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MICROBIOLOGY

Microbial inoculation and fertilizer application on growth of cowpea and spore-based assemblages of arbuscular mycorrhizal fungi in its rhizophere

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Abstract: In this study, the effect of microbial inoculants and fertilizer application on cowpea (BRS Pujante) growth and on the structure and composition of arbuscular mycorrhizal fungi (AMF) assemblages were evaluated. A completely randomized experiment was set up involving 17 treatments: four with AMF, three with nodulating bacteria, six with AMF + nodulating bacteria, two with phosphorus, one with nitrogen and one control (reference) in five replicates. Plant growth and nutritional content, mycorrhizal colonization, glomerospores number, spore-based AMF assemblages and ecological indices were evaluated. Mycorrhizal inoculants associated with *Bradyrhizobium* BR3267 strain were more effective than the *Microvirga* BR3296 strain. Multidimensional scaling analysis showed that *Acaulospora longula* treatments were more similar among themselves, and distinct from the other treatments. A difference was observed in the structure of AMF community assemblage between treatments with *G. albida* + *Bradyrhizobium* BR 3267 and *A. longula*, with greater Shannon diversity and Pielou equitability indices in the first treatment and greater dominance in the treatment with *A. longula* only. Long-term studies are required to determine if the successful establishment of *A. longula* among indigenous species persists over time and if its dominant behavior is not deleterious to the AMF native community.

Key words: Ecology, Fabaceae, mycorrhizae, nodulating bacteria.

INTRODUCTION

Soils of arid and semi-arid regions generally have low fertility, which promotes a greater dependence of plants on microorganisms capable of mitigating environmental stresses (Kongpun et al. 2011, Oyewole et al. 2017). The use of microorganisms for the purpose of promoting greater availability of nutrients and water to plants is an important practice in agriculture (Basu et al. 2018, Igiehon & Babalola 2017, Oyewole et al. 2017). Among these microorganisms, nitrogen-fixing bacteria (NFB) and arbuscular mycorrhizal fungi (AMF) stand out for their importance in fundamental soil and crops processes.

Cowpea [*Vigna unguiculata* (L.) Walp (Fabaceae)] plants associate with soil microorganisms and are of significant agricultural importance in semi-arid areas of Brazil as a source of protein in the diet of local communities (Ballesteros-Almanza et al. 2010, Marinho et al. 2017). This leguminous plant is one of the most important in the world agricultural scenario, allowing Brazil to stand out as one of the largest producers and consumers of cowpea in the Latin American continent (FAO 2016). The Brazilian Northeast is the largest producer and

consumer of cowpea in the whole country, with an estimated production of 177.527 thousand tons (EMBRAPA 2016). Productions take place in rural areas, where cultivation plays an important socioeconomic role in family farming, specifically (Costa et al. 2013, Rego et al. 2015, Cruz et al. 2017).

Rhizobia induce the formation of nodules in leguminous roots, promoting biological fixation of N and making this macronutrient available to the plant. The use of rhizobia can reduce or even cancel N limitation in crops. This makes the use of N-fixing bacteria (NFB) an alternative for the application of nitrogen fertilizers, which, besides being costly, can harm the environment if handled inadequately (Carvalho & Moreira 2010, Moura et al. 2009).

Like rhizobia, arbuscular mycorrhizal fungi (AMF) are highly relevant to the functioning and maintenance of terrestrial ecosystems, forming symbiotic, mutualistic associations called arbuscular mycorrhizae with roots of most terrestrial plants (Basu et al. 2018, Bonfante & Genre 2010). In this association, the external mycelia absorb soil nutrients and transfer a portion to the plant, especially the elements that are limiting to plant growth such as nitrogen and phosphorus. In turn, fungi receive lipids and carbohydrates produced by the host plant (van der Heijden et al. 2015, Keymer et al. 2017, Rich et al. 2017). Co-inoculation with these different organisms (rhizobia and AMF) can lead to positive improvement in plant and soil conditions, justifying the search for efficient combinations of agricultural interest (Haro et al. 2018, Yasmeen et al. 2012).

The majority of reports test exotic isolates to select AMF species efficient in promoting plant growth, and few studies are carried out with indigenous AMF isolates. Among the works carried out with indigenous AMF isolates, we can highlight the study by Tchabi et al. (2010), who demonstrated that the majority of indigenous AMF isolates (14 of 27) promotes an increase in the weight of the yam tuber cultivated in West Africa. Although indigenous AMF isolates can be favorable to plant growth, their use is unusual due to the difficulty in isolating it from the field. Tchabi et al. (2009) demonstrated that some species are susceptible to isolation due to fast and abundant sporulation, *e.g. Claroideoglomus etunicatum* (= *Glomus etunicatum*) and *Acaulospora scrobiculata*. Thus, most studies still evaluate the efficiency of exotic AMF isolates in promoting the development and protection of plants against biotic and abiotic adversities.

On the other hand, the effects of introducing exotic microorganisms into the local microbial diversity is still a matter of concern (Antunes et al. 2009, Koch et al. 2011, Trabelsi & Mhamdi 2013), especially with respect to the composition of the AMF soil community. Negative ecological impacts on the mycorrhizal community might change the productivity of the ecosystem, including plant establishment and growth (Koch et al. 2011, Rillig 2004, Rodriguez & Sanders 2015, Sanders & Rodriguez 2016).

Changes in structure and composition of indigenous AMF communities due to the introduction of rhizobia have been rarely discussed (Omirou et al. 2016) and in general only the plant response to AMF and rhizobia co-inoculation has been considered (Haro et al. 2018, Yasmeen et al. 2012). Shifts in the mycorrhizal community as a function of AMF soil inoculation has received more attention (Antunes et al. 2009, Balota & Lopes 1996, Koch et al. 2011, Köhl et al. 2016). In these cases, the main goal has been to identify efficient mycorrhizal inoculants to improve plant growth without suppression of indigenous AMF species. Since inoculants are composed of living organisms, the introduction of exotic AMF in an environment could cause unexpected effects on the soil (Koch et al. 2011,

Pellegrino et al. 2012). Verbruggen et al. (2018) have addressed the importance of studying the interaction between AMF taxa as a way of predicting the composition and distribution of these communities. Therefore, it is important to perform studies on the effects of inoculation on the soil's indigenous microbiota, mainly the AMF, under controlled conditions prior to going into the field.

