Glo08
Glo09
Glo10
Glo11
Glo12
Glo13
Glo14
Glo15
Glo16
Glo17
Glo18
Glo19
Glo20
Glo21
Glo22
Glo23
Gig01
Gig02
Glomeraceae
Gigasporaceae
Acaulosporaceae
Claroideoglomeraceae
Paraglomaceae

◀ **Fig. 5** Neighbor-joining (NJ) tree representing AM sequences obtained from rhizosphere soils (*S*) (*in brown*) and mycorrhizal cowpea roots (*R*) (*in green*). *Mortierella chlamydospora* was used as the outgroup. Bootstrap values were estimated from 1,000 replications. *Red-colored branches* Bootstrap values of >97.5 %. Operational taxonomic units (OTUs), defined as a sequence group showing bootstrap values of≥95 % are indicated to the *right* of the NJ tree

some morphospecies include more than one sequence type. A high proportion of the analyzed sequences (90 %) belong to unknown or non-identified OTU. The growing number of unidentified fungal sequences without corresponding morphospecies recorded in international nucleotide sequence databases over the past years has led some mycologists to conclude that fungal diversity is larger than the current estimates (Pennisi 2008) and that presumably a large number of AMF species present in tropical soils still remain to be identified (Öpik et al. 2013; Holland et al. 2014).

The dominance of OTUs belonging the Glomeraceae family in the rhizosphere soil of cowpea (96 %) is in accordance with the results of our previous survey based on morphological characters of AMF spores (Johnson et al. 2013), indicating that this observation is not caused by a bias related to the molecular analysis. Predominance of Glomeraceae has also been reported in some studies based on AMF morphotypes in various agricultural soils (Oehl et al. 2003; Mathimaran et al. 2005).

The absence in this study of AMF OTUs belonging to the genera *Acaulospora* and *Scutellospora*, which were detected in a previous study of morphospecies (Johnson et al. 2013), could be explained by the fact that the number of clones

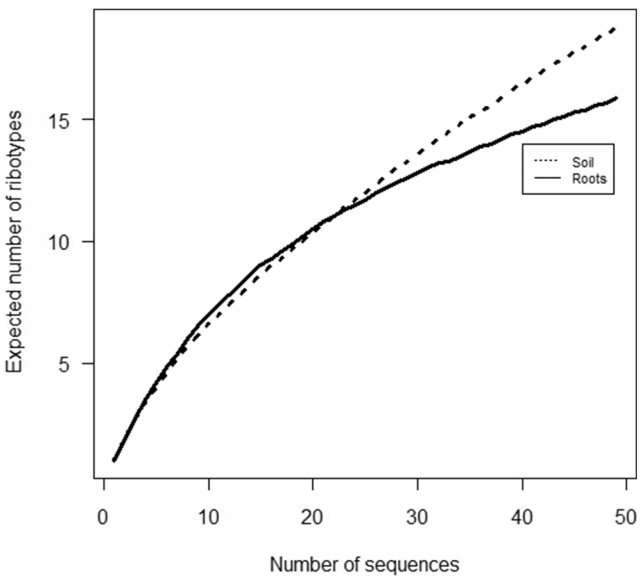

Fig. 6 Rarefaction curves of large subunit (LSU) rDNA libraries from rhizosphere soil (*dotted line*) and mycorrhizal cowpea roots (*continuous line*). Fitted formulas are as follows: $y = \frac{36.67x}{49.21+x}$ (R^2=0.996) for rhizosphere soil DNA and $y = \frac{23.19x}{23.86+x}$ (R^2=0.998) for mycorrhizal cowpea roots DNA

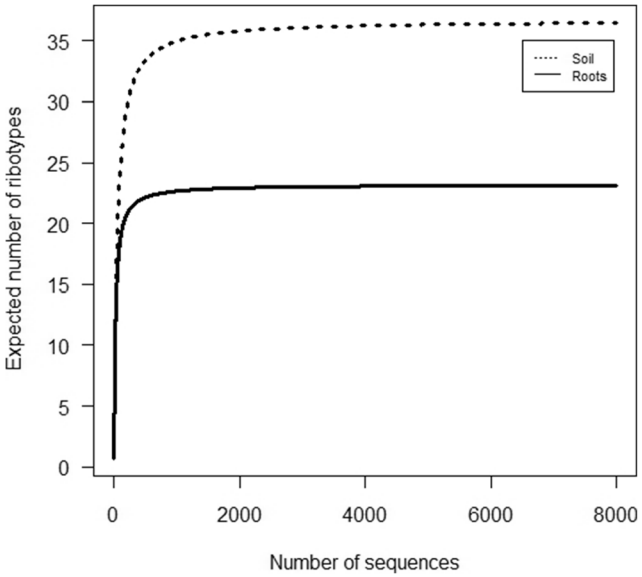

Fig. 7 Extrapolation of rarefaction curves of LSU rDNA libraries from rhizosphere soil (*dotted line*) and mycorrhizal cowpea roots (*continuous line*)

sequenced was insufficient for a comprehensive assessment of Glomeromycota diversity in the rhizosphere soils. In addition, the overall number of OTUs has been estimated to be 32 ±10; consequently, approximately 13 AMF OTUs in the cowpea rhizosphere soils were not observed during this study. A more exhaustive assessment of AMF diversity associated with cowpea would certainly reveal other rare taxa.

