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SYMBIOSIS AND ECOLOGICAL ADAPTATION OF THE COMMERCIAL NITROGEN FIXING BACTERIA ON UNDERUTILISED BAMBARA GROUNDNUT CROP

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Abstract

The efficiency of the natural symbiotic relationship between communally produced legumes and nitrogen fixing bacteria has been reported to be very poor, mainly due to poor soils in communal farming areas. With the use of synthetic fertilizers to increase soil fertility being both environment-unfriendly and unaffordable for majority of communal farmers, strategies that are cost-effective and climate smart need to be put in place to improve the productivity of communal legumes such as Bambara groundnuts. A 5 x 3 factorial arrangement experiment in a randomised complete block design with five replications was established under greenhouse conditions. Factors consisted of bacterial species: [Bradyrhizobium japonicum (Bj), Rhizobium loti (Rl), Rhizobium meliloti (Rm), Rhizobium leguminosarum biovar phaseoli (Rlt) and a negative control (untreated)], and local Bambara groundnut varieties: black, creamy white-eye, and red. Bambara groundnut seeds were inoculated with levels recommended for cowpea and soyabean. Nitrogen fixing bacteria application on Bambara groundnuts increased plant growth and nodulation variables, with the effects varying with Bambara groundnut variety and rhizobium type. Bj outperformed the other tested bacteria on nodulation variables, with the red cultivar performing better in all growth variables, making it suitable for pod and vegetative production.

Key words: *Bambara groundnut, Bradyrhizobium japonicum, Nitrogen fixing bacteria, Inoculum, Rhizobium spp., soil fertility.*

INTRODUCTION

An ever-growing population and climate change are the main factors that have negatively impacted agricultural production (Olabanji et al., 2020). The two calls for the utilization of an increased number of food crops which were previously deemed underutilized to ensure that food security is met (Cook, 2017). Bambara groundnut (*Vigna subterranea* L.) is an underutilized, indigenous, drought-tolerant legume crop mostly grown by subsistence farmers in sub-Saharan Africa for its grain. It is the third most significant food legume in Africa after cowpea (*Vigna unguiculata*) and groundnuts (*Arachis hypogaea*) both in land cultivation and consumption (Ibny et al., 2019). The edible grain of this legume contains high carbohydrates (55.6%), protein (21.2%), fats (7%), and fibre (6.3%), which makes it a comprehensive meal (Pouzaa et al., 2017). Due to Bambara groundnut's great nutritional value, the crop is labelled as 'a native key solution to African's food crisis' (Temegne et al., 2018). Harvested seeds of Bambara groundnut, can be consumed fresh or dried for future consumption (Cook, 2017). The leaves contain high K and N, therefore can serve as an excellent protein rich feed for animals.

Low soil nitrogen (N) is a major constraint for sustainable crop production in communal farming systems in Africa (ref). Farmers mostly rely on synthetic fertilizers to improve soil fertility and these fertilizers are environment-unfriendly and very expensive for the smallholder farmers who are resource-poor (Gomoung et al., 2017). Due to colonial history, most communal farmers who depend on the crop are found in degraded soils very low in nutritional status and a natural population of the beneficial organisms resulting in reduced crop productivity (Hasan et al., 2018; Nyamador et al., 2016).

Grain legumes such as Bambara groundnuts can form symbiotic association with nitrogen fixing root nodule bacteria called 'rhizobia'; in a process that can supply sufficient N for the legume and other crops growth in the same land under intercrop or crop rotation (Azman-Halimi et al., 2019; Ibrahim et al., 2018). This symbiotic relation is mainly of economic and agronomic

significance due to its N contribution to the total balance of N in the terrestrial ecosystems (Stagnari et al., 2017). Most of the research on the symbiotic relationship between Bambara groundnut and nitrogen-fixing bacteria have focused on its promiscuous tendencies to pick bacteria in soils (Akpalu et al., 2013).

The compatibility of Bambara groundnut with commercially produced nitrogen-fixing bacterial could help improve the yields of the crop and quality of the soils and are less expensive, when compared to synthetic fertilisers, at the same time promote sustainable farming (Nelwamondo, 2020). Hence the study sought to determine the effectiveness and adaptability of the comercially produced nitrogen-fixing bacteria on Bambara groundnuts growth and nodulation.