Our hypothesis is that introduced AMF species interact in a distinct way with indigenous AMF communities, resulting in differential growth of cowpea plants and changes in the microbial community structure. We evaluated the potential impact of different soil management on growth of cowpea and in the structure and composition of indigenous AMF communities under microcosm conditions, discussing the main ecological aspects related to the sporulating AMF community.

MATERIALS AND METHODS

Experimental conditions

The experiment was carried out in a greenhouse (9°19'31.7"S 40°33'34.5"W), at an average temperature of 32 °C and 37% relative humidity. After collection the soil was sifted, standardized and distributed in 2 kg black plastic bags. This soil was of the Flossic Neosol type, with the following characteristics: 21 mg/dm^3 P; 4.4; 1.7; 0.1; 0.0 cmol$_c$/dm^3 Ca, Mg, Na and Al, respectively, and a pH of 6.7.

The AMF isolates (*A. longula* URM-AMF 07, *C. etunicatum* URM-AMF 03 and *G. albida* URM-AMF 11) were provided by the Mycorrhizal Laboratory of the Department of Mycology at the Federal University of Pernambuco (UFPE). The inoculants were cultivated for three months in a greenhouse in 2 kg pots, using sterilized sand and soil (1:1 v/v) as substrate and corn (*Zea mays* L.) as the host plant. The AMF inoculation consisted of soil-inoculum with about 2,000 infective propagules per pot applied below the seeds. The number of AMF propagules was estimated in a bioassay according to Feldmann & Idczak (1994). We equalized the inoculum by the most probable number (MPN) of infective propagules because the species tested present different life strategies and differ in their infective propagules (Hart & Reader 2002). For each species we calculated the amount of inoculum to reach 2,000 infective propagules applied per pot, and it was: *Acaulospora longula* - 25 mL, *Claroideoglomus etunicatum* - 14 mL and *Gigaspora albida* - 12 mL.

The nodulating bacteria (*Bradyrhizobium* BR3267 and *Microvirga* BR3296) were supplied by Embrapa Agrobiology, in a peat substrate in a concentration of 10^9 cells/g. Inoculation of cowpea (BRS Pujante) seeds consisted of 10 g of peat per kg of moistened seeds.

In order to evaluate the effects of different inoculation practices on the AMF community we used: three AMF isolates (taxonomically different at the family level: *Acaulospora longula* (Acaulosporaceae), *Claroideoglomus etunicatum* (Entrophosporaceae) and *Gigaspora albida* (Gigasporaceae), either combined or not with two nodulating bacteria isolates specific for cowpea (*Bradyrhizobium* BR3267 and *Microvirga* BR3296); three treatments with fertilizers (using the recommended amount for the culture), and a control treatment (without application of inoculants and fertilizers).

The experimental design was completely randomized with 17 treatments: T1- inoculated with *A. longula* (AL), T2- inoculated with *C. etunicatum* (CE), T3- inoculated with *G. albida* (GA), T4- inoculated with a Mix of AMF (AL+CE+GA), T5- inoculated with *Bradyrhizobium* BR3267, T6- inoculated with *Microvirga* BR3296, T7- inoculated with a Mix of bacteria (BR3267+BR3296), T8- co-inoculated with *A. longula*+BR3267,

T9- co-inoculated with *A. longula*+BR3296, T10- co-inoculated with *C. etunicatum*+BR3267, T11- co-inoculated with *C. etunicatum*+BR3296, T12- co-inoculated with *G. albida*+BR3267, T13- co-inoculated with *G. albida*+BR3296, T14- 50% of amount of P recommended for the crop (0.0825 g superphosphate), T15- amount of N recommended for the crop (0.0225 g urea), T16- control (without inoculation and without fertilization), T17- amount of P recommended for the crop (0.165 g superphosphate), in five replicates.

Plant growth and nutrient analyses

Forty-five days after sowing, when the plants were in the blooming stage, bacterial nodules were harvested and quantified, and the following parameters were determined: a) height and stem diameter with the aid of a measuring tape and calipers, respectively; b) leaf area (the leaves were photographed and analyzed using the Quant 2002-2 program); c) shoot and root dry biomass (samples of plant material were dried in a convection oven at 70 °C for 72 h); d) levels of chlorophyll *a*, *b*, and total chlorophyll (determined *in vivo* using an electronic ClorofiLOG meter); e) P and N contents (plant material samples, after drying, were ground and digested in a mixture of sulfuric acid with oxygen peroxide). P was determined by colorimetry (Silva et al. 1999) and total N by distillation (Bremner & Mulvaney 1982).

Microbial analyses

The bacterial nodules were manually separated and counted per plant. For evaluation of mycorrhizal colonization, 0.5 g samples of roots from each treatment were washed with water, cleared in 10% (w/v) KOH, stained with 0.05% (v/v) trypan blue in lactoglycerol (Phillips & Hayman 1970 - modified) and analyzed by the intersection quadrant method (Giovannetti & Mosse 1980). Glomerospores were extracted from 50 g of soil via wet sieving (Gerdemann & Nicolson 1963), followed by centrifugation in water and sucrose (Jenkins 1964) and counted in a channeled slide using a stereomicroscope (40x). Later, the glomerospores extracted from native soil (initial sample) and treatments kept in pots were mounted on slides with PVLG (polyvinyl alcohol-lactic acid-glycerol) and/or with Melzer Reagent+PVLG and analyzed under a microscope. Identification manuals were consulted (Blaszkowski 2012, Schenck & Pérez 1990), in addition to the database of INVAM (International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi; http://invam.caf.wvu.edu) and publications with species descriptions. The following AMF species were identified in the fallow soil (before the experiment): *Acaulospora elegans*, *Acaulospora longula*, *Cetraspora pellucida*, *Claroideoglomus etunicatum*, *Dentiscutata cerradensis*, *Gigaspora margarita*, *Glomus* sp.1, *Glomus* sp.2 and *Septoglomus constrictum*.

