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Nodulation and nitrogen fixation of two cowpea (*Vigna unguiculata*) varieties in response to indigenous Rhizobial inoculation in the Rainforest of Nigeria

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Abstract

Legume-rhizobia symbiosis is an efficient source of soil nitrogen for sustainable agriculture. However, the abilities of rhizobial strains differ in terms of nodule formation and biological nitrogen fixation. Symbiotic nitrogen fixation depends mainly on the host plant genotypes, the rhizobial strains, interactions of these symbionts with environmental conditions and pedo-climatic factors. Rhizobial inoculation of cowpea IT89KD-288 and IT97K-568-18 varieties were evaluated at five levels (IDC8, R25B+IRj2180A, TRC2, O1a6(c3a) and no inoculation) in three different locations: the University of Ibadan Teaching and Research Farm (UITRF), Idi-Ayunre (IA) and Orile-Ilugun (OI) in a randomized complete block design. Numbers of nodules were significantly ($P<0.05$) higher in cowpea variety IT97K-568-18 (55.0) compared to cowpea variety IT89KD-288 (43.0) and also in rhizobial strains IDC8 and O1a6 (c3a) inoculated plants compared to the control with more than 200 % increase. The % nitrogen derived from the atmosphere (% NDFA) ranged between 50 – 74 % with no significant effect ($P<0.05$) of inoculation. However, the total N fixed (kg/ha) was significantly higher in rhizobial strains O1a6 (c3a) and TRC2, 72.8 kg N/ha and 68.4 kg N/ha, respectively, compared to the control (49 kg N/ha). Indigenous rhizobial strains have the potential to increase nodulation and biological N fixation in cowpea in the rainforest region of Nigeria.

Keywords: Cowpea; Inoculation Nodulation; Rhizobial strains; Nitrogen fixation.

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1.0 Introduction

Grain legumes are widely grown by farmers and research has proved that grain legumes also fix a large amount of nitrogen and improve soil nitrogen. Research findings have also proved that cowpea has the potentials to contribute to soil nitrogen through BNF , thereby increase the yield of subsequent crops. However, a wide variation in nodulation and N-fixation of legumes is observed in the tropical cropping system, which is an indication that the legumes involved may add or deplete soil nitrogen (Cardoso and Kuyper, 2006). Giller *et al.* (1994) estimated nitrogen fixed by cowpea to be in the range of 47 – 201 kg N ha^{-1} and common bean 3 – 91kg N ha^{-1}

Generally, legumes often increase the number of soil Rhizobia by multiplication and the release from plant nodules (Kumar *et al.,* 1997). The population of indigenous Rhizobia in most tropical soils is very low and these indigenous strains are less efficient in fixing nitrogen (