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Isolation of Rhizobia From the Nodules of Bambara Groundnuts for Inoculant Production

Abdul-Wahab M. Imoro[1], Jonas Pobee[1] & Fortune Akabanda[2]

[1] Department of Applied Biology, C. K. Tedam University of Technology and Applied Sciences, Navrongo, Ghana

[2] Department of Food Science and Technology, University for Development Studies, Tamale, Ghana

Correspondence: Abdul-Wahab M. Imoro, Department of Applied Biology, C. K. Tedam University of Technology and Applied Sciences, Navrongo, Ghana. E-mail: aimoro@cktutas.edu.gh

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Abstract

Rhizobia symbiotic interactions with legumes fix atmospheric nitrogen into the soil, which is essential in amending the characteristically low-nitrogen soils in most farming communities in northern Ghana. A high potential for improvement of Bambara groundnuts production in low-nitrogen soils is by the exploitation of colonization of the plant roots with rhizobial inoculation. This experiment sought to isolate Legume Nodulating Bacteria (LNB) obtained from root nodules of Bambara groundnut (*Vigna subterranea*) plants and to identify effective strains for improved production of the crop. Roots nodules of Bambara plants used in this study were obtained from preserved plants and the isolates were authenticated for their symbiotic effectiveness under screen house conditions. Nodulation of the isolates was examined in plastic pots containing sterile river sand and test crop (Bambara seeds). The experiment included reference strains, a positive nitrogen control and an un-inoculated control. The results were obtained after two months of data collection. The difference in results was explained via nodulation capacity. Out of the two isolates obtained, 2CL showed a high nodulation capability, rating it as highly effective. The outcome of this study provides stakeholders with the prospect for the use of effective isolates as inoculants to improve Bambara groundnut yield in general and in northern Ghana in particular.

Keywords: Bambara groundnuts, rhizobia, inoculant, nodules

1. Introduction

Low crop productivity due to poor soil fertility is a general problem facing most farming systems in Sub-Sahara Africa, especially in the northern parts of Ghana. The high cost of agrochemicals such as fertilizers and other farm inputs further compound the problem (Agyenim-Boateng et al., 2006; McArthur & McCord, 2017; Hebbebrand & Laborde, 2022), because farmers are unable to purchase adequate quantities to meet their demands. This is particularly serious in the case of the subsistence farmers who form a majority of the farming population in Ghana (Ghana Statistical Service, 2013). This problem of soil fertility could be reduced or to some extent curbed if the cost of agrochemicals and other sustainable technical solutions could be lowered to meet the demand of smallholder farmers (Somasegara & Hoben, 1994). Chemical or synthetic fertilizers give short-term high yield product, but cause a long-term negative impact on farm lands. Besides this, the use of these fertilizers causes ecological problems as well as affects human health.

Legumes' symbiotic interactions with microorganisms in the rhizosphere affect the crops positively on many of the plants' attributes including yields. The beneficial effect of rhizobium on legumes in terms of biological nitrogen fixation (BNF) is well known and nitrogen fixing can range from 266-673 n moles (Werner, 1992; Farrukh et al., 2004). In legume-microorganism relations, root colonization is an essential first step in the interaction of beneficial bacteria with plants (Kloepper & Beauchamp, 1992). Biological nitrogen fixation is the cheapest and most environmentally friendly procedure in which rhizobia interact with leguminous plants and fix nitrogen into the soil (Mohammedi et al., 2012). Rhizobia belong to a huge bacteria group and *Rhizobium* is one of the main genera of this group capable of fixing nitrogen in symbiotic association with legume plants. It has been proven that the presence of rhizobia increases plant productivity without causing any harm to human health as well as the environment. In maintaining soil fertility, the cultivation of leguminous plants is important because

they replenish soil nitrogen through symbiosis with rhizobia in rotation with non-leguminous plants (Gaur et al., 1980; Chabot et al., 1996). Leguminous plants possess a unique ability in establishing symbiosis with nitrogen-fixing bacteria of the family Rhizobiaceae from the genera *Rhizobium*, *Bradyrhizobium*, *Allorhizobium*, *Mesorhizobium* and *Sinorhizobium* (Martínez-Romero, 2003) which are collectively referred as rhizobia. These organisms help in biological nitrogen fixation through the formation of root nodules (Peoples et al., 1995). Within the root nodules, these bacteria convert atmospheric nitrogen to ammonia and provide the plants with an organic nitrogenous compound, this leads to plant development and grain yield improvement (Kummerer, 2004). The effectiveness of rhizobia populations in fixing nitrogen is correlated with soil fertility status.