Ecological indices

In order to analyze the AMF community structure, the following ecological indices were estimated: Shannon diversity (H'), Pielou equitability (J'), and dominance (C) per sample. The Shannon diversity index (H') was calculated according to the equation: $H' = -\Sigma (Pi \ln [Pi])$; where $Pi = ni/N$, ni = number of individuals of species i, and N = total number of individuals of all species (Shannon & Weaver 1949). The Pielou equitability index (J') was obtained by the equation: $J' = H'/Log (S)$ where H' is the value obtained by the Shannon index and S is the total number of species (Pielou 1975). The Simpson dominance index (C) was calculated by the equation $C = \Sigma (ni (ni - 1)/N(N - 1))$.

Data analysis

The data (growth, nutritional content, ecological indices and microbial analyses) were submitted to the Shapiro-Wilk normality test, and when they did not meet the normal distribution, they were transformed before the analysis of variance. Data were submitted to analysis of variance and means were compared by the Scott-Knott test ($p<0.05$). The analyses were performed using the Assistat program, version 7.7 beta (Silva & Azevedo 2016). Ecological indices were calculated using the Primer 6.0 program (Clarke & Gorley 2006). The community abundance data were relativized, and changes in the community were analyzed by multidimensional scaling (MDS), using the Sorensen distance, also with the Primer 6.0 program.

RESULTS

All treatments inoculated with AMF alone or mixed, and some with nodulating bacteria, co-inoculated (AMF + *Bradyrhizobium*), or fertilized had a larger leaf area (LA) than the control plants (Table I). Among the AMF, the co-inoculation of *A. longula* with nodulating bacteria did not improve the leaf area, but the treatments with *C. etunicatum* or *G. albida* benefited from co-inoculation with the BR3267 strain. Similarly, inoculation with the *Bradyrhizobium* BR 3267

Table I. Leaf area, P and N contents and N accumulation in the shoot of cowpea (BRS Pujante) plants inoculated with arbuscular mycorrhizal fungi and nodulating bacteria and fertilized, 45 days after sowing in a microcosm.

Treatments	Leaf area (cm^2)	P ($g\ kg^{-1}$)	N ($g\ kg^{-1}$)	N ($mg\ plant^{-1}$)
A. longula (AL)	814.63 a	1.73 c	41.24 b	129.37 a
C. etunicatum (CE)	794.65 a	1.97 b	45.76 a	141.3 a
G. albida (GA)	771.29 a	1.70 c	47.24 a	141.74 a
Mix AMF (AL+CE+GA)	765.39 a	1.57 c	41.80 b	140.75 a
Bradyrhizobium BR3267	834.07 a	1.90 b	43.64 a	140.48 a
Microvirga BR3296	731.01 b	1.79 c	41.50 b	131.88 a
Rhizobia Mix (BR3267 + BR3296)	805.98 a	1.76 c	41.78 b	138.86 a
AL longula+BR3267	707.09 b	1.72 c	39.84 b	135.17 a
AL longula+BR3296	668.74 b	2.02 b	43.20 a	146.78 a
CE etunicatum+BR3267	786.12 a	2.02 b	41.42 b	125.08 a
CE etunicaum+BR3296	721.06 b	1.93 b	44.08 a	142.29 a
GA albida+BR3267	776.77 a	1.94 b	43.78 a	131.62 a
GA albida+BR3296	743.66 b	1.84 c	43.48 a	132.86 a
P (50% of the recommended dose)	701.07 b	1.92 b	41.42 b	125.44 a
N (urea)	788.40 a	1.96 b	41.84 b	129.99 a
Control	684.85 b	2.03 b	44.54 a	131.31 a
P (100% of the recommended dose)	868.32 a	2.42 a	44.10 a	144.07 a
CV (%)	11.29	10.81	6.77	10.35

CV: Coefficient of variation; Means followed by the same letter in the column do not differ by the Scott-Knott test at the 5% probability level. P (50% of recommended dose to the crop - 0.0825 g); Control= Uninoculated and unfertilized; P= fertilized with P (100% of recommended dose to the crop - 0.165 g).**

strain alone promoted significant increase in leaf area of cowpea. The indigenous AMF community did not promote growth of leaf area compared to the control treatment.

The bacterial isolates differed in their effects on cowpea, with the *Bradyrhizobium* BR3267 strain performing better in terms of improving leaf area than the *Microvirga* BR3296 strain either alone or in presence of AMF inoculants (Table I).

The stem diameter (4.14 cm), height (37.5 cm), shoot dry biomass (3.16 g), the levels of chlorophyll *a* (37.1 ICF), *b* (17.8 ICF) and total chlorophyll (59.9 ICF) and the number of root nodules (75.4 nodules plant^{-1}) did not differ statistically ($p > 0.05$) among treatments (data not shown). Inoculation and fertilization did not increase the P content of the plants, except for the treatment with the highest amount of P (100% of the recommended dose - Table I), which increased the concentration of this element in the shoot. Accumulation of N (mg/plant) did not differ among treatments, and although the N content (g kg^{-1}) did vary, with some treatments showing higher values than others, statistically they did not differ from the control (Table I).

The highest rates of mycorrhizal colonization were observed in plants inoculated only with AMF or co-inoculated with *C. etunicatum* + BR3267 and *G. albida* + BR3267 (Figure 1a). Although mycorrhizal colonization rates were generally lower in the co-inoculated plants and in those associated with nodulating bacteria alone, they were significantly higher than the control (uninoculation and unfertilized) and the fertilized (P and N recommended for the crop) treatments.

