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Prospecting of efficient rhizobia for peanut inoculation in a Planosol under different vegetation covers

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Peanut (*Arachis hypogaea* L.) is capable of establishing an efficient symbiosis with a wide range of rhizobia, nevertheless, the inoculation of this legume is under used due to lack of information on the bacteria naturally established in soils and the agronomic and economic viability of the practice. The aim of this study was to evaluate the diversity and the symbiotic efficiency of peanut rhizobia from a Planosol under different vegetation covers (native vegetation, sugarcane cultivation and fallow after sugarcane cultivation), located in Zona da Mata, Northeast of Brazil. A total of 177 isolates were obtained from peanut nodules, most of them having the characteristic of rapid growth and acidification of the Yeast Mannitol Agar (YMA) medium. The isolates obtained from soil samples with native vegetation cover showed higher relative abundance, higher species richness and greater uniformity of morphological groups of rhizobia. Eighteen isolates were evaluated for their ability to nodulate peanuts, of which 13 have been authenticated and submitted to evaluation of symbiotic efficiency in pots with soil. The isolate 23M showed the best symbiotic performance in pots with soil. In the field, plants inoculated with the isolate 23M showed better performance than non-inoculated plants, in addition to a grain yield (kg ha^{-1}) similar to that of plants inoculated with recommended bacteria or receiving 200 kg ha^{-1} N.

Key words: Bacteria of legume nodules, biological nitrogen fixation, diversity, grain legume, sugarcane field reform.

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is a legume of major global importance, being grown in Asia, Africa, North America and South America, where it originated (Sujay et al., 2012; Wang et al., 2012; Mokgehle et al., 2014; Pozzi et

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al., 2014). In the peanut production system, one of the factors that most influence yield is an appropriate management of fertilization, especially nitrogen. To obtain high yields, this crop removes a high amount of the nutrient from the soil, and accumulates it in its biomass (Ambrosano et al., 2013). Hence, practices that enable the nitrogen supply are essential for a rational cultivation of peanuts. Inoculation with N_2-fixing bacteria (rhizobia) is an economical and environmentally advantageous option to favor the supply of N to plants. The symbiosis of the peanut with these bacteria can promote an increased grain yield, in addition to being an alternative for the partial or total replacement of nitrogen fertilizers (Torres Júnior et al., 2014). However, in Brazil, the peanut inoculation is not widely used, mainly due to lack of technical information aimed at the characterization of the agronomic and economic viability of the practice. The reason for this is that the peanut has the ability to nodulate with a wide variety of native rhizobia, which often reduces the responses to inoculation (Borges et al., 2007; Marcondes et al., 2010). In this sense, obtaining bacteria with high efficiency in the combination with peanut plants may represent a strategy to increase the interest of producers for the adoption of the inoculation technique (Torres Júnior et al., 2014).

In recent years, prospecting of effective rhizobia for the inoculation of various legumes has been carried out in Brazilian soils, yielding promising results for calopo (Calheiros et al., 2013; Calheiros et al., 2015), velvet bean (Lima et al., 2012) and cowpea (Chagas Junior et al., 2010), among others. Nonetheless, few studies have evaluated the efficiency of peanut isolates (Hoffman et al., 2007; Torres Júnior et al., 2014; Sizenando et al., 2016).

The obtention of rhizobia strains efficient in biological nitrogen fixation (BNF) comprises different test conditions, making the bases for recommendation of strains, which is divided into four stages: 1) selection under controlled conditions in the laboratory; 2) selection under sterile conditions in Leonard jars; 3) selection in pots with unsterilized soil; and 4) selection under field conditions (Howieson and Dilworth, 2016). The obtention of the collection of native isolates and the pre-selection of effective bacteria, generate genetic resources for implementation of programs for the recommendation of new strains (Rufini et al., 2013).

Given these considerations, this work aimed to select promising isolates for peanut inoculation in the region of Zona da Mata, Pernambuco state, Brazil. The soils of this region are home to a great diversity of bacteria capable of nodulating this legume, including the possibility of yet unknown species (Lyra et al., 2013). Thus, it was initially carried out, the prospecting of rhizobia in a Planosol with three different vegetation covers, in order to obtain a collection of isolates adapted to the edaphoclimatic conditions of the region and to assess the diversity of naturally established populations. With the obtained isolates, successive experiments were performed under sterile conditions, in pots with soil and under field conditions to evaluate the symbiotic capacity of the rhizobia obtained.