Grain legumes, particularly cowpea, groundnut, soybean and Bambara groundnut are an important component of the cropping system in northern Ghana. These crops require soil rich in nutrients, especially nitrogen to fully express their yield potentials. Their growth and yields are constrained by poor and degraded soils that are characteristic of northern Ghana (Brookman-Amissah et al., 1980; Runge-Metzger, 1993; Baudoin & Mergeai, 2001; Brink el al., 2006). Over the years, the production of inoculants for cowpea and groundnuts have been successful and indeed, inoculating legumes with commercial rhizobial inoculants is a common agricultural practice to increase crop production. However, a study of this nature is very limited in Bambara groundnuts production, which is a common legume crop cultivated by indigenous smallholder farmers. This study, therefore, sought to isolate rhizobium strains for the production of inoculants used in assessing the nodulation capabilities of Bambara groundnut variety (purple colored), the test isolates compared to the nitrogen control and the reference strains.

2. Methodology

2.1 Study Area

The experiment was conducted at the greenhouse and the Inoculant laboratory of the Savanna Agriculture Research Institute (SARI), Nyankpala, which is located in the Guinea Savanna Agro-ecological zone, at latitudes 9º25′ and 10° north and longitude 1° west at altitude 183 m (Kombiok, 2013). The temperature and the relative humidity in the Greenhouse during the experimental period were 25±2 °C and 79±3%, respectively. The study area has an average annual rainfall of about 1200 mm, which is unimodal and distributed uniformly from May to October. The daily temperature ranges from a minimum of 26 °C to a maximum of 39 °C with a mean temperature of 32 °C (Kombiok et al., 2000).

2.2 Source of Bambara Plant Nodules and Its Preparation

Healthy Bambara plants with root nodules attached were uprooted from the pots in the plant house of SARI. The plants were carefully placed in a rubber bag and brought to the Inoculant laboratory where the isolation was done. The roots were washed free of soil. The nodules were detached from the roots and then put in water until ready for isolation. Desiccated nodules were re-hydrated by immersion in water for three to four hours in labeled Petri dishes.

2.3 Isolating Bacteria From the Nodules

The nodules were surface sterilized to remove contaminants. A pair of forceps was used to transfer the nodules from the sterilizing solution to the rinsing solution. Sterilizing solutions of 70% (v/v) ethanol and 4% (v/v) sodium hypochlorite, and six changes of sterile water in vessels that accommodated the forceps (*e.g.*, 20 mL) or tea strainer (100 mL) were prepared. The nodules were surface sterilized to remove microorganisms on the surface by immersing them for one minute in 70% (v/v) ethanol, followed by up to three minutes in sodium hypochlorite. The nodules were carefully rinsed six times in sterile water by transferring the locked forceps from vessel to vessel, ensuring that the solution was drained from the forceps each time. From the last rinse, the nodules were aseptically crushed individually with blunt-nosed forceps held directly over the growth medium, allowing the contents to drop onto the plate. A new plate was used for each nodule. The drop was then streaked onto plates containing an appropriate growth medium. A dilution streaking pattern was used to isolate single colonies. The inoculated plates were then incubated at 28 °C and checked every 24 hours to observe the growth of the rhizobia and contaminant bacteria. A high percentage of isolations resulted in mixed cultures and care was taken to identify and purify rhizobia. Single colonies that resembled rhizobia for subculture were selected and purified by touching the edge of the loop to the colony by streaking on a fresh plate. After the single colony subcultures had grown, the colonies were stored for the short term at -80 °C in a glycerol medium (Howieson & Dilworth, 2016).