The number of glomerospores in the soil was significantly lower in the treatments with nodulating bacteria and *C. etunicatum* singly (Figure 1b) compared to the control treatment (uninoculated and unfertilized); however, this

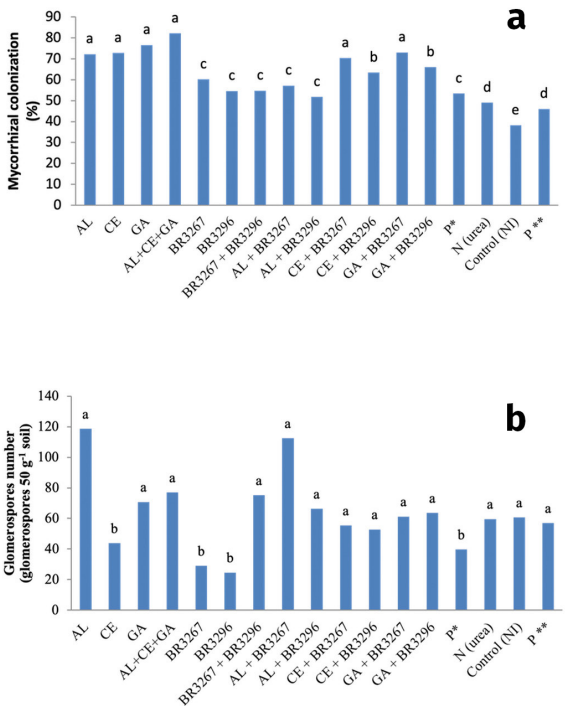

Figure 1. a) Percentage of mycorrhizal colonization in cowpea (BRS Pujante) plants inoculated with arbuscular mycorrhizal fungi and/or nodulating bacteria, or fertilized b) Number of glomerospores in soil with cowpea (BRS Pujante) plants inoculated with arbuscular mycorrhizal fungi and/or nodulating bacteria or fertilized.

was not observed when nodulating bacteria and AMF were co-inoculated.

Among the correlations (Table II) it was observed: 1) negative correlation between mycorrhizal colonization and soil P content; 2) positive correlation between mycorrhizal colonization and the shoot dry biomass/root dry biomass ratio; 3) positive correlation between P and N levels, indicating that the increase in P levels in the plant also favors the increase of N content and vice-versa.

After 45 days of cowpea cultivation in the microcosm, the initial AMF community changed: some treatments enabled sporulation, while others did not enhance sporulation of these fungi. Of the nine AMF species found in the initial soil (before growing cowpea), only *Glomus* sp. 1 and *Glomus* sp. 2 were found in all 17 treatments

Table II. Pearson correlation coefficient between the variables.

	LA	SD	PH	CL a	CL b	CL total	SDB	RDB	SDB/ RDB	P	N	MC	GLOM	NOD
LA														
SD	0.11^{ns}													
PH	0.20^{ns}	-0.05^{ns}												
CL a	0.00^{ns}	-0.05^{ns}	-0.16^{ns}											
CL b	-0.08^{ns}	-0.07 ns	-0.18^{ns}	0.90**										
CL total	-0.03^{ns}	-0.06^{ns}	-0.16^{ns}	0.84**	0.92**									
SDB	0.24 *	0.20^{ns}	0.24 *	0.12^{ns}	0.14^{ns}	0.13^{ns}								
RDB	0.07^{ns}	0.10^{ns}	-0.42 **	0.25 *	0.35 **	0.32 **	0.06^{ns}							
SDB/RDB	0.01^{ns}	-0.04^{ns}	0.46 **	-0.15^{ns}	-0.25 *	-0.23 *	0.26 *	-0.91 **						
P	0.14^{ns}	0.12^{ns}	0.10^{ns}	0.04^{ns}	-0.04^{ns}	-0.01^{ns}	-0.11^{ns}	-0.15 ^{ns}	0.07^{ns}					
N	0.08^{ns}	0.16^{ns}	-0.16^{ns}	-0.08^{ns}	-0.17^{ns}	-0.21 *	-0.16 ^{ns}	0.03 ^{ns}	-0.07^{ns}	0.33 **				
MC	0.16^{ns}	0.03^{ns}	0.04 ^{ns}	-0.12^{ns}	-0.17 ^{ns}	-0.18^{ns}	0.00 ^{ns}	-0.21 ^{ns}	0.24 *	-0.36 **	0.01 ^{ns}			
GLOM	0.01 ^{ns}	-0.10 ^{ns}	-0.03 ^{ns}	0.04 ^{ns}	-0.01^{ns}	0.05 ^{ns}	0.12 ^{ns}	0.19 ^{ns}	-0.09 ^{ns}	-0.18 ^{ns}	-0.05^{ns}	0.06^{ns}		
NOD	0.10 ^{ns}	0.14^{ns}	-0.02 ^{ns}	-0.11^{ns}	0.00 ^{ns}	0.03 ^{ns}	0.02^{ns}	0.17 ^{ns}	-0.12 ^{ns}	0.02^{ns}	-0.03 ^{ns}	-0.17^{ns}	0.10^{ns}	

** significant at the 1% probability level (p <0.01); * Significant at the 5% probability level (p <0.05); ns= not significant (p >0.05) LA: leaf area; SD: stem diameter; PH: plant height; CL a: chlorophyll a; CL b: chlorophyll b; CL total: total chlorophyll; SDB: shoot dry biomass; RDB: root dry biomass; MC: mycorrhizal colonization; GLOM: glomerospores; NOD: bacterial nodules.

and *C. pellucida* was in 14 of these treatments at the end of the experiment, while the other AMF were less frequent. Treatments with *A. longula* alone or combined with bacteria, and those with 50% of amount of P recommended for the crop had seven or six of the initial nine AMF species. In the other treatments the number of AMF species that remained until the end of the experiment decreased to five or four. In general, 29 AMF taxa (including those inoculated) belonging to 11 genera were identified at the end of the experiment (Table III). *Acaulospora* was the most representative with 13 species; *Gigaspora*, *Glomus*, and *Ambispora* were represented by four, three and two species, respectively; *Cetraspora*, *Claroideoglomus*, *Corymbiglomus*, *Dentiscutata*, *Entrophospora*, *Racocetra* and *Septoglomus* were represented by one species each (Table III). The greatest AMF richness (14 taxa distributed in seven genera) was recorded in the treatment inoculated with the AMF mix, while the lowest richness (six taxa in five genera) was observed in the co-inoculated treatment with GA + BR3296 (Table III). Considering only the co-inoculations, AMF richness was higher in the treatment with AMF + BR3267 than the one with AMF + BR3296.