MATERIALS AND METHODS

Capture of naturally established rhizobia in soil with three different vegetation covers

To obtain the nodules and isolate the bacteria, an experiment was conducted in a greenhouse growing peanut (*Arachis hypogaea* L.) in pots with a capacity of 4.5 kg of soil. Samples were collected in 0 to 20 cm layer of a Haplic Planosol (Embrapa, 2013), with three different vegetation covers: 1) sugarcane cultivation; 2) fallow after sugarcane cultivation; and 3) native vegetation (Atlantic Forest). The samples were collected in the municipaility of Itambé (7°26' 49" S and 35°14' 27"W, 179 m), located in the region of Zona da Mata, Pernambuco state, Brazil. The climate, according to Köppen classification, is tropical rainy As', with average annual rainfall of 1400 mm and temperature of 24.2°C.

The collected soil samples were air-dried, sieved (5 mm) and distributed in the pots. Subsamples were taken to determine the chemical and physical characteristics of the soil under each vegetation cover (Table 1), following the methods recommended by Embrapa (2009). The peanut seeds (cultivar BR-1) were surface-sterilized (ethanol 70%, for 30 seconds, and sodium hypochlorite, for 1 min) and then washed in sterile distilled water. Each pot received four seeds, leaving one plant per pot after 10 days. A completely randomized block design was adopted; the treatments consisted of three types of vegetation covers, with 7 replications each. Plants were harvested at 50 days after emergence. The roots were separated from the shoots and the nodules were detached and packed in tubes with silica gel for drying and conservation.

For the rhizobia isolation, 6 nodules of each plant were randomly selected. These nodules were rehydrated in autoclaved distilled water for 40 min, being surface-disinfected (70% ethanol, for 1 min, and 5% sodium hypochlorite, for three minutes) and then washed 10 times with sterile distilled water. The disinfected nodules were pressed into a Petri dish containing yeast mannitol agar medium (YMA, pH 6.8) (Vincent, 1970) with 25 mg kg^{-1} (w/v) of Congo red (Somasegaran and Hoben, 1994). The plates were incubated at 28°C until the appearance of bacterial colonies. For purification of isolates, the colonies were inoculated successive times in YMA medium containing bromothymol blue indicator, until the obtention of pure culture.

The following characteristics of the isolates were observed in YMA medium with bromothymol blue: time required for the appearance of isolated colonies (rapid: within three days; intermediate: four to five days; slow: six days or more); change in the pH of the medium after cell growth (acid, neutral or alkaline); colony size (< 1 mm, 1 to 2 mm and > 2 mm); transparency (translucent or opaque); elevation (flat or convex); color (white or yellow); shape and edge of the colony; appearance of the mucus of colonies (homogeneous or heterogeneous) and type of mucus, which was sorted as butyric or viscous (Vicent, 1970).

The characterization data were encoded in a binary matrix. With this, the simple matching coefficient was calculated and the clustering of strains was performed through NtsysPC program, using the UPGMA algorithm and the Jaccard similarity matrix. A dendrogram was made with the purpose of separating the isolates in groups with higher similarities (Rohlf, 2000). The results of the cluster analysis were used to calculate diversity (Shannon H), richness (Margalef), and evenness (Pielou) indices for the soils and species, where each morphological group, at 75% similarity, was considered as one operational taxonomic unit. The PAST

Table 1. Characteristics of the Planosol under different vegetation cover in the States of Pernambuco (PE), Brazil.

Soil characteristics	Vegetation cover		
	Native vegetation	Sugarcane	Fallow
pH (H_2O)	6.1	6.2	6.2
Ca^{2+}(cmolc dm^{-3})	6.8	1.65	1.5
Al^{3+}(cmolc dm^{-3})	0	0	0
Mg^{2+}(cmolc dm^{-3})	6.2	2.6	2.5
Na^{+}(mg dm^{-3})	0.002	0.001	0.003
K^{+}(mg dm^{-3})	0.010	0.023	0.022
P (mg dm^{-3})	1.08	0.04	4.30
Dg (kg.dm^{-3})	1.22	1.18	1.24
Sand (%)	70.44	52.58	87.56
Clay (%)	17.58	34.40	7.24
Silt (%)	11.99	13.03	5.20

pH in water (1:2.5); P, K and Na extracted by Mehlich-1; Ca, Mg and Al extracted by 1 mol L^{-1}. All determinations according to Embrapa (2009).