MATERIALS AND METHODS

Description of the study site

The study was conducted in a greenhouse located at the University of Mpumalanga farm (25°27′06.18″S 30°58′5.21″E) Mbombela campus, South Africa. The temperature and humidity in the greenhouse were set at 25 ± 2°C and 70 ± 10%, respectively.

Experimental treatments and design

The experimental treatments were arranged in a 5 x 3 factorial arrangement in a Randomised Complete Block Design (RCBD) with five replications (Figure 1).

Figure 1: Experimental layout in greenhouse

The first factor comprised of five bacterial inocula: *Bradyrhizobium japonicum* (Bj), *Rhizobium loti* (Rl), *Rhizobium meliloti* (Rm), and *Rhizobium leguminosarum* biovar phaseoli (Rlt) inoculum at a manufacturer recommended rates for cowpeas and soyabean, and a negative control (untreated seeds), whereas the second factor was the Bambara groundnut varieties; black (C1), and creamy white-eye (C2), and red (C3).

A 25 cm diameter pots were filled with steam-pasteurised sandy loam soil. Before inoculation, seeds were mixed with 48% sugar solution in order to get a thin uniform coating of inoculum before sowing. A 15 g of seeds was used for each inoculation following manufacturer's recommendation of 6.5 x 10^7 live cells/seed of *Bradyrhizobium japonicium*, 5 x 10^7 live cells/seed of *Rhizobium loti* and 2.4 x 10^7 live cells/seed of *Rhizobium leguminosarum* biovar *phaseoli* and *Rhizobium meliloti*. Seeds were sown an hour after inoculation and were irrigated with 300 ml of tap water per plant when soil moisture levels were below 50 %. Monitoring of seed germination and scouting for pests was done daily.

Data collection

Plant growth variables

Seedling emergence and days to flowering were recorded every day from the first sign until 100%. After 110 days, the number of leaves and number of runners were counted per plant and recorded. Plant height measured from the soil level to the tip of the flag leaf using a ruler. Length of the longest runner was measured using a 30 cm ruler. Chlorophyll content was measured from two mature topmost leaves utilizing a Chlorophyll Content Meter. The plants were then cut at soil line and stem diameter measured using Vernier Callipers (C.C, Johannesburg) 5 cm from the severed end. Plant roots and pods were removed from the pots immersed in water to wash off soil particles and blotted dried using paper towel and number of fresh pods per plant were recorded, the weight per plant of fresh pod, shoot, and roots per plant were measured and recorded using electronic weighing balance. The shoots were then oven-dried for 72 hours at 55 °C and weighed to determine dry shoot mass (DSM). Plant growth vigour score as a measure of plant nitrogen access levels was assessed based on the following previously described scoring system (Corbin et al., 1977) where plants that are vigorous and green were scored at 5, those relatively small and green were given a score of 4, plants that were losing the green pigment and were small had a score of 3, plants that were slightly chlorotic had a score of 2, and finally plants that were very chlorotic were given a score of 1.

Plant Nodulation variables

Abundance and presence of nodules were assessed from the root system of each plant. The number of active nodules (strong pink internal colour), and nonactive (brown, green, or white internal colour) were taken. Nodule colour and abundance were assessed using a previously described scoring system (Somasegaran & Hoben, 1994; Corbin et al., 1977), where more than five clusters of pink pigmented nodules were scored at 5, four to five clusters of mostly pink nodules were scored 4, three to four cluster of less pink nodules 3, some pink or whitish with green area 2, less than three clusters of nodules or green or white nodules 1, and roots with no nodules or green or white nodules were scored 0. Nodule position score was assessed as follows: where there were both crown and lateral nodulation a score of 3 was given, crown nodulation only 2, and where lateral nodulation only occurred a score of 1 was given. Nodulation efficiency and nitrogen fixation potential were determined by adding the scores for plant vigour, nodule colour & abundance score, and nodule position score. Nodulation efficiency and nitrogen fixation potential were then rated using total scores as follows: 11-13 score indicate excellent nodulation and nodule nitrogen fixing potential, 7-10 score indicate good still effective nodulation with nodules having limited nitrogen fixing potential, 1-6 score indicated poor nodulation with nodules with very little to no nitrogen-fixation potential.