The lowest values of AMF richness, P levels, mycorrhizal colonization and leaf area were recorded in treatments inoculated with the *Microvirga* BR 3296 strain. It was also observed that AMF richness was favored in the treatment with a half strength of P (13 taxa) unlike that observed at the higher P dosage (8 taxa) (Table III). Although richness was higher in the treatment with half strength of P, the AMF community structure, represented by diversity, abundance, and equitability did not differ among the fertilized treatments.

Acaulospora longula was the most abundant species among those recorded and produced more than half of the glomerospores extracted from the soil (Table III). Multidimensional scaling analysis (MDS) showed that the treatments inoculated with *A. longula* were more similar among them and distinct from the other treatments (Figure 2). Differences in AMF community structure were observed between treatments inoculated with *G. albida* + BR 3267 and *A. longula* ($p > 0.05$). Greater Shannon diversity and Pielou equitability indices were observed in the treatment with *G. albida* + BR 3267, while greater dominance was found in the treatment inoculated only with *A. longula* (Table IV).

DISCUSSION

Although differences among treatments for some variables (dry biomass, stem diameter, chlorophyll index, and number of root nodules) were not found, inoculation with AMF and the *Bradyrhizobium* BR 3267 strain promoted an increase in cowpea leaf area. This indicated that mycorrhizal inoculation alone or associated with a specific nodulating bacteria strain promoted an increase in this parameter in a similar way to that obtained with the application of fertilizers.

Differences in the benefits provided by AMF inoculation reported between this and other studies may be due to factors such as the use of indigenous AMF isolates, and the duration and conditions of the experiment. In our study, the experiment was harvested after the beginning of flowering (45 days) of the cultivar 'BRS pujante' while other studies were longer (Silva et al. 2018). Siqueira et al. (2003), for example, reported that inoculation with *Claroideoglomus etunicatum* (= *Glomus etunicatum*) produced a significant increase in leaf area and shoot dry biomass of cowpea plants. Silva et al. (2009) recorded a 33 to 148% increase in plant growth compared to uninoculated plants, testing 51 AMF isolates and observed that 95% of these isolates favored the

Table III. Relative abundance (%) of glomerospores from soil of the experimental microcosm treatments 45 days after inoculation with AMF and/or nodulating bacteria or fertilization with P or N.

Taxa of AMF	AL	CE	GA	AMF mix	BR3267	BR3296	Rhizobia Mix	AL + BR3267	AL + BR3296	CE + BR3267	CE + BR3296	GA + BR3267	GA + BR3296	P*	N	Control	P**
Acaulospora elegans	-	-	0.10	-	-	-	-	-	-	-	0.05	-	-	-	-	-	-
Acaulospora longula	11.80	-	0.10	1.52	0.05	-	-	6.71	6.96	-	-	-	-	0.10	-	-	-
Acaulospora mellea	-	-	0.10	-	-	-	0.05	-	-	-	-	-	-	0.10	-	-	-
Acaulospora morrowiae	-	-	0.10	-	-	-	0.05	0.39	-	-	-	-	-	0.24	-	-	-
Acaulospora reducta	0.05	-	0.10	-	-	-	-	-	-	-	-	-	-	0.05	-	-	-
Acaulospora scrobiculata	-	-	0.10	-	-	-	-	-	-	0.05	-	-	-	-	0.05	0.05	0.10
Acaulospora sp1	0.05	-	0.10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acaulospora sp2	-	-	0.10	-	-	-	-	-	-	-	-	-	-	-	-	0.05	-
Acaulospora sp3	-	0.05	0.05	0.05	-	-	-	-	-	-	-	-	-	-	-	0.05	-
Acaulospora sp4	-	-	-	0.10	-	-	-	0.10	0.05	-	-	0.06	-	-	-	0.10	-
Acaulospora sp5	-	0.05	-	-	0.05	-	-	0.10	-	0.05	-	-	-	0.05	-	-	-
Acaulospora sp6	-	-	-	0.05	-	0.10	-	0.05	-	0.05	0.05	-	-	-	0.15	-	0.05
Acaulospora sp7	-	-	0.05	0.05	-	-	-	-	-	-	-	-	-	-	-	-	-
Ambispora appendicula	-	0.05	-	-	-	-	0.05	-	-	-	-	-	-	-	-	-	-
Ambispora sp.	-	-	-	-	-	-	-	0.10	-	-	-	-	-	-	-	-	-
Cetraspora pellucida	0.20	-	0.10	0.05	0.10	0.05	0.05	0.25	0.05	0.05	-	0.12	-	0.05	0.05	0.05	0.15
Claroideoglomus etunicatum	0.05	0.44	-	0.05	-	-	-	-	-	0.49	0.54	-	-	-	-	-	-
Corymbiglomus tortuosum	-	-	-	0.05	0.05	-	-	-	-	-	-	-	-	-	-	-	-
Dentiscutata cerradensis	0.05	-	-	-	-	0.05	0.10	-	-	0.20	0.14	0.12	0.05	0.10	0.10	0.05	0.05
Entrophospora infrequens	-	-	-	-	-	-	0.05	-	-	-	-	0.06	-	0.05	-	0.05	-
Gigaspora albida	-	-	0.49	-	-	-	-	-	-	-	-	0.86	1.08	-	-	-	-
Gigaspora gigantea	0.24	0.15	-	0.15	0.10	0.10	0.34	-	0.05	0.05	0.39	-	-	0.10	0.05	0.25	-
Gigaspora margarita	0.05	0.05	-	-	-	-	-	0.15	0.05	-	-	-	-	-	0.05	-	-
Gigaspora sp.	-	0.10	-	0.10	0.05	0.05	-	0.10	0.15	0.25	0.10	-	-	-	0.10	-	-
Glomus sp1	0.34	2.11	1.27	1.81	1.37	1.18	4.12	1.86	1.27	2.55	2.89	2.08	2.11	2.16	3.97	1.37	2.01
Glomus sp2	0.10	0.78	1.57	1.57	1.18	0.49	1.37	2.60	1.03	1.27	1.08	1.22	2.40	1.32	2.01	1.71	1.71
Glomus sp3	-	-	0.05	0.05	-	-	-	-	-	-	-	-	-	-	-	-	-
Racocetra coralloidea	-	-	-	-	0.05	-	0.10	-	-	-	-	-	0.10	0.05	0.05	-	0.05
Septoglomus constrictum	-	0.05	-	0.10	-	-	-	0.10	0.05	0.15	-	0.49	0.05	0.05	-	0.15	0.39
Total species richness	10	10	7	14	9	7	10	12	9	11	8	8	6	13	10	11	8

