Applied Soil Ecology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Bacteria related to *Bradyrhizobium yuanmingense* from Ghana are effective groundnut micro-symbionts

Ophelia Osei[a], Robert C. Abaidoo[b,c], Benjamin D.K. Ahiabor[d], Robert M. Boddey[e], Luc F.M. Rouws[e,*]

[a] Department of Crop and Soil Sciences, Faculty of Agriculture, Kwame Nkrumah University of Science and Technology, PMB, Kumasi, Ghana
[b] Department of Theoretical and Applied Biology, Kwame Nkrumah University of Science and Technology, PMB, Kumasi, Ghana
[c] International Institute of Tropical Agriculture, PMB 5320, Ibadan, Nigeria
[d] CSIR, Savannah Agricultural Research Institute, P.O. Box 52, Tamale, Ghana
[e] Embrapa Agrobiologia, Rodovia BR 465 km 07, Seropédica, Rio de Janeiro 23891-000, Brazil

ARTICLE INFO

Keywords:
Native isolates
Biological nitrogen fixation
Genetic diversity
Arachis hypogaea L.

ABSTRACT

The identification of locally-adapted rhizobia for effective inoculation of grain legumes in Africa's semiarid regions is strategic for developing and optimizing cheap nitrogen fixation technologies for smallholder farmers. This study was aimed at selecting and characterising effective native rhizobia, from Ghanaian soils for groundnut (*Arachis hypogaea* L.) inoculation. From surface-disinfected root nodules of cowpea and groundnut plants grown on farmers' fields, 150 bacterial isolates were obtained, 30 of which were eventually found to nodulate groundnut plants. After testing the symbiotic potential of these isolates on groundnut on sterilized substrate, seven of them, designated as KNUST 1001–1007, were evaluated in an open field pot experiment using ^{15}N-labelled soil. Although ^{15}N dilution analyses did not indicate differences among treatments in the proportion of nitrogen (N) derived from the atmosphere (%Ndfa), all seven strains increased total N derived from N_2 fixation by inoculated groundnut plants as compared to the non-inoculated control. Inoculation with KNUST 1002 led to total N accumulation as high as that of the groundnut reference strain 32H1. Genetic characterisation of the isolates by sequence analysis of 16S rRNA gene, 16S – 23S rRNA intergenic transcribed spacer (ITS) region and *nodC* gene revealed that isolates KNUST 1003 and 1007 were related to *Rhizobium tropici*, a common bean symbiont. The other five isolates, including KNUST 1002 belonged to the *Bradyrhizobium* genus, being closely related to *Bradyrhizobium yuanmingense*. Therefore, this study revealed novel native Ghanaian rhizobia with potential for the development of groundnut inoculants.

1. Introduction

Groundnut (*Arachis hypogaea* L.) is a multipurpose grain legume, which is considered a nutritious component in diets and a source of income for smallholder farmers in developing countries (Carlberg, 2012). In Ghana, about 94% of the groundnut production is concentrated in the northern region; a place considered as one of West Africa's main groundnut production areas (Tsigbey et al., 2003). In terms of symbiotic nitrogen fixation, groundnut has been found to form effective association with both fast and slow growing 'rhizobia' of the *Rhizobium* and *Bradyrhizobium* genera, respectively (Taurian et al., 2002). Among the *Bradyrhizobium* strains identified to nodulate groundnut are: *Bradyrhizobium arachidis, Bradyrhizobium japonicum, Bradyrhizobium elkanii, Bradyrhizobium lablabi, Bradyrhizobium yuanmingense and Bradyrhizobium iriomotense* (Taurian et al., 2006; El-Akhal et al., 2008; Chang et al., 2011; Muñoz et al., 2011; Wang et al., 2013). Other species that nodulate groundnut include *Rhizobium gardinii* and *Rhizobium tropici* (Taurian et al., 2006). Despite the nitrogen-fixing ability of groundnut, yields are often below their maximum potential (Nutsugah et al., 2007). These low yields have been partially attributed to low inherent soil fertility and nutrient deficiencies in N and P mostly limit productivity of this crop (Maheswar and Sathiyavani, 2012; Mohamed and Abdalla, 2013).