Data analysis

Data collected were tested for normal residual distribution using the Shapiro-Wilk normality test before subjecting it to analysis of variance (ANOVA) (Gomez & Gomez, 1984). The data that were not normally distribution were transformed using $Log_{10}(x+1)$ then subjected to ANOVA through Statistix 10 software. The mean separation was achieved using Fisher's Least Significant Differences (LSD) at 5% probability level.

RESULTS AND DISCUSSIONS

Shapiro-Wilk test for normality distribution revealed that most plant growth variables and all nodulation variables were normally distributed ($P \leq 0.05$), except for plant height, length of longest runner, fresh shoot mass, dry shoot mass, and fresh root mass hence were transformed accordingly. The interaction between Bambara groundnut variety and bacteria inoculum were not significant in all tested variables.

Plant growth variables

Rhizobia inoculation is an important technology used to address soil fertility problems and application of insufficient fertilizer in legume production (Saharan & Nehra, 2011). Effective application of rhizobia strains as bio-fertilizers to improve production is a significant approach in sustainable agriculture (Saharan & Nehra, 2011). Glasshouse inoculation experiments on three Bambara groundnut varieties with four isolates and one reference strains (bacteria in untreated soil) from this study resulted in varying effects on all tested variables. The current study reports that there were no interactions between varieties and bacteria inoculum. However, bacteria treatments had highly significant effects on the number of leaves, fresh shoot mass, and dry shoot mass, contributing 43, 48, and 47% to the total treatment variable (TTV) (Table 1). Moreover, bacteria also had significant effect on stem diameter and fresh root mass, contributing 22 and 24% to TTV, respectively (Table 1). varieties had highly significant effect on the number of runners, length of longest runner, number of leaves, and pod mass, contributing 75, 81, 39, and 78% ($P \leq 0.01$) (Table 1).

The black variety was statistically different from the red and creamy white-eye varieties on number of runners, length of the longest runner, chlorophyll content, pod mass, and number of leaves expect for the number of pods which was similar to the red variety (Table 2). Additionally, the black variety had the highest pod number, pod mass, and chlorophyll content whereas the red variety had the least of the same variables (Table 2). The red variety had the highest number of runners with the black variety having the least. Bacteria in untreated soil outperformed the commercial inoculum in all plant growth variables (Table 3). The inferiority of the seed coated with commercial rhizobacteria inoculant could be attributed to the texture and big seed size of Bambara groundnut relative to cowpea and soyabean were these are recommended. Bambara groundnut seeds might not have

imbibed optimum quantity of the liquid inoculum suspension that is capable of effectively promoting plant growth. Milus and Rothrock (1993) also noted that the bacteria population in the soil dependent on the initial stack of inoculums on the seeds coat. In contrary, Nelwamondo (2020) observed that inoculation of cowpea with *Bacillus subtills* strain BD233 *and Bradyrhizobium japonicum* significantly increased number of leaves and plant fresh weight, having a weight of 175.18 g, followed by *Bradyrhizobium japonicum* inoculation having a plant fresh weight of 165.38 g. Allito et al. (2015) reported that the response of legumes to inoculum differs with the degree of promiscuity of legume species. These results were in line with Yakubu et al. (2011), Manisha and Bhadoria (2008), who observed significant responses of groundnut to Rhizobia inoculation.

Nodulation variable

All measured variables were significant ($P \leq 0.05$), except for plant growth vigor and total assessment score. Bacteria treatments had highly significant ($P \leq 0.01$) effects on the total number of nodules and non-active nodules contributing 80% and 78% in TTV (Table 4). Bacteria treatments also had significant ($P \leq 0.05$) effects on nodule position, number of active nodules, and nodule color and abundance score, contributing 46, 66, 48% in TTV of the respective nodulation variables (Table 4). Inoculation of Bambara groundnut seeds resulted in a significant variation in all nodulation variables particularly in enhanced nodule formation (Figure 2A) (Table 5).