Treatments: Inoculated with: *A. longula* (AL); *C. etunicatum* (CE); *G. albida* (GA); AMF mix (AL+CE+GA); BR3267; BR3296; rhizobia Mix(BR3267+BR3296); *A. longula*+BR3267 (AL+BR3267); *A. longula*+BR3296 (AL+BR3296); *C. etunicatum*+BR3267 (CE+BR3267); *C. etunicatum*+BR3296 (CE+BR3296); *G. albida*+BR3267 (GA+BR3267); *G. albida*+BR3296 (GA+BR3296); P*= fertilized with P (50% of recommended dose to the crop - 0.0825 g); N= fertilized with urea; Control= Uninoculated and unfertilized ; P= fertilized with P (100% of recommended dose to the crop - 0.165 g).**

absorption of P, with maximum efficiency of *Acaulospora* and *Glomus* species.

Since the germination speed of glomerospores and recognition events of the symbiosis may vary between AMF and plant species, the response provided by the mycorrhizal inoculation is affected by these factors. Another aspect that should be emphasized is that, in sterilized soils, the introduced AMF isolates can colonize the roots and establish a symbiosis without competitors in the soil while on unsterile soils the introduced AMF need to be more efficient than indigenous AMF. Moreover, the sterilization process can alter the soil properties, generating an artificial environment with favorable conditions to some AMF isolates, of which few may be adapted to the existing interactions in the rhizosphere or even be uncompetitive compared to indigenous AMF (Carvalho & Moreira 2010).

Among the inoculants tested, three treatments of co-inoculation with AMF isolates and the *Bradyrhizobium* BR3267 strain produced an increase in the leaf area of cowpea plants, indicating their potential to be applied under field conditions. The BR 3267 strain (*Bradyrhizobium yuanmingense*) was used in this study because it was isolated from the semi-arid region of Brazil and recommended by the Ministry of Agriculture, Livestock and Supply (MAPA), as a commercial inoculant (Brasil 2011). Studies confirm its symbiotic and agronomic efficiency in soils of this region in association with cowpea (Marinho et al. 2017, Martins et al. 2003, Sena et al. 2020, Xavier et al. 2017). The less obvious effects of inoculation with the *Microvirga* BR3296 strain together with AMF on cowpea plants demonstrate the importance of knowing efficient combinations (nodulating bacteria and AMF) for plants of interest since inadequate combinations may not promote the desired effects.

In the present study, no differentiated benefits were observed as a function of the AMF isolate on plant growth. However, Andrade et al. (2009) reported a greater increase in shoot dry biomass of cowpea (cultivar IPA 206) provided by *C. etunicatum* when compared to *Gigaspora*

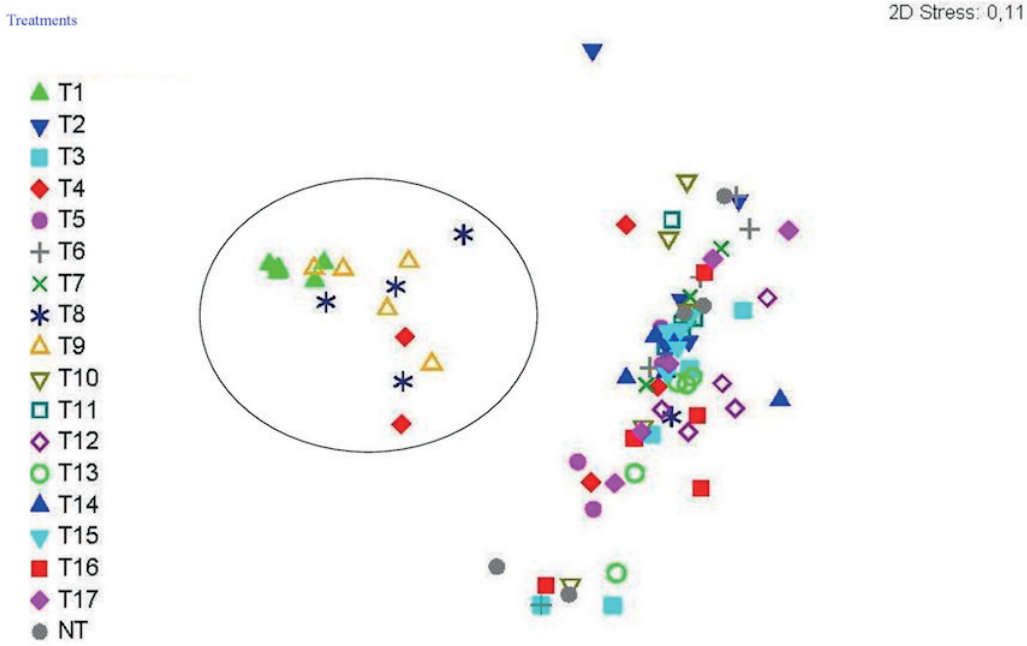

Figure 2. Multidimensional scaling (MDS) analysis of arbuscular mycorrhizal fungi (AMF) in the rhizosphere of cowpea from soil inoculated with AMF and/or nodulating bacteria 45 days after sowing in a microcosm.

albida, under unsterilized soil conditions. These authors did not provide quantitative data of AMF propagules in the 10 mL of soil-inoculum, and it is possible that differences on inoculum concentration has resulted in differences in the growth promoted by these two AMF species on cowpea plants. Other factors that should be considered for understanding the differences between ours and Andrade et al. (2009) results are the cultivars chosen (IPA 206 x BRS pujante), the low P content in the soil (2.7 mg/kg x 21 mg/dm^3) and the types of soils used, since the efficiency of the AMF is influenced by these factors.

