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Molecular and symbiotic characterization of peanut bradyrhizobia from the semi-arid region of Brazil

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ABSTRACT

Peanut (*Arachis hypogaea* L.) is a legume crop native from South America and grown worldwide. This is well adapted to the unfavorable edaphoclimatic conditions found in Brazilian Semi-Arid region, where is cultivated mainly by smallholders. The selection of new rhizobial isolates is important to increase the rates of biological nitrogen fixation and yield. Furthermore, the diversity evaluation of new bacterial isolates may help us to understand the ecology of these groups in regions where little information are available. In this context, this study aimed to evaluate the phylogenetic relationships of peanut bradyrhizobia from Brazilian Semi-Arid soils by analysis of 16S rRNA, *rec*A, *nod*C and *nif*D gene sequences, as well as their symbiotic performance. A trap-host experiment was performed using two soil samples from the municipality of Barbalha, Ceará, Brazil, taken under peanut cropping and one soil sample from the municipality of Juazeiro, Bahia, Brazil, covered by the native *Arachis triseminata*. Seeds of peanut cv. BR1 were sown and the plants were grown until 52 days after emergence. The nodules were inoculated in YMA medium for isolation of slow-growing bacteria. The Box-PCR fingerprinting and the 16S rRNA gene sequences was evaluated for all bacteria and *rec*A, *nod*C and *nif*D were sequenced for the six selected isolates. Selected bacteria were evaluated according to their symbiotic characteristics in a greenhouse experiment. Ten slow-growing isolates were obtained and classified within the genus *Bradyrhizobium*, by the 16S rRNA sequence analysis and showed divergent Box-PCR profiles. All bacteria were clustered within the *B. japonicum* clade. The bacteria from Juazeiro were closely related to *B. yuanmingense*, and those from Barbalha showed similarity to *B. yuanmingense*, *B. kavangense* and *B. guangxiense*. The sequences of *rec*A showed partial congruence to the 16S rRNA gene analysis but the phylogeny trees based on the symbiotic genes were not related, indicating horizontal gene transfer. All bacteria were able to efficiently nodulate the peanut and the isolate ESA 83, pointed out regarding the nitrogen fixation ability and were selected for further analysis in non-sterile soils and field conditions.

1. Introduction

Peanut (*Arachis hypogaea* L.) is a legume from South America. This species is an important oilseed crop cultivated in several tropical countries for oil, culinary, food and pharmaceutical industry applications. According to FAOSTAT (2017), China is the main peanut producer in the world, with more than 1.6 million tons produced in 2014. Brazil is the 18th peanut producer, with the total production of more than 400 thousand tons at the same year, indicating an opportunity to increase the Brazilian yields according to the technology applied in the different crop systems in the country.

In Southeastern Brazil, mainly in São Paulo State, is located the major peanut production region, although the crop is grown in other Brazilian locations with quite different environmental conditions. The Northeastern is the second largest peanut producer and consumer region in the country (CONAB, 2017). In Brazilian northeast peanut

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growers are smallholders often located at the Semi-Arid environment, adopting the rain-fed agricultural systems (Santos et al., 2010). In these systems, the yields are frequently low due to scarce investment in technological resources such as fertilization (Sizenando et al., 2016).

Peanut plants have wide adaptation to several soil conditions, although pod yield is largely dependent on phosphorous and nitrogen sources. For N supply, the biological nitrogen fixation (BNF) is an economic and ecological alternative (Melo et al., 2016; Sizenando et al., 2016) that can be maximized by the use of inoculants containing efficient rhizobial strains. In this context, the use of efficient rhizobial inoculants can increase the peanut yield favoring crop management by smallholders (Sizenando et al., 2016; Valetti et al., 2016).

The *Bradyrhizobium elkanii* strain SEMIA 6144, native from Zimbabwe, is officially recommended for production of peanut rhizobial inoculants for more than 25 years in Brazil (Brasil, 2011). Despite of the longtime of official recommendation, the adoption of the inoculation technology for peanut by farmers is low, mainly due to the low efficiency of commercial inoculants in the field (Santos et al., 2017; Sizenando et al., 2016). Nevertheless, recent studies showed that the Brazilian Semi-Arid soils can harbor efficient *Bradyrhizobium* isolates for peanut (Sizenando et al., 2016), indicating the potential of those soils as sources of promising new rhizobia.

Peanut is a promiscuous crop nodulating with a wide range of rhizobia isolates affiliated to the *Rhizobium* (Taurian et al., 2006) and *Ensifer* (Yang et al., 2005) genera. However, the main peanut microsymbionts partner are those classified within the *Bradyrhizobium* genus (Chen et al., 2003; Yang et al., 2005). *Bradyrhizobium* is the type genus of the Bradyrhizobiaceae family, described in 1982 to receive the slow-growing strains formerly classified as *Rhizobium japonicum* (Jordan, 1982). In September 2017 there were 36 described and validated *Bradyrhizobium* species in the List of Prokaryotic Names with Standing in Nomenclature (LPSN, available at http://www.bacterio.net/bradyrhizobium.html) (LPSN, 2017). Among this species, *B. lablabi* (Chang et al., 2011), *B. arachidis* (Wang et al., 2013), *B. guangdongense* and *B. guangxiense* (Li et al., 2015) had their type strains isolated from peanut nodules in China, whilst *B. subterraneum* and *B. kavangense* type strains were isolated from peanut root nodules in Namibia (Grönemeyer et al., 2015a,b). In the last few years, new species of *Bradyrhizobium* have been described by means of the polyphasic characterization of Brazilian bacteria (da Silva et al., 2014; Delamuta et al., 2015; Michel et al., 2017; Zilli et al., 2014), but none of them were originated from peanut nodules or isolated in a Semi-Arid region.