2.4 Identification of Native Rhizobia Strain

A prelude screening for authentic rhizobia was performed using Bambara groundnut in growth pots under screen house conditions. Two strains of root nodulating bacteria were isolated from the nodules of one Bambara groundnut variety obtained from Nyankpala. These two isolates were IB2 and 2CL strains. The identification process was achieved by authentication and evaluation of the symbiotic effectiveness of selected native Bambara Isolates in potted sterile river sand under screen house conditions. The two isolates (*i.e.*, IB2 and 2CL strains) were assessed using one Bambara groundnut variety (purple colored) as test crop. Control treatments (un-inoculated control (N-) and a nitrogen control (N+) - 0.05% KNO_3) and reference strains were included in the experiment. Thus, the treatments consist of two native rhizobia isolates namely, IB2 and 2CL strains, reference/commercial strains, NC92 and BR3267, and control treatments, *i.e.*, un-inoculated control (N-) and a nitrogen control (N+) - 0.05% KNO_3). The seeds of the test crop were surface sterilized using 95% ethanol and rinsed in six changes of sterile distilled water.

2.5 Plant Treatment in the Greenhouse

Three seeds of the Bambara groundnuts were planted per pot and this was thinned to two, a week after germination. The seedlings in each pot were inoculated with 2 ml of each bacterial isolate and standard strain. In all, 480 pots were used and the experiment was laid out in a Completely Randomize Design with three replications (Dilworth & Broughton, 1970). The set-up was monitored for eight weeks after which nodulation and shoot biomass yield were assessed. Three randomly selected plants from each treatment at each sampling date were uprooted for the following: Determination of nodule number, nodule fresh and dry weights, shoot fresh and dry weights and effectiveness index and relative effectiveness of the isolates.

2.6 Determination of Nodule Number

The number of nodules was counted manually and record taken for each treatment.

2.7 Determination of Nodule Fresh and Dry Weights

The nodule fresh and dry weight of Bambara plants treated with test rhizobium isolates, nitrogen control, un-inoculated control and the reference strains were recorded**.** The fresh weight of the nodules of each Bambara plant was measured with an electronic balance whilst the dry weight was obtained after drying to a constant weight and then measured with an electronic balance. Records were taken for three replications.

2.8 Determination of Shoot Fresh and Dry Weights

The shoot fresh weight was measured using an electronic balance whilst the shoot dry weight of the Bambara plants was measured using the oven-dry method to constant weight at 80^{o}C for 48 hours.

2.9 Determination of Effectiveness Index and Relative Effectiveness of the Isolates

The effectiveness index and relative effectiveness of the isolates compared to the N-fed plants and the reference strains were calculated using the formula described by Ferreira and Marques (1992) as follows: *Effectiveness index* = $(x - y)/(z - y)$. Where, x is the shoot dry weight of the test isolate, y is the shoot dry weight of the un-inoculated control, and z is the shoot dry weight of nitrogen control plants. *Relative effectiveness = Shoot dry weight of inoculated test isolate*/*Shoot dry weight of inoculated reference strain*.

2.10 Data Analysis

The results from the data are presented as means±standard deviations. The data were analysed using inferential statistics involving Analysis of variance (ANOVA) in the General Statistical Software Package of GenStat, 2017 and where significant differences occurred, Fisher test was used to compare the means.

3. Results

3.1 The Effects of the Inoculum on the Number of Nodules of the Bambara Groundnuts

The number of nodules obtained from Bambara plants treated with rhizobium test isolates, reference strains, un-inoculated control, and a nitrogen control showed that, strain 2CL+N significantly ($P < 0.001$) produced the highest number of nodules (213.7±21.48), whereas IB2+N recorded the least number of nodules (81.0±34.4) among the two rhizobium test isolates (Table 1). The reference strain BR3267 also produced a high nodule number (143.0±13.7) confirming its viability in this test.

Table 1. Nodule number of each Bambara treatment

Treatment	Nodule Number
NC92	53.0±14.00[a]
IB2-N	104.7±17.32[a]
IB2+N	81.3±34.42[a]
2CL-N	149.7±11.26[b]
2CL+N	213.7±21.48[b]
N-	9.7±2.62[a]
N+	68.3±5.44[a]
BR3267	143.0±13.70[b]

Note. Means accompanied by the same superscript do not differ significantly (Fisher, $P < 0.001$).

3.2 The Effects of the Inoculum on the Nodules Dry Weight of the Bambara Groundnuts

The dry weight of the nodule of test plants treated with rhizobium test isolates (obtained from the root nodules of Bambara groundnut), nitrogen control, un-inoculated control and reference strains showed that 2CL-N treatment recorded the highest nodule dry weight whereas IB2+N treatment was the least nodule dry weight among the test treatments (Figure 1). There was a significant ($P < 0.001$) increased in the dry weight of nodules produced by the 2CL-N treatment over the IB2+N treatment of Bambara plants.