All treatments that were inoculated with *Bradyrhizobium japonicum* isolate had high number of nodules but with less pink internal nodule colours and accumulation of plant biomass (Table 5). These results indicate a higher rate of symbiotic efficiency and N-fixation. The current observation support report by Onyango and Ogolla (2019), that *Bradyrhizobium* spp. isolate may have acquired both *nif* and *nod* genes within the *Sym* plasmid which enabled it to produce the high number of nodules. Bacteria isolates in untreated soil had the highest number of active nodules when compared to the commercial inoculum (Figure 2B) (Table 5).

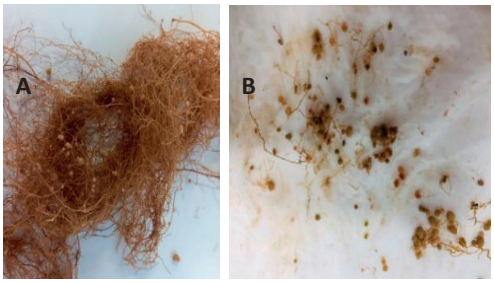

Figure 2. Nodules on roots of Bambara groundnut (A); Interior colour of the nodules (B)

McLoughlin et al. (1984) reported that inoculation efforts failed to enhance legume productivity because the indigenous strains inhabited the root nodules rather than the inoculum strains. This was related to a more competitive nature and adaptability of the indigenous population. *Rhizobium meliloti*, *Rhizobium loti*, and *Rhizobium leguminosarum* had higher number of active nodules, colour and abundance of the nodules (Table 5). On the number of active nodules *Rhizobium meliloti*, *Rhizobium loti*, and *Rhizobium leguminosarum* were statistically different from *Bradyrhizobium japonicum* and isolates from untreated soil (Table 5). These strains have the potential to be use as bio-fertilizers in Bambara groundnuts production, hence they well distributed in most soil types. Ikenganyia, Anikwe and Ngwu (2018) stated that when bacteria isolates are introduced into the rhizosphere, they have the potential to change microbial populations in the rhizosphere and influence nutrient availability, transformation, and uptake by plants. Furthermore, *Rhizobium* and *Agrobacterium* are known as the fast-growing genera which have better rates of competition for nodulation occupancy (Howieson, Nutt, and Evans, 2000). Consequently, Bambara groundnuts are mostly more compatible with the genus *Bradyrhizobium* and *Burkholderia* spp. but are outcompeted for nodulation colour and abundance as well as the total nodulation for effectiveness in the local soils by the *Rhizobium* spp. Conversely, the ability of the isolates to thrive and result in more nodule occupancy, and interior pink colour is influenced by the prevailing status of the soil (Slattery, Pearce and Slattery, 2004).

Table 1: Partitioning mean sum of squares for number of runners (NR), length of longest runner (LLR), chlorophyll content (CC), number leaves (NL), stem diameter (SD), pod number (PN), pod mass (PM), fresh shoot mass (FSM), dry shoot mass (DSM) and fresh root mass (FRM) for Bambara groundnut growth in response to cultivar and commercial nitrogen fixing bacteria.

		NR		LLR		CC		NL		SD	
Source	DF	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)
Replication	4	0.163	17	8.170	4	0.008	4	27.180	8	2.949×10^{-3}	53
varieties	2	0.732	75^{***}	149.467	81^{***}	0.109	53^{**}	127.960	39^{***}	8.762×10^{-4}	16^{ns}
Bacteria	4	0.034	3^{ns}	7.300	4^{ns}	0.054	26^{ns}	140.313	43^{***}	1.243×10^{-3}	22^{**}
Varieties*Bacteria	8	0.014	1^{ns}	6.691	4^{ns}	0.004	2^{ns}	8.043	2^{ns}	1.928×10^{-4}	3^{ns}
Error	56	0.036	4	12.776	7	0.030	15	26.544	8	3.279×10^{-4}	6
Total	74	0.980	100	184.403	100	0.206	100	330.04	100	5.589×10^{-3}	100
		PN		PM		FSM		DSM		FRM	
Source	DF	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)	MSS	TTV (%)
Replication	4	0.200	10	0.047	-6	38.076	31	5.741	41	86.751	61
Varieties	2	1.203	61^{**}	0.064	78^{***}	10.993	9^{ns}	0.412	3^{ns}	1.145	1^{ns}
Bacteria	4	0.301	15^{ns}	0.077	9^{ns}	58.647	48^{***}	6.522	47^{***}	33.556	24^{**}
Varieties* Bacteria	8	0.075	4^{ns}	0.016	2^{ns}	6.718	5^{ns}	0.339	2^{ns}	7.699	5^{ns}
Error	56	0.178	9	0.038	5	7.802	6	0.933	7	13.629	10
Total	74	1.957	100	0.823	100	122.236	100	13.950	100	142.780	100