Although nitrogen-fixing bacteria promote, in most cases, positive effects on growth of leguminous plants, this has not been the case for the *Microvirga* BR 3296 strain. Probably this strain is not efficient enough to compete against the diversity of bacteria found in tropical soils. Alcântara et al. (2014) reported that cowpea was more benefited by inoculation with *Bradyrhizobium* strains, presenting low specificity with other strains. This explains the better results for leaf area obtained with the *Bradyrhizobium* BRS 3267 strain, both inoculated alone and with AMF.

In general, AMF inoculation contributes to higher rates of mycorrhizal colonization (Cozzolino et al. 2013, Pellegrino et al. 2012). Mycorrhizal colonization rates higher than 50% in treatments with inoculation of AMF isolates may result from the joint establishment of native and introduced AMF. These values are similar to

Table IV. Shannon diversity, dominance and Pielou equitability indices for the AMF community in fallow soil and in soil inoculated with AMF and/or nodulating bacteria, 45 days after sowing in a microcosm.

Inoculation treatment	Shannon	Dominance	Pielou
A. longula (AL)	0.50 b	0.76 a	0.37 b
C. etunicatum (CE)	0.97 ab	0.43 ab	0.72ab
G. albida (GA)	0.86 ab	0.47 ab	0.82 ab
AMF mix (AL+CE+GA)	1.14 ab	0.37 ab	0.78 ab
Bradyrhizobium BR3267	1.03 ab	0.35 ab	0.81 ab
Microvirga BR3296	0.61 ab	0.63 ab	0.78 ab
Rhizobia mix (BR3267 + BR3296)	0.95 ab	0.48 ab	0.63 ab
AL + BR3267	1.02 ab	0.44 ab	0.66 ab
AL + BR3296	0.81 ab	0.56 ab	0.59 ab
CE + BR3267	1.09 ab	0.39 ab	0.76 ab
CE + BR3296	1.15 ab	0.36 ab	0.76 ab
GA + BR3267	1.36 a	0.26 b	0.84 a
GA + BR3296	1.07 ab	0.36 ab	0.82 ab
P* (50%)	1.21 ab	0.30 b	0.81 ab
N (urea)	0.96 ab	0.43 ab	0.78 ab
Control (NI)	1.10 ab	0.39 ab	0.74 ab
P** (100%)	0.86 ab	0.45 ab	0.75 ab

Treatments: Inoculated with: *A. longula* (AL); *C. etunicatum* (CE); *G. albida* (GA); AMF mix (AL+CE+GA); BR3267; BR3296; rhizobia Mix(BR3267+BR3296); *A. longula*+BR3267 (AL+BR3267); *A. longula*+BR3296 (AL+BR3296); *C. etunicatum*+BR3267 (CE+BR3267); *C. etunicatum*+BR3296 (CE+BR3296); *G. albida*+BR3267 (GA+BR3267); *G. albida*+BR3296 (GA+BR3296); P*= fertilized with P (50% of recommended dose to the crop - 0.0825 g); N= fertilized with urea; Control= Uninoculated and unfertilized ; P= fertilized with P (100% of recommended dose to the crop - 0.165 g).**

those observed by Haro et al. (2018) after 60 days of inoculation with AMF and *Bradyrhizobium* spp strains in cowpea (var. KVX 396-4-5-2D) in an experiment conducted in the field. Mycorrhizal colonization percentages may vary considerably as observed by Silva et al. (2018), which found amplitude from 1 to 82% in cowpea roots (cv. BRS 17 Gurgueia) due to AMF isolates and the soils types tested. Another aspect that should be considered is the phenological phase of the plant species, since in the study conducted by Johnson et al. (2016), it was found that mycorrhizal colonization in cowpea (cv. IT96D-610) was lower in the flowering period (13.6 to 70%) than fruiting (48 to 82%). These results confirm that cowpea is likely to be associated with AMF and reinforce that the variation in the percentage of mycorrhizal colonization can be influenced by several factors, from experimental conditions to cowpea cultivars and AMF isolates studied.

The lower rates recorded in the majority of co-inoculated plants compared to AMF inoculated plants indicate that establishment of a symbiosis affects the outcome of the other (Catford et al. 2003, Omirou et al. 2016). Low mycorrhizal colonization (mean of 15%) of bean plants (*Phaseolus*) was also observed by Ballesteros-Almanza et al. (2010) after co-inoculation with nodulating bacteria and *Rhizophagus intraradices* (= *Glomus intraradices*). Ordoñez et al. (2016) warned about the influence of bacteria (*Pseudomonas*) on intra- and extra-radicular colonization by AMF, considering that the occurrence of mycorrhizal symbiosis can be directly impacted by the inoculated bacteria. Root colonization is one of the first benefits the plant receives when associated with symbionts since the availability to sites for pathogen infection can decrease significantly (Denison & Kiers 2011).