(palaeontological statistics) program was used to perform cluster analysis and diversity indices calculation (Hammer et al., 2001).

The ability of nodulate the original host was confirmed for two representative isolates of each of the phenotypic groups obtained, by cultivating peanut in monoxenic conditions, using Leonard jars (Vincent, 1970) containing sterile substrate (sand and vermiculite in a 1:1 (v/v) ratio). It was adopted a randomized complete block design with 20 treatments (18 isolates, a control inoculated with the strain SEMIA 6144, *Bradyrhizobium* sp., and an absolute control without inoculation) and three replications.

Before sowing, peanut seeds were surface-sterilized, as described in the step of obtention of nodules. To prepare the inoculants, the rhizobia isolates and the control strain were cultured in YM medium for a suitable period of growth for each bacterium. At the time of sowing, 1 mL of culture broth with approximately 10^9 viable cells was applied to each seed. After the fall of the cotyledon leaves, a nitrogen-free nutrient solution was applied weekly (Norris and T'Mannetje, 1964). Plants were harvested at 45 days after emergence, being assessed the nodulation ability of the inoculated isolates. The isolates that were able to form viable nodules (pink or red color in the interior, showing the presence of leghemoglobin) were authenticated as peanut rhizobia (bacteria capable of nodulating and fixing N in symbiosis with legumes).

Symbiotic efficiency of isolates in pots with soil

Three independent and simultaneous experiments were conducted under greenhouse conditions to evaluate the symbiotic efficiency of the authenticated peanut isolates, in Leonard jars with sterile substrate, as in the experiment described above. In each experiment, the isolates were tested, by cultivating peanuts in pots containing soil samples, collected in areas with one of the three types of vegetation cover (sugarcane cultivation, fallow and native vegetation) of the Planosol as described above.

A completely randomized block design was adopted, in which the effects of both the non-inoculation of peanut and the inoculations with different strains of rhizobia (the isolates obtained and authenticated as described above and the recommended strains BR426 and SEMIA 6144, applied alone) on the nodulation and shoot biomass production of peanut (cultivar BR-1) were compared with the effects of fertilization with nitrogen fertilizer (dose equivalent to 100 kg ha^{-1}). Nitrogen fertilization was provided in the form of urea in a single dose. All treatments received phosphate and potassium fertilization in the form of triple superphosphate and potassium chloride, respectively, according to the fertilization recommendation for the peanut crop in the state of Pernambuco (Ipa, 2008). The same procedures were adopted for disinfection of seeds, preparation and application of inoculants and supply of nutrient solution as described above.

Plants were harvested at 45 days after emergence, being evaluated; the nodulation (number and biomass of nodules) and the shoot biomass production. Statistical analyses were performed using the computer program Sisvar (Ferreira, 2008). The variables were submitted to analysis of variance by F test , where F was significant, the means were compared by Tukey test at 5% probability.

Symbiotic efficiency in the field

An experiment was conducted under field conditions in the area of fallow after sugarcane cultivation, to test the isolate that provided the best dry biomass yield in the experiments under controlled conditions. This area was chosen because the cultivation of legumes that are able to perform BNF during soil fallowing at the sugarcane field reform is interesting to restore the natural soil fertility (Ambrosano et al., 2013).

In addition to this isolate, treatments with inoculations strains SEMIA 6144 and BR 426 were used, plus two controls without inoculation, being one with nitrogen (200 kg ha^{-1} of urea in planting). The experimental design was randomized blocks with six replications, in plots of 2 m x 2 m. The peanut cultivar BR-1 was sown in rows spaced 0.5 m apart, with 8 to 10 plants per linear meter. The experiment was harvested 90 days after germination and at harvest, the dry biomass of shoots and the grain yield were evaluated.

Statistical analyses were performed using the computer program Sisvar (Ferreira, 2008), the variables were submitted to analysis of variance by F test where the F was significant, the means were compared by Tukey test at 5% probability.