^{ns}Not significant at $P > 0.05$, **Significant at $P \leq 0.05$, ***Highly significant at $P \leq 0.01$. $^{\times10}$Exponents beginning with three zeros before the comma.

Table 2: Effect of varieties on number of runners (NR), length of longest runner (LLR), chlorophyll content (CC), pod number (PN), pod mass (PM), and number of leaves (NL) for Bambara groundnut.

Varieties	NR	LLR	CC	PN	PM	NL
Cream-white eye	$0.805^{a}(5.720)$	9.912^{a}	$1.288^{b}(19.684)$	$0.263^{b}(1.400)$	$0.031^{c}(0.078)$	17.000^{a}
Red variety	$0.818^{a}(5.800)$	8.568^{a}	$1.286^{b}(19.492)$	$0.571^{a}(5.000)$	$0.164^{b}(0.624)$	16.560^{a}
Black variety	$0.515^{b}(2.960)$	5.168^{b}	$1.402^{a}(26.284)$	$0.688^{a}(6.520)$	$0.350^{a}(1.716)$	12.880^{b}
F-value	20.210	11.700	3.620	6.750	16.860	4.820
P-value	0.000	0.001	0.033	0.002	0.000	0.012
$LSD_{0.05}$	0.108	2.025	0.099	0.239	0.1108	2.919

Values that are found in brackets are means which are not transformed [$Log_{10}(x+1)$].

Table 3: Effect of *Bradyrhizobium japonicum* and *Rhizobium* spp. on the number of leaves (NL), stem diameter (SD), fresh shoot mass (FSM), dry shoot mass (DSM), and fresh root mass (FRM) for Bambara groundnut cultivars.

Bacteria	NL	SD	FSM	DSM	FRM
5	20.267^{a}	0.223^{a}	12.432^{a}	4.659^{a}	16.223^{a}
4	11.733^{b}	0.157^{c}	7.185^{c}	2.876^{c}	13.799^{ab}
3	15.000^{b}	0.191^{abc}	8.234^{bc}	3.299^{bc}	14.637^{ab}
2	15.400^{b}	0.197^{ab}	9.823^{b}	3.666^{b}	12.299^{b}
1	15.000^{b}	0.171^{bc}	9.465^{b}	3.645^{b}	13.179^{b}
F-value	5.290	3.790	7.520	6.990	2.460
P-value	0.001	0.009	0.001	0.000	0.056
$LSD_{0.05}$	3.769	0.0132	2.043	0.707	2.700

1= *Bradyrhizobium japonicum*, 2= *Rhizobium loti*, 3= *Rhizobium leguminosarum biovar phaseoli*, 4= *Rhizobium meliloti*, and Control.

Nodulation variable

All measured variables were significant ($P \leq 0.05$), except for plant growth vigor and total assessment score. Bacteria treatments had highly significant ($P \leq 0.01$) effects on the total number of nodules and non-active nodules contributing 80% and 78% in TTV (Table 4). Bacteria treatments also had significant ($P \leq 0.05$) effects on nodule position, number of active nodules, and nodule color and abundance score, contributing 46, 66, 48% in TTV of the respective nodulation variables (Table 4). Inoculation of Bambara groundnut seeds resulted in a significant variation in all nodulation variables particularly in enhanced nodule formation (Figure 2A) (Table 5).