Considering the correlation data, the lowest plant benefits resulted from lower mycorrhizal colonization. Positive correlations between the degree of colonization and AMF efficiency have been reported by Melloni et al. (2003) and Silva et al. (2009) in bean plants [*Phaseolus vulgaris* L. cv. Carioca and *Vigna unguiculata* (L.) Walp cv. Caupi, respectively], evidencing the importance of mycorrhizal symbiosis for the development and growth of these plants. In this way, phosphate fertilization of the soil should be parsimonious, because negative correlation was observed between soil P content and mycorrhizal colonization, demonstrating that mycorrhizal colonization is compromised as the availability of this nutrient increases in the soil. Similar result was obtained by Solaiman et al. (2019), who demonstrated that sources of biochar with higher P concentration reduced mycorrhizal colonization in wheat and subterranean clover roots. Although weak, positive correlation (0.33) was found between N and P in cowpea experiment. Both elements are essential for development of plants and some works showed that P fertilization increases N uptake (Graciano et al. 2006). This fact was corroborated by Jia et al. (2004), who found out that an adequate P level is essential for the accumulation of N in leaves of *Vicia faba* L. associated with AMF and nodulating bacteria.

Plants inoculated only with nodulating bacteria had higher percentages of mycorrhizal colonization than the control (uninoculated and unfertilized) plants. In this case, it is possible that the introduction of nodulating bacteria stimulated the indigenous mycorrhizal symbionts to colonize the roots. The low number of glomerospores (~ 25 glomerospores/50 g of soil) after the introduction of the nodulating bacteria strains suggests that the AMF passed from an asymbiotic condition to a symbiotic one, considering that mycorrhizal colonization in these treatments was higher than in control plants. Little is known about the factors that

shape the interactions between these two important groups of microorganisms and how they influence one another's growth and establishment (Catford et al. 2003, Omirou et al. 2016, Yasmeen et al. 2012). In general, these interactions are less studied compared with effects on plant growth and productivity (Cozzolino et al. 2013, Haro et al. 2018). The AMF community is influenced by different abiotic and biotic factors, which in turn interfere with survival and germination of infective propagules, thus altering the process of fungal colonization and its effects on the root system (Carrenho et al. 2010, Mohammad et al. 2003, Ordoñez et al. 2016, Rodriguez & Sanders 2015, Xu et al. 2017). Nodulating bacteria are also influenced by several factors, such as the availability of nutrients in the soil, pH, host plant, and use and management of the soil. In this study, strains of the *Bradyrhizobium* BRS 3267 promoted great benefit, alone or mixed with AMF, while *Microvirga* BR 3296 was not beneficial for the growth of cowpea and mycorrhizal colonization, suggesting a potential negative effect of this strain.

The management of soil with AMF inoculation might change the structure of community. In our study higher AMF richness was recorded with application of the AMF Mix, showing the importance of inoculation with a mixture of species to potentially increase the functional diversity of AMF in the soil (Maherali & Klironomos 2007). Fungal genotypes are ecologically distinct, which contributes to the maintenance of AMF diversity and this may provide greater resilience to the associated plants (Bever et al. 2001). In addition, the increase in AMF species richness is important because it affects plant community structure, improving its performance (Bever et al. 2001, van der Heijden et al. 1998).

Differences in the structure of AMF communities between treatments with inoculation of *G. albida* + BR 3267 and *A. longula* may be related to the life strategy of the fungal species. Gigasporales species are considered K-strategists, producing a small number of large spores (>200 mm), adapted to live in stable and predictable environments and investing in the formation of mycelial biomass outside the root system (Denison & Kiers 2011, Maherali & Klironomos 2007). The dominance of *A. longula,* confirming its successful establishment in the presence of indigenous AMF species, may be related to an intrinsic characteristic of the group, considered to be an r-strategist, which is the production of large number of small spores (Balota & Lopes 1996, Denison & Kiers 2011).

The abundance of *A. longula* glomerospores in treatments where this species was inoculated indicates fast establishment of symbiosis and consequent sporulation, mainly when inoculated alone. Although prevailing in the microcosm, treatments inoculated only with *A. longula* still allowed the detection of six of the eight indigenous AMF species identified in the initial characterization of the soil, suggesting that: i) competition between these species is weak (Martignoni et al. 2020) or ii) temporal analysis has demonstrated a niche of succession (Pacala & Rees 1998). If the competition among these species is weak, it is possible that, although in this treatment the diversity was reduced in comparison to the *G. albida* + BR3267 treatment, there may not be a loss of functionality. Maynard et al. (2017) suggested that communities with weak competitors may exhibit a positive relationship between diversity and functionality. On the other hand, the data on the AMF community after 45 days of introduction of the bioinoculants may indicate a succession niche, corroborated by the characterisation of *A. longula* as r-strategist, with rapid root

colonization and resource allocation for the formation of small glomerospores (< 90 µm diameter), characteristic of this species. This result serves as a call for long-term studies to determine if the successful establishment of this *Acaulospora* species among indigenous members persists over time and if its dominance is not deleterious to indigenous species. Studies on the dynamics of AMF populations are still scarce and can make a major contribution to understanding the ecology of AMF, as well as aiding the choice of fast-establishing isolates, especially for application in annual agricultural crops.

CONCLUSION

It was confirmed that the microbial isolates (nodulating bacteria and AMF) differ in their effects on growth and nutrient contents in cowpea plants, which in the present study were more beneficial with mycorrhizal inoculants associated with the *Bradyrhizobium* BR3267 strain compared to the *Microvirga* BR3296 strain. The structure of the sporulating AMF community differs as a function of the inoculants, and this can be observed even in a short period of time. In general, the isolate of *A. longula* was more effective at establishing symbiosis with cowpea plants than the other AMF species, but long-term studies are required to determine if the successful establishment of this species among indigenous species persists over time and if its dominance in the soil is not deleterious to the other AMF species. In addition, the introduction of exotic isolates into the environment should be evaluated with caution, especially when the species does not occur in the local community, which reinforce the importance of experimental studies in greenhouse conditions.

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AMYM, DKAS, IANL, LMVM and LCM designed the study. IANL performed the experiment and analysis. DKAS supported morphological taxonomy analysis. AMYM, DKAS and IANL conducted the statistical analysis and IANL coordinated writing of the paper with input from all authors.