RESULTS AND DISCUSSION

From the nodules collected in the capture experiment, 177 bacterial isolates were obtained from the soil

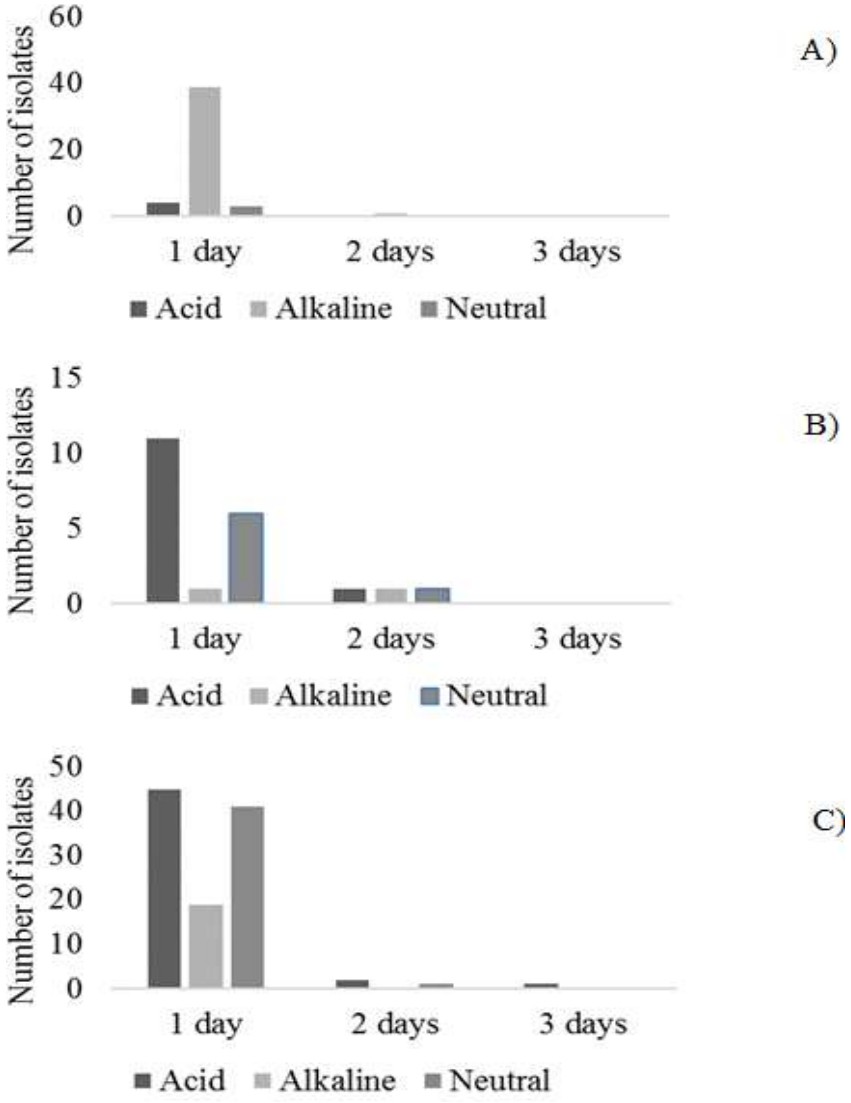

Figure 1. Number of bacterial nodule isolates from peanut grown in a Planosol under different vegetation covers.A= native vegetation, B = sugarcane and C= fallow.

samples of different vegetation covers, 47 of these from the area with native vegetation cover, 109 from the fallow area and 21 from the area with sugarcane cultivation.

Most studies report that peanut-nodulating rhizobia are belonging to the genus *Bradyrhizobium* (Steenkamp et al., 2008), which shows characteristics of slow growth and alkaline reaction in YMA culture medium (Wong et al., 1988). However, populations of isolates with rapid growth and acidification are apparently more common in soils from different regions of Brazil (Lyra et al., 2013; Torres Júnior et al., 2014). In contrast to these results, all isolates obtained in the Planosol showed rapid growth (up to three days in YMA culture medium with bromothymol blue incubated at 28°C), while majority (61%) presenting a metabolism which alkalizes the culture medium. This shows that the populations of peanut rhizobia naturally established in different soils, have variable characteristics according to the edaphoclimatic conditions to which they have adapted.