Options such as mineral nitrogen application and rhizobium inoculation have been considered as means to supply legumes with N (Mweetwa et al., 2014). Apart from the possible adverse environmental consequences of excessive mineral nitrogen application (Trindade et al., 2001; Flechard et al., 2007), farmers are unable to exploit this option due to financial constraints. Thus, the more feasible alternative is the use of rhizobium inoculants. The practice of inoculation with highly

* Corresponding author.
E-mail address: luc.rouws@embrapa.br (L.F.M. Rouws).

https://doi.org/10.1016/j.apsoil.2018.03.003
Received 10 January 2018; Received in revised form 26 February 2018; Accepted 2 March 2018

effective rhizobium strains has been identified, among other factors, as an essential means to promote biological nitrogen fixation (BNF) with subsequent increases in grain yields (Unkovich and Pate, 2000). The usefulness of this BNF process is made evident when legumes depending on atmospheric N_2 produce increased yields in soils in which non-legume crops would require a substantial amount of mineral nitrogen. In addition, inoculation of groundnut has led to considerable increases in nodulation, growth and productivity (Sajid et al., 2010; Sharma et al., 2011; Mohamed and Abdalla, 2013). Despite the potential benefits of inoculation, farmers rarely apply inoculants to groundnut because of the consensus that the association between groundnut and native soil rhizobia is usually adequate. Another factor that could contribute to the limited use of inoculants by farmers is the low awareness of the higher economic returns from the use of inoculants relative to mineral nitrogen (Ndakidemi et al., 2006). Limited availability of groundnut inoculants (i.e. of exotic origin) and lack of local strains for inoculating the crop particularly in Ghana, further exacerbate the limited use of inoculants.

To improve inoculation response of tropical legumes, Nkot et al. (2008) suggested the use of indigenous rhizobia as inoculants. For example, improvement in nodulation and N_2 fixation was reported when groundnut was inoculated with native rhizobia (Bogino et al., 2008). Shishido and Pepper (1990) and Sattar et al. (1995) suggested that strains isolated from a particular region are the most effective for a given crop in that same region. In addition, rhizobia that are moderately to highly effective have been found to be well represented among the native population (Herridge et al., 2008) and could serve as a source of elite strains for local inoculant production. This emphasizes the need to identify elite isolates adapted to the prevailing environmental conditions for improved BNF. In selecting rhizobia strains for use as inoculants, the characteristics competitiveness in nodule formation and effectiveness in nitrogen fixation are considered (Stephens and Rask, 2000).

Conversely, the symbiotic potential and genetic diversity of groundnut-nodulating rhizobia is yet to be investigated, particularly in the context of Ghanaian agriculture. Previous reports based on the analyses of 16S rRNA and RFLP revealed a large diversity within cowpea- and soybean-nodulating strains only at the genus level (Abaidoo et al., 2000; Fening et al., 2004). Therefore, it is imperative to assess the diversity within the native rhizobium populations that nodulate groundnut and to estimate their contribution to N_2 fixation in grain legumes. The aim of this study was to characterise rhizobia capable of nodulating groundnut using molecular tools and to identify elite strains for groundnut inoculation. To this end, symbiotic potential and phenotypic tests in addition to sequence analyses of 16S rRNA gene, 16S – 23S rRNA intergenic transcribed spacer (ITS) region and symbiotic genes; *nodC* and *nifH* were carried out to reveal the diversity within groundnut nodulating rhizobium and identify elite strains for improved inoculation response.

2. Materials and methods

2.1. Recovery and authentication of Rhizobium isolates

Groundnut and cowpea nodules were collected from farmers' fields across the three regions in northern Ghana at the flowering stage and sampling points were located using a GPS (Supplementary Fig. S1). Recovered nodules were kept on desiccated silica gel and transported to the microbiology laboratory, Kwame Nkrumah University of Science and technology (KNUST) in Kumasi, Ghana, for isolation. Dried nodules were rehydrated in sterile distilled water overnight. After rehydration, whole nodules were surface sterilised using 95% ethanol for 10 s and transferred into a 3% hydrogen peroxide solution for 3 min. The nodules were then rinsed in several changes of sterilised distilled water to remove the remaining hydrogen peroxide as described by Somasegaran and Hoben (1994). Sterilised nodules were carefully crushed onto YMA (yeast mannitol agar) plates (Fred and Waksman, 1928) under aseptic conditions using heat-sterilised forceps. The resulting plates were incubated at 28 °C and monitored for 10 days. Bacterial colonies were repeatedly streaked on YMA medium to obtain pure cultures.