The characterization of peanut bradyrhizobia applying a molecular approach with the concomitant evaluation of housekeeping and symbiotic gene sequences was recently carried out in countries such as China (Chen et al., 2016) and South Africa (Jaiswal et al., 2017), revealing a large diversity of bacterial isolates. Jaiswal et al. (2017) showed that the bacterial isolates evaluated from two different regions of South Africa were classified as *B. kavangense* (2), *B. stylosanthis* (2), *B. elkanii* (1), *B. manauense* (1) along with four bacterial isolates less close to *B. diazoefficiens*. Chen et al. (2016) showed that among 36 bacterial isolates, 11 of them were closely related to *B. japonicum* and other 21 to *B. guangxiense,* in addition to other four bacteria related to *B. iriomotense*. The evaluation of symbiotic genes sequences is important to indicate the range of hosts nodulated by the bradyrhizobial isolates due to the determination of their “symbiovar” affiliation (Hungria et al., 2015). In addition, the concomitant evaluation of the sequences of housekeeping and symbiotic genes lead to a better understanding of the taxonomic position and phylogenetic relationships of the bacterial isolates (Delamuta et al., 2012; Hungria et al., 2015).

Despite their importance, only the sequence analyses cannot indicate the bacterial symbiotic efficiency (Muñoz et al., 2011; Ribeiro et al., 2015). Recent reports showing the peanut *Bradyrhizobium* diversity based in gene sequences without results about the symbiotic performance of the isolates are available in the literature (Chen et al., 2016; Jaiswal et al., 2017). Moreover, studies reporting in more detailed the symbiotic characteristics of peanut rhizobia do not achieve a better understanding of their phylogenetic relationships (Santos et al., 2017; Torres-Júnior et al.,2014). Furthermore, despite the importance of peanut for the agricultural systems in the semi-arid region at the Brazilian northeast, there is little information regarding both phylogenetic diversity and symbiotic efficiency for peanut bradyrhizobial native for this region.

Due to the lack of knowledge in this field for the Brazilian Semi-arid region, this study aimed, for the first time, to evaluate the phylogenetic relationships of slow-growing peanut rhizobia from soils of the Semi-Arid region of Brazil, using housekeeping and symbiotic genes sequences analyses and assessing the symbiotic performance.

2. Materials and methods

2.1. Soil sampling for trap-host experiment and bacterial isolation

Composite samples (fifteen sub-samples) of the soils A horizon were collected at two locations in January, 2016. For each sampling site, three composite samples were collected and transported to the Laboratory of Soil Microbiology at Embrapa Semiárido (Embrapa Semi-Arid), Petrolina, PE, Brazil, and stored at 10 °C for one week until the experiment implementation.

The first soil sampling was carried out at the experimental field of Barbalha (07°13′50″S; 35°52′52″W), at the facilities of Embrapa Algodão (Embrapa Cotton), Barbalha, CE, Brazil. At this location samples were collected in two sites: at the first site, the soil was characterized as a Red Ultisol and, at the second site, about 2000 m away from the first, the soil was classified as a Haplic Vertisol. At both sites, the soils have been used for experiments with peanut (*A. hypogaea*) during summer (from December to March) and with cotton (*Gossypium hirsutum* L.) during winter (from July to September). At moment of the soil sampling, both sites were covered by non-inoculated peanut.

The second soil sample was taken in a Haplic Vertisol at the experimental field of Mandacaru (09°24′50″; 40°26′52″W), at the facilities of Embrapa Semiárido, located in Juazeiro, BA, Brazil. In this area, there was natural occurrence of wild peanut *Arachis triseminata* surrounding the irrigated crop experiments. Soil samples were collected from the sites where *A. triseminata* occurred. The samples from the soils used in the trap-host experiment where chemically analyzed and the results are shown in Table S1.

For the trap-host experiment, the soil samples were manually crushed, sieved (5 mm), homogenized and used to fill 500 mL polystyrene pots in three repetitions. Peanut seeds (cv. BR1) were previously disinfected with 96° GL ethanol for 30 s, 3% (v/v) NaClO for ten minutes, followed by ten washes in distilled autoclaved water (DAW). Four seeds were sowed per pot and seven days after emergence (DAE) the spare plants were thinned and one plant per pot was left. The pots were irrigated with 50 mL of distilled water on every morning. The plants were collected at 52 DAE, separating the roots and shoots. The roots were carefully washed with tap water. The nodules were detached and immediately used for rhizobial isolation.

For the isolation, three to four nodules were selected from each plant (Chen et al., 2014). The nodules were surface disinfected with 96° GL ethanol for 30 s, NaClO (2,5% v/v) for 5 min and followed by ten washes DAW (Vincent, 1970). The nodules were crushed in Petri dishes containing YMA medium with congo red. The plates were incubated in a growth chamber in the dark, at 28 °C for ten days, and the rise of slow-growing colonies was monitored daily. The colonies with typical characteristics of *Bradyrhizobium*, were transferred to YMA medium with bromothymol blue and incubated as described above. Successive inoculations in the same medium were carried out until obtaining pure cultures. Each bacterial isolate was stored in liquid YM medium supplied with glycerol (25% v/v) at −80 °C in the Culture Collection of Micro-Organisms of Agricultural Interests of Embrapa Semiárido (CMISA).