This type of original soil cover is conditioned to the differences in the pH reaction, of the culture medium of isolates. Among the isolates from the area covered with native vegetation, there was predominance of bacteria which promote alkaline reaction (Figure 1A). In the area with sugarcane cultivation, there was predominance of rhizobia of acid reaction (Figure 1B). Finally, in the fallow area, the proportion of acidifying, alkalizing and neutral bacteria were similar (Figure 1C). As for the size of the colonies, 75 isolates (42%) had colonies with a diameter less than 1 mm, 84 isolates (47%) showed colonies

Figure 2. Similarity dendrogram constructed from the cultural characteristics of rhizobia isolated from peanut grown in a Planosol under different vegetation covers in the States of Pernambuco (PE), Brazil.

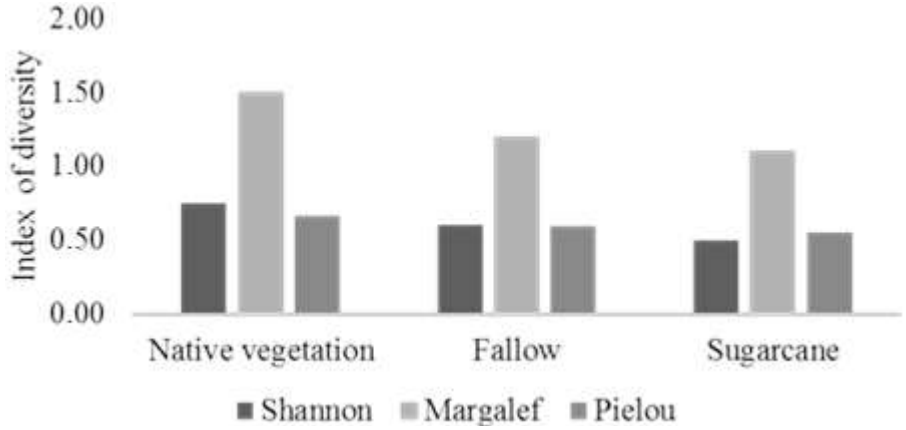

Figure 3. Shannon, Margalef and Pielou indices for bacterial nodule isolates from peanut grown in a Planosol under different vegetation covers.

between 1 and 2 mm, and 18 isolates (11%) had colonies with more than 2 mm diameter. All colonies showed white color, among which predominated the producers of medium and high amounts of exopolysaccharides.

The clustering of isolates from the characteristics thereof in the culture medium resulted in the formation of 9 groups with 75% similarity (Figure 2). This clustering based on phenotypic characterization is an important step in the process of selection of new isolates, as it enables a reduction in the number of bacteria, that will be evaluated in the process of authentication and symbiotic efficiency in sterile substrate. Furthermore, it makes possible to estimate the diversity of rhizobial populations in each studied area, from the indices calculation.

The type of soil cover conditioned differences, is in the diversity of bacterial populations of peanut nodules (Figure 3). According to the indices of Shannon (1948), Margalef (1958) and Pielou (1977), the populations naturally established in the area under native vegetation had greater diversity, richness and evenness of morphological groups in relation to the populations of the other two areas. The effects of changes in the land use

Table 2. Growth characteristics or authenticated bacterial nodule isolates from peanut grown in a Planosol under different vegetation covers.

Isolates	Vegetation cover	Growth time	pH reaction	Production of exopolysaccharides
6A1D	Fallow	Fast	Neutral	Medium
23M2A	Native vegetation	Fast	Neutral	Medium
16M1B	Native vegetation	Fast	Neutral	Medium
6M1E	Native vegetation	Fast	Alkaline	Medium
6A1C	Fallow	Fast	Neutral	Medium
16M1C	Native vegetation	Fast	Neutral	Medium
22M2B	Native vegetation	Fast	Neutral	Medium
23M2C	Native vegetation	Fast	Neutral	Medium
11C1A	Sugarcane	Fast	Acid	Medium
4A1F	Fallow	Fast	Acid	High
18C1C	Sugarcane	Fast	Acid	Medium
6A1A	Fallow	Fast	Neutral	Medium
23M	Native vegetation	Fast	Alkaline	Medium

system on populations of soil microorganisms are conditioned by the edaphoclimatic particularities of each environment. Contrary to the results found in this research, many studies point to the existence of populations with lower diversity in soils under native vegetation. For example, in the Amazon, the bacterial communities of grazing and agricultural areas showed greater diversity among different land use systems, even when considering different species of macrosymbionts (Jesus et al., 2009). In a study conducted in northeastern China, communities of soybean-nodulating bacteria had higher diversity in soils covered with grassland compared with those coming from fallowing soils, or making use of monoculture or crop rotation (Yan et al., 2014).