To authenticate isolates as true rhizobia, a nodulation test was carried out under aseptic and controlled conditions using cowpea (*Vigna unguiculata* L. Walp, cv. Asontem) as the test host. Cowpea was selected for this initial screening because of its highly promiscuous nodulation pattern and for being easily cultivable in growth pouches. Cowpea seeds were prepared (Section 2.4) and pre-germinated on moist sterile tissue paper in Petri dishes and incubated at 28 °C for three days. Seedlings with equal radicle length (2 cm) were selected and aseptically transferred into plastic growth pouches (Mega International, USA) containing N-free plant nutrient solution (Broughton and Dilworth, 1970). After seeding, the growth pouches were arranged on a wooden rack and placed in the greenhouse at KNUST, Kumasi, Ghana. A week after transplanting, broth cultures of each of the isolates were used to inoculate the cowpea seedlings. At 28 days after inoculation, the seedlings were assessed for nodulation and isolates that induced nodule formation on the test host were considered as true rhizobia. Where no nodules were observed, the isolate was not subjected to further studies. Isolates confirmed as true rhizobia were maintained on agar slants and also in 25% (w/v) glycerol (at −20 °C) for short term and long term (−80 °C) storages, respectively.

2.2. Symbiotic potential of native isolates on groundnut in sterilised sand in Ghana

The sixty-five isolates, that were considered true rhizobia based on the authentication test on cowpea, were evaluated for their symbiotic potential together with recommended/commercial strains namely: *Bradyrhizobium diazoefficiens* USDA 110 (soybean strain from Florida, USA) (Delamuta et al., 2013), and two Brazilian elite-strains, *Bradyrhizobium pachyrhizi* strain BR 3262 and *Bradyrhizobium yuanmingense* strain BR 3267 (Leite et al., 2017). USDA 110 is a strain widely used in commercial inoculants for soybean in Africa and in characterising newly cultured isolates.

The groundnut variety 'Chinese' (an early maturing variety preferred by most farmers in Ghana) was used. For the first experiment, four-litre capacity pots were filled with 3 kg of sterilised river sand and arranged in the greenhouse at KNUST, Kumasi, Ghana. Prior to filling the pots, the sand was sterilised in an autoclave at 121 °C for 1 h (Lupwayi and Haque, 1994). Broughton and Dilworth (Broughton and Dilworth, 1970) N-free nutrient solution was used to irrigate the plants weekly. The strains were classified by a symbiotic effectiveness index (SEI) that was calculated from the shoot dry matter (SDM) of the groundnut plants inoculated with a specific isolate divided by the SDM of groundnut plants inoculated with the reference strain BR 3267, expressed as a percentage (Yates et al., 2016).

2.3. Nitrogen fixation contribution of isolates on groundnut in ^{15}N labelled soil in Brazil

The second experiment was conducted in pots in the open field at Embrapa Agrobiologia, Seropédica, Brazil. The planting medium used was soil classified as an Alfisol (US Soil Taxonomy Classification) obtained from Piracicaba, São Paulo State, Brazil, with a history of ^{15}N enrichment since the 1980s through the application of ^{15}N labelled organic matter (Tsai, Siu Mui, CENA, Piraicicaba, personal communication). Due to the compacted nature of the soil, it was mixed with 50% sand to improve drainage. Prior to the experiment, the chemical properties of the soil were analysed using the methods of Souza and Nogueira (2005): pH in H_2O, 5.3; exchangeable Al, Ca and Mg: 0.04, 0.96 and 0.18 $cmolcd^{-1}$, respectively; and P and K were 16.2 and 21.7 mgL^{-1}, respectively. The soil had sand, silt and clay fractions of 14%, 22% and 64% respectively and fell within the clay textural class. Seven effective isolates identified from the first experiment alongside