The diversity of AMF colonizing roots of cowpea in different agro-ecosystems of Benin was found to be high (*H′*= 2.45). A high diversity of AMF colonizing roots of *Prunus africana* in a dry Ethiopian forest has been reported (Wubet et al. 2003), and Pivato et al. (2007) reported a high diversity of AMF colonizing roots in four annual *Medicago* species cultivated in France (*H′*=1.94–2.24). To the contrary, the diversity of AMF colonizing roots of potato (*Solanum tuberosum*) in an Italian agricultural area was low (*H′*= 0.188–0.287) (Cesaro et al. 2008). In our study, the OTUs belonging to the family Glomeraceae predominated in cowpea

Table 2 Number of observed operational taxonomic units (OTUs) or ribotypes, estimated OTU richness, diversity indices and Pielou's evenness index of arbuscular mycorrhizal fungi OTUs from rhizosphere soil and cowpea root at fruition

Ecological system	S	$\hat{S}_{Chao1}$	*H′*	*D*	*E*
Rhizosphere soil	19	32 (10)	2.41	7.49	0.82
Cowpea root	16	23 (7)	2.45	9.06	0.88

S, Number of observed AMF OTUs or ribotypes; $\hat{S}_{Chao1}$, estimated AMF OTU richness; *H′*, Shannon–Wiener diversity index; *D*, Simpson diversity index; *E*, Pielou's evenness index

Values are presented as the mean with the standard deviation in parenthesis

roots (92 %). This observation is in line with reports showing its wide distribution (Oehl et al. 2003; Öpik et al. 2006; Vallino et al. 2006) and its prevalence in the roots and nodules of various legumes (Scheublin et al. 2004). The predominance of few Glomeraceae species is probably a consequence of the strong selection pressure imposed by agricultural practices which lead to the predominance of fast root-colonizing species (Oehl et al. 2004b) and of AMF species able to tolerate the repeated disruption of external hyphal networks and the application of mineral fertilizers and pesticides (Gosling et al. 2006). Moreover, physiological conditions (flowering or fruiting) may also affect AMF diversity within roots.

It has nevertheless to be taken into account that the primers used in our study have a positive bias towards the Glomerales (Krüger et al. 2009; Kohout et al. 2014). However, that these primers have a certain level of selectivity and do not target all the fungi belonging to the Glomeromycota (Kohout et al. 2014) does not affect studies where differences between contrasting situation are analysed and also does not affect the sole diversity description of the whole Glomeromycota phylum. These primers have been successfully applied in many studies for the characterization of AMF associated with roots of different plant species with the cloning sequencing approach (van Tuinen et al. 1998; Gollotte et al. 2004; Stukenbrock and Rosendahl 2005; Farmer et al. 2007; Mummey and Rillig 2007) and, more recently, for the Next-Generation Sequencing approach (Lekberg et al. 2012; Holland et al. 2014).

Distribution of AMF types

The classic Jaccard index of similarity (J_{clas}) calculated in our study indicates that the composition of the AMF community in the rhizosphere soil of cowpea had a low similarity (40 % identity) to that found in roots. Chao's estimator for Chao's Jaccard Abundance-based similarity index ($\widehat{J}_{abd}$) between AMF community composition in rhizosphere soil and in cowpea roots was also moderate (46 % identity). Ellenberg (1956) stated that for plant communities belonging to the same association, the classic Jaccard index is usually between 0.25 and 0.5. Keeping in mind that we are studying fungal communities and not plant communities, a meaningful comparison is difficult and based on this index, it is not possible to conclude that the AMF communities in the rhizosphere soil and the roots were similar. However, the existence of a very high significant dependence between AMF OTUs and ecological systems (rhizosphere soil or cowpea roots) indicate that Glomeromycota species were not randomly distributed in rhizosphere soil and in cowpea roots—but instead they preferentially choose their ecological system. Hence, the studied cowpea cultivar (IT96D-610) may establish preferential symbiosis with some Glomeromycota species. The preference of fungal partners for some plants has previously been observed in various ecosystems, including agricultural systems (Scheublin et al. 2004; Cesaro et al. 2008). Similarly, the results of Bidartondo et al. (2002) indicate that some AM associations are specific, supporting the notion of functional diversity within Glomeromycota species. A semi-quantitative PCR analysis with specific primers could be used to confirm and quantify AMF abundance observed by cloning (Pivato et al. 2008).

This study is the first comprehensive assessment of AMF diversity in cowpea roots from the cowpea-growing region of West Africa. Overall results on the molecular diversity of AMF communities in rhizosphere soil and in cowpea roots from the studied fields suggest that preferential symbiosis between cowpea (*Vigna unguiculata*) and AMF may exist. Future investigations are needed to establish whether AMF OTUs preferentially associating with cowpea improve its growth, its tolerance to parasites and/or drought and its productivity. It is vital that soil scientists and agronomists pay more attention to AMF in order to increase, restore or manage soil fertility.

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