The lower diversity found in the area cultivated with sugarcane in the present study may be due to the large anthropic interference and the consequent process of degradation suffered by this ecosystem, with deforestation of the Atlantic Forest and cultivation of the sugarcane monoculture for several years. Despite the low diversity, the fact that some rhizobia groups persist in this ecosystem, as in others in a degraded state, is of fundamental importance, since the biological nitrogen fixation process mediated by these microorganisms can provide environmental resistance against an impact or stress, contributing to the resilience of degraded areas (Saturno et al., 2015).

Among the isolates tested in monoxenic conditions, 13 re-nodulated the peanut plants, confirming to be rhizobia. All of these isolates are fast growing and producing exopolysaccharides, with differences in relation to the pH change in YMA medium (Table 2) and high variability as regards its symbiotic efficiency in the peanut cultivated in pots with soil (Table 3). In general, the peanut plants showed better development, when grown in the soil collected in area under sugarcane cultivation, with an average 21% higher than the average biomass yield of the peanut cultivated in the soil of the Atlantic Forest and 25% higher than the average yield in the fallowing soil. The highest peanut yield in the soil with sugarcane production could be attributed to the considerable chemical fertilization normally used in the system, however there were no differences in the availability of major nutrients in the soil of this area in relation to others (Table 1). Fertilezers and lime represent an important secondary source of micronutrients for the soil and can be an important in the soil fertility (Carvalho et al., 2012). Possibly, the availability of some efficient nutrient absent in our parameters analysed, such as Mn, Zn, Cu and Fe, conditioned the highest yield of peanut grown in soil with sugarcane production.

The symbiotic performance of strains, assessed by the shoot biomass production of inoculated plants, varied according to the soil in which the peanut was grown. Bacteria from the soil covered with native plants were more efficient when inoculated in peanuts cultivated in the soil, collected in the same location (isolates 23M and 23M2A), and also grown in the soil collected in the area with sugarcane cultivation (16M1B). However, when the peanut was grown in soil cultivated with sugarcane, the best performance was obtained in plants inoculated with the isolate 11C1A, native of sugarcane area. Considering the overall average yield of each isolate in the three land use systems, it appears that the isolate 23M had the highest average shoot biomass yield (2.118 g plant^{-1}), which is approximately 10% higher than the control and received nitrogen fertilization which is a promising isolate for the production of inoculants.

Santos et al. (2005) evaluated the efficiency of peanut-nodulating rhizobia, isolated from soils in northeastern Brazil, and obtained a dry matter accumulation ranging from 1.54 to 2.59 g plant^{-1}, values which is close to the results of this study.Torres Júnior et al. (2014) evaluated the shoot biomass yield of peanut cultivars, inoculated

Table 3. Dry biomass productivity of peanut inoculated with diffetent isolates in a Planosol under different vegetation covers.

Inoculation	Covers vegetation			
	Native Vegetation	Sugarcane	Fallow	Overall average
	----------Dry biomass productivity t ha^{-1}----------			
Control	1.908^{ab}	2.108^{ab}	1.772^{ab}	1.929
Control with N	1.678^{b}	2.422^{ab}	1.65^{b}	1.917
6A1D	1.8a^{b}	2.182^{ab}	1.978^{ab}	1.987
23M2A	1.93^{ab}	2.567^{ab}	1.71^{ab}	2.069
16M1B	1.783^{ab}	2.254^{ab}	2.008^{a}	2.015
6M1E	1.663^{b}	1.99^{b}	1.777^{ab}	1.810
6A1C	1.848^{ab}	2.44^{ab}	1.745^{ab}	2.011
16M1C	1.888^{ab}	2.192^{ab}	1.91^{ab}	1.997
22M2B	2.024^{a}	2.534^{ab}	1.575^{b}	2.044
23M2C	1.865^{ab}	2.408^{ab}	1.725^{ab}	1.999
11C1A	1.827^{ab}	2.785^{a}	1.712^{ab}	2.108
4A1F	1.82^{ab}	2.232^{ab}	1.72^{ab}	1.924
18C1C	2.067^{a}	2.567^{ab}	1.622^{b}	2.085
6A1A	2.068^{a}	2.298^{ab}	1.555^{b}	1.974
23M	2.353^{a}	2.103^{ab}	1.898^{ab}	2.118
BR 426	1.468^{c}	2.098^{ab}	1.815^{ab}	1.794
SEMIA 6144	1.54^{b}	1.973^{b}	1.928^{ab}	1.928
Average	1.855	2.338	1.771	1.988