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Furthermore, *Rhizobium* and *Agrobacterium* are known as the fast-growing genera which have better rates of competition for nodulation occupancy (Howieson, Nutt, and Evans, 2000). Consequently, Bambara groundnuts are mostly more compatible with the genus *Bradyrhizobium* and *Burkholderia* spp. but are outcompeted for nodulation colour and abundance as well as the total nodulation for effectiveness in the local soils by the *Rhizobium* spp.

Conversely, the ability of the isolates to thrive and result in more nodule occupancy, and interior pink colour is influenced by the prevailing status of the soil (Slattery, Pearce and Slattery, 2004).

Table 4: Partitioning mean sum of squares for nodule position (NP), nodule number (NN), active nodules (AN), non-active nodules (NNA), and nodule color and abundance (NCA) for Bambara groundnut nodulation in response to cultivars and commercial nitrogen-fixing bacteria

Source	NP		NN		NA		NNA		NCA	
	MSS	TTV (%)	MSS	TTV (%)	MSS	TVV (%)	MSS	TTV (%)	MSS	TTV (%)
Replication	0.041	26	0.069	7	0.049	4	0.193	10	0.056	18
Varieties	0.006	4^{ns}	0.002	0^{ns}	0.085	7^{ns}	0.028	1^{ns}	0.024	8^{ns}
Bacteria	0.074	46^{**}	0.747	80^{***}	0.779	66^{**}	1.576	78^{***}	0.149	48^{**}
Varieties*Bacteria	0.024	15^{ns}	0.054	6^{ns}	0.161	13^{ns}	0.103	5^{ns}	0.051	17^{ns}
Error	0.015	9	0.060	6	0.116	10	0.118	6	0.032	10
Total	0.160	100	0.930	100	1.190	100	2.020	100	0.310	100

^{ns}Not significant at P >0.05, **Significant at P ≤ 0.05, ***Highly significant at P ≤0.05

Table 5: Effect of commercial nitrogen-fixing bacteria on nodule position (NP), number of nodules (NN), number of active nodules (AN), number of non-active nodules (NNA), nodule colour and abundance (NCA) for Bambara groundnut cultivars

Bacteria	NP	NN	AN	NNA	NCA
5	0.442^{b}(1.933)	1.693^{b}(50.870)	1.400^{a}(25.867)	1.369^{b}(25.000)	0.778^{a}(5.000)
4	0.453^{b}(2.000)	1.514^{c}(35.930)	1.107^{b}(13.400)	1.258^{b}(22.530)	0.758^{a}(4.800)
3	0.457^{b}(2.000)	1.596^{bc}(43.870)	1.042^{b}(13.400)	1.379^{b}(30.470)	0.686^{a}(4.133)
2	0.433^{b}(1.867)	1.519^{bc}(35.270)	1.044^{b}(12.600)	1.235^{b}(22.600)	0.721^{a}(4.467)
1	0.602^{a}(3.000)	2.052^{a}(135.600)	0.759^{c}(9.667)	2.021^{a}(125.930)	0.527^{b}(3.267)
F-value	4.770	12.420	6.700	13.330	4.620
P-value	0.002	0.000	0.000	0.000	0.003
$LSD_{0.05}$	0.091	0.179	0.249	0.252	0.132

1= *Bradyrhizobium japonicum*, 2= *Rhizobium loti*, 3= *Rhizobium leguminosarum biovar phaseoli*, 4=*Rhizobium meliloti,* 5= Control.
Values that are found in brackets are means which are not transformed [$Log_{10}(x+1)$].

CONCLUSIONS

There is variation in the response of the different varieties of Bambara groundnuts to the different treatments with species of bacteria. The black variety could be recommended for yield production as it gave the highest pod mass and numbers, whereas the red variety can be used for vegetative production as it had the highest vegetative growth. Natural occurring bacteria outperformed the commercial species in Bambara groundnut nodulation giving the highest number of active nodules, hence identification of this bacteria is recommended for future study. *Bradyrhizobium japonicum* had the high number of nodules yet the lowest number of active nodules.

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