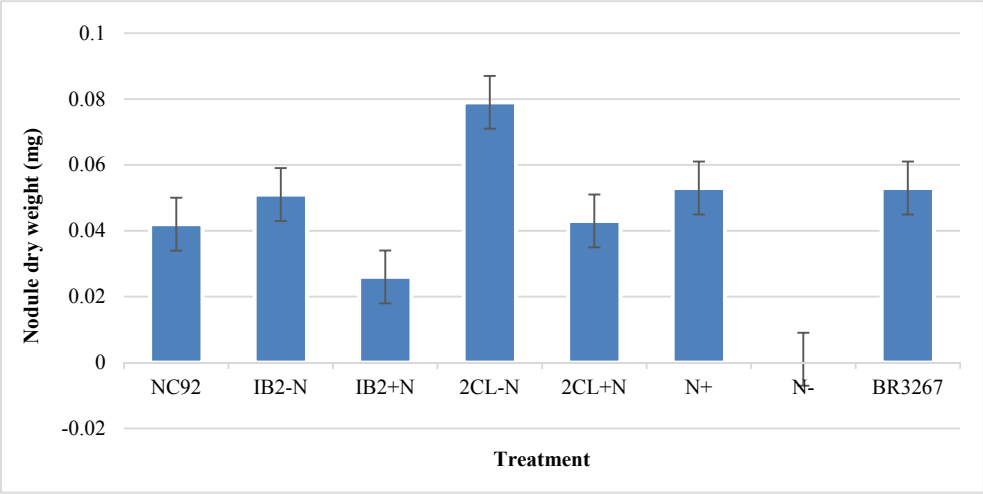

Figure 1. Nodule dry weight of different treatments of Bambara groundnuts

3.3 The Effects of the Inoculum on the Shoot Dry Weight of the Bambara Groundnuts

The results obtained for the shoot dry weight of Bambara plants treated with rhizobium test isolates, reference strains, un-inoculated control and a nitrogen control indicate that there were no variations ($P > 0.001$) amongst the various treatments. However, the trend showed that the shoot mean values are in this order: 2CL-N, 2CL+N, NC92, and BR3267. The IB2-N and un-inoculated control (N-) treated plants in terms of the trend showed very low shoot dry weight among the various rhizobium treatments (Table 2).

Table 2. Shoot dry weight of Bambara treatments

Treatment	Shoot Dry Weight (mg)
NC92	3.24±0.15[a]
IB2-N	2.67±0.15[a]
IB2+N	2.92±0.07[a]
2CL-N	3.45±0.44[a]
2CL+N	3.43±0.21[a]
N+	4.07±0.05[a]
N-	2.60±0.23[a]
BR3267	2.68±0.06[a]

Note. Means accompanied by the same superscript do not differ significantly (Fisher, $P < 0.001$).

3.4 The Effects of the Inoculum on the Relative Effectiveness and Effective Index of the Bambara Groundnuts

In addition, the relative effectiveness and effective index of the rhizobium isolates obtained from the nodules of Bambara groundnut indicated that the 2CL-N treatment recorded a higher percentage in terms of the symbiotic effective index with a corresponding higher relative effectiveness, whereas IB2-N turn up with the least (Table 3).

Table 3. Relative effectiveness and effective index of test rhizobium isolates

Treatment	Relative Effectiveness (R.E.)	Effective Index (E.I.) %
IB2-N	1.0	4.7
IB2+N	1.1	21.7
2CL-N	1.3	57.7
2CL+N	1.3	56.6

4. Discussion

The results demonstrate a higher number of production of nodules in 2CL+N treated plants and a greater nodule dry weight in treatment 2CL-N strain of Bambara groundnuts in this study with the use of the native strains inoculum. A study by Gaur et al. (1980) in cereal-legume crop rotation systems by inoculating the preceding cereal crop with rhizobia found a significant increase in the nodule number, its volume, dry weight of shoots, number of pods and grain yield in groundnuts. Höflich et al. (1994) also reported significant shoot dry weight matter yield increases by inoculating many plant species with rhizobia. A similar study by Chabot et al. (1996) obtained significant increases in shoot dry matter yield by inoculating some plant species. The general increase in the number of nodules and the dry weight of the Bambara groundnuts in the present study may be attributable to the efficacy of the inoculum used.

Generally, in assessing the relationship between rhizobia and their host, infectivity and symbiotic effectiveness are the two essential features commonly considered (Brockwell, 1998). The symbioses between legumes and rhizobium must be effective for enhanced Biological Nitrogen Fixation and subsequent yield improvement to be realized (Hubbell & Kidder, 2009; Osei, 2019). The results in this study revealed that the 2CL+N treatment recorded the highest nodule number with a corresponding nodule weight compared with the reference strains and control treatment. Farrukh et al. (2004) reported a higher nodule dry weight of 4.27 mg per plant in fodder legumes. Martins et al. (2003) reported a significant increase in the nodule number of cowpea after inoculating with a rhizobium inoculant. 2CL-N treatment also recorded a high nodule number and a corresponding nodule dry weight. Nodule dry weight is an important parameter for strain evaluation as it acts as an indicator of symbiotic effectiveness. Treatment with the reference strain (BR3267) recorded a high number of nodules and a nodule dry weight as well. Similar results were obtained by Martins et al. (2003) as they used BR3267 and found a significant grain increase compared to the control treatments. This justifies the assertion that BR3267 is effective and confirms why it is used as a commercial strain for inoculant production (Martins da Costa et al., 2014). IB2-N treatment recorded a nodule number of 104.7±17.32 and a corresponding nodule dry weight of 0.051±0.013, which is not up to the reference strain BR3267 but more than the treatment with NC92 strain. In general, environmental factors influence a plant growth and development and in this experiment, the environment influence was uniform

as all the treatments were subjected to the same environmental variables. Tate (1995) however, reported environmental factors including moisture stress accounting for low nodule numbers and nodule dry weight in legumes. Nutrient deficiencies could not have arisen in this study as well but Friedericks et al. (1990) and Sanginga et al. (1995) reported these variables to affect nodule numbers. For now, it would be speculative to assign any cogent reason why the NC92 strain did not record high nodule numbers and nodule dry weight as its counterpart reference/commercial strain, BR3267.

The treatment with isolate 2CL outperformed its counterpart native isolate IB2 in nodule numbers. Treatment with isolate IB2+N produced 81.3±34.42 nodules. The low nodulation in IB2 could be attributable to possible genotypic differences in the two strains and their interactions with the test crop. For instance, rhizobia compete for nutrients (Singleton & Tavares, 1986) and host plant competing rhizobial strains show their high specificity for the respective crop (Hafeez et al., 2000). However, further study is required to clarify this assertion. Additionally, low concentration of ammonium results in poor nodulation (Gulden & Vessey, 1998). Nitrate and ammonium induce differential effects on nodulation (Salah et al., 2012). Even at low concentrations, nitrate is known to cause reductions in nodule number in a concentration-dependent fashion, whereas ammonium has the opposite effect (Salah et al., 2012). Bambara plants treated with nitrogen control (N+) and the un-inoculated (N-) both did not yield any positive response to the treatment. This implies that the concentration of ammonia in these treatments could not dominate the effect induced by nitrate. The effect induced by mineral N will also depend on the dominant N-species; that is, nitrate vs ammonium present.

5. Conclusion

Among the isolates tested, 2CL recorded the highest nodulation, performing more than the commercial strains, BR3267 and NC92, which were used as reference strains for the experiment. Both treatments, with or without nitrogen (N+ and N- respectively) nodulated effectively. This implies that isolate 2CL is highly effective and reveals it as an important source of local inoculant strains. In addition, the close association between identified *Bradyrhizobium* isolates from the study and *Bradyrhizobium yuanmingense* strain confirms the identified isolate as an important micro-symbiont of Bambara groundnut. Test isolate IB2 was observed as not effective for the treatment due to its inability to nodulate well. This indicates that test isolate IB2 is not feasible for enhancing Bambara groundnut growth. In all, it can be concluded that test isolate 2CL is an effective native isolate which can be used to improve Bambara groundnut nodulation in the northern region and the nation in general.

6. Recommendation

The study, therefore, recommends that in order to improve grain yields of Bambara groundnut in an environmentally friendly manner and a more cost reduce way, the symbiotic potential of isolate 2CL should be assessed under field conditions and the Isolate (2CL) should be further studied for local inoculant production for farmers in the northern regions.

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