Means followed by the same letter do not differ significantly by the Tukey test at 5% probability.

with rhizobia isolates, which originated in the southeast of Brazil and, similar to our results found in a wide variation, in the efficiency of isolates. This has also been observed for rhizobia isolates, inoculated in other legumes (Lima et al., 2012; Calheiros et al., 2013). Such variability in symbiotic system which has the ability of collections of rhizobia, is a result of high biodiversity of these microorganisms, associated with biotic and abiotic factors. Studies have shown that the symbiotic association is affected by several factors such as soil acidity, aluminum toxicity (Campanharo et al., 2010), salinity (Medeiros et al., 2008), low soil fertility (Sulieman and Tran, 2015), phosphorus and molybdenum deficiency (Matoso and Kusdra, 2014), high soil temperatures (Ferrari et al., 1967) and low water availability in the soil (Andres et al., 2012). The use of microsymbionts adapted to different environmental conditions, combined with the correction of factors related to soil fertility, which may potentiate the effects of inoculation with diazotrophic bacteria.

Despite these promising results of some of the isolates obtained until the phase of screening tests in pots with unsterilized soil in the field test, only the strain BR 426 and the nitrogen treatment (200 kg ha^{-1}) showed shoot biomass production which is statistically higher than the control without inoculation and nitrogen fertilization. The plants inoculated with the test isolate (23M) did not differ statistically neither, from the treatment that received inoculation with the strain SEMIA 6144, nor from non-inoculated plants. According to Souza et al. (2008), the biomass accumulation is a strong indicator of the nutritional status of the plants and has been one of the variables used in studies for selecting rhizobia isolates with potential for BNF (Marcondes et al., 2010; Torres Júnior et al., 2014; Valetti et al., 2016).

However, the grain yield results revealed that, in the conditions under which the experiment was conducted, all inoculated treatments (with the recommended strains SEMIA 6144 and BR 426 and also with the isolate originated from the forest, 23M) were statistically superior than the control without inoculation and nitrogen fertilization. Moreover, the inoculation promoted the same grain production than the fertilization with 200 kg ha^{-1} urea. These results reveal that the test isolate (23M), when used as inoculant in peanuts, was able to increase grain yield by more than 780 kg ha^{-1} when compared to the treatment without inoculation and nitrogen fertilization (Table 4). Interestingly, this isolate features fastgrowth the alkaline metabolism in YMA medium; unusual features in bacteria of legume nodules which is different from the previously reported for peanut rhizobia in tropical soils (Lyra et al., 2013; Torres Júnior et al., 2014).

The practice of nitrogen fertilization has been increasingly used in production systems. The replacement

Table 4. Above groundbiomass and grain yield of peanut cv. BR 1, cultivated in a Planosol under fallow.

Treatment	Dry biomass productivity of aerial part (kg ha^{-1})	Productivity of grains (kg ha^{-1})
Control	509^{b}	1387^{b}
Urea 200 kg ha^{-1}	569^{a}	1802^{a}
Semia 6144	525^{ab}	1774^{a}
BR 426	588^{a}	1766^{a}
23 M	480^{b}	2180^{a}

Means followed by the same letter do not differ significantly by the Tukey test at 5% probability.

of chemical fertilizers by the use of efficient inoculants, contributes to the reduction of environmental impacts and production costs. Therefore, the lack of statistical difference between nitrogen control and test isolate (23M) suggests that this isolate can be used as an alternative to the use of nitrogen fertilizers in the northeast of Brazil.

Conclusions

Bacterial isolates of peanut nodules coming from soils of the Brazilian Zona da Mata have high morpho-physiological diversity. These isolate 23M showed a high potential for evaluation in network, testing for the recommendation of new strains for peanut inoculation.

Conflict of interest

The authors have not declared any conflict of interest.

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