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Genetic diversity and symbiotic efficiency of rhizobial strains isolated from nodules of peanut (*Arachis hypogaea* L.) in Senegal

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1. Introduction

Cultivated peanut (*Arachis hypogaea* L.) was domesticated in South America. The value of the crop was quickly perceived and it has been successfully adapted to many other tropical and subtropical zones worldwide (Hammons, 1994). Today peanut is cultivated on approximately 25 million hectares from which 45 million tons are harvested annually (FAOSTAT, 2014) making peanut one of the most cultivated grain legumes and a crucial source of protein, oil and revenue. In Senegal peanut cultivation started in the 1850s. Since the 1960s, peanut is cultivated as an industrial export-oriented crop, representing up to 80% of Senegalese exports and providing the majority of cash income for rural populations (Sene et al., 2010; Noba et al., 2014).

Like many other legumes, *A. hypogaea* is able to establish a mutualistic symbiotic association with soil bacteria collectively known as rhizobia. Rhizobia form symbioses with legumes and fix atmospheric nitrogen, converting it into a form that can be assimilated by plants (Mylona et al., 1995). Grain legumes generally assimilate around 80% of their nitrogen from the air, but peanut only acquires by this way about 55% of its total nitrogen requirement (Hardarson, 1993). In addition, fixation efficiency is highly variable between peanut genotypes, rhizobia strains, and environmental conditions (Hardy et al., 1968; Stalker, 1991; Stalker et al., 1994; Mokgehle et al., 2014). This variability is certainly linked to the intrinsic characteristics of each partner and to the capacity of host plants to recognize and retain the best (Frederickson, 2013; Ibáñez et al., 2016, 2015), but could also reflect a complex evolutionary history of hosts and symbionts including horizontal transfer of symbiotic genes that created genotypes adapted to particular geographic zones or hosts (Laguerre et al., 2001; Muñoz et al., 2011).

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Although peanut has long been adapted to the Senegalese agro-systems, little is known about the native symbiotic components that allow for biological nitrogen fixation (Sene et al., 2010). *A. hypogaea* forms effective nodules with *Bradyrhizobium* sp. (Urtz and Elkan, 1996; El-Akhal et al., 2008; Ngo Nkot et al., 2008; Chen et al., 2014), but data on nitrogen fixation efficiency and taxonomic diversity of rhizobial strains in peanut fields in Senegal are scarce (Sene et al., 2010). Thus, understanding the process that favoured peanut adaptation in Senegal by mining the extent of the diversity of the native strains found in peanut nodules and their efficiency in fixing atmospheric nitrogen is an important step to identify elite strains that can be used as potential inoculants to improve peanut productivity in Senegal through BNF.

In this work, we studied the phenotypic and genetic diversity of rhizobia isolated from *A. hypogaea* grown in different agroecological zones in Senegal and compared these strains with efficient strains from Argentina and Zimbabwe.

2. Material and methods

2.1. Sampling sites

This study focused on three agro-ecological zones of Senegal (Fig. S1): the Coastal zone, the Sylvopastoral area and the Groundnut basin. The Coastal zone is located in the western part of the country and runs along the Atlantic coast. Fruits and vegetables are produced in this region during the dry season. The average rainfall rarely exceeds 1000 mm. The Sylvopastoral area is located in the northern part of the country and corresponds to a Sahelian climate with a rainfall generally below 600 mm. The Groundnut basin is the main peanut growing area and is mainly located in the Sahelian Sudanese and Sudanian climatic domain with a mean rainfall between 500 and 1200 mm.

2.2. Soil sampling

Sixty-eight soil samples were collected from 53 different locations during the dry season. Soil have been sampled to a 20-cm depth from fields which have no history of peanut inoculation.

2.3. Plant material

Experiments were conducted using the Fleur11 cultivar of *Arachis hypogaea* L. Ssp. Fastigata. Fleur11 is an improved cultivar, Spanish type, high yielding with short cycle (90 days). The cultivar is one of the parents of several interspecific (cultivated x wild) populations and widely grown in the Senegalese Groundnut basin and other West-African countries. (Clavel et al., 2005; Fonceka et al., 2012; Nguepjop et al., 2016).

2.4. Bacterial strains

2.4.1. Reference strains

Three strains able to nodulate peanut: *Bradyrhizobium* sp. SEMIA6144 which was isolated in Zimbabwe (Germano et al., 2006) and two efficient strains isolated in Argentina: *Bradyrhizobium* sp. CH81 and *Bradyrhizobium* sp. LH237 (Muñoz et al., 2011; Valetti et al., 2016)

2.4.2. Trapping experiment to isolate indigenous strains

A. hypogaea seeds were sterilized with 1.81% calcium hypochlorite for 8 min, washed five times with sterile distilled water and germinated in the dark for 72 h at 28 °C in Petri dishes containing 0.9% agar. The seedlings were sown in 11-cm diameter pots by containing 1 kg of each of the sampled soils and one seedling was grown per pot. The pots were placed in a shade house in a completely randomized block design with three replicates. Plants were watered daily.

Plants were harvested at 40 days after planting. Five nodules were randomly collected per plant and bacteria were isolated from nodules on YEM medium supplied with 0.002% Bromothymol blue as described by Andrés et al. (1997). Isolates were stored in 20% glycerol at −80 °C since 1990.

2.4.3. Infectivity of bacterial strains

The ability of the isolated strains to induce nodule formation in *A. hypogaea* was first tested in semi-axenic conditions as follows: seeds were surface sterilized and pre-germinated as described above. After germination, seedlings were transferred to glass tubes containing a sterile nitrogen-free Jensen solution (Jensen, 1942) as described by Vincent, (1970). Bacterial cultures were grown at 28 °C in YM (yeast extract and mannitol) liquid medium for 2–5 days until absorbance measured at 600 nm reached 0.6. The bacterial cultures were centrifuged at 6,480 × *g* for 5 min at 10 °C and resuspended in phosphate-buffered saline buffer (Sambrook and Russell, 2001). For 4 mL of bacterial cultures, the centrifuged bacterial cells are resuspended in 4 mL of saline buffer. In each tube, 1 mL of bacterial suspension (corresponding approximatively to 10^8 bacterial cells) was used to inoculate seedling radicles. Four replicates were done for each strain. The experiment was carried out in a growth chamber at 28 °C with a 16 h photoperiod at 54 µmol m^{-2} s^{-1}. Plants were harvested 28 days after planting and the number of nodules was visually scored. Non-inoculated plants were used as negative controls.

2.4.4. Molecular analysis

Total genomic DNA was isolated from 1 mL of a bacterial culture using the blood genomic-Prep Mini Spin Kit (GE Healthcare). The intergenic spacer (IGS), corresponding to the region between 16S and 23S rDNA and the symbiotic gene *nodC* were amplified using the primers listed in supplemental Table S6. PCR was performed in an Applied Biosystems 2720 thermocycler using the following program for both the IGS and *nodC* gene: an initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final elongation step at 72 °C for 7 min.

Samples were sequenced by Genoscreen (Lille, France). Nucleotide sequences were edited manually using the software SeqMan II version 8.0 (DNASTAR, Wisconsin, WI, USA). The closest sequences were retrieved from GenBank using a BLASTN search (Altschul et al., 1990). IGS sequences corresponding to strains isolated from *Arachis* were also retrieved from GenBank using a keyword search. The IGS sequences described in Grönemeyer et al., 2016 and Muöoz et al., 2011 were also included. Maximum likelihood phylogenies were constructed using the phylogeny.fr platform (Dereeper et al., 2008) applying the default settings, except for GBLOCKS, which was configured to be less stringent. The bootstrap support for each node was evaluated with 500 replicates. All sequences were submitted to NCBI and accessions number are available in Table S7.

2.5. Setting up an experimental system to evaluate symbiotic phenotypes

To assess the symbiotic phenotype of peanut symbionts a growth substrate was obtained by wet-sieving (2 mm) a Loam in three changes of water each of 2.4 L as to prevent inhibition of nodulation by excess soil Nitrogen (Streeter, 1985). The locally available commercial substrate that we used in this study contained 65% of organic matter and chemical fertilizers. Peanut seeds were surface sterilized and germinated as described above. After germination, the seedlings were transferred to 800 mL pots containing 200 g of leached-out loam sterilized at 120 °C for 20 min. The pots were previously disinfected with a solution containing 1.81% of calcium hypochlorite. A total of 45 plants were transplanted and 39 young plants were inoculated three days after seedling transplantating by adding 5 mL of a SEMIA6144 suspension prepared as above. Six young non-inoculated plants were used as negative controls. The plants were cultivated in a shade house and watered every two days. We used a completely randomized design, with 3 repetitions per treatment (inoculated and uninoculated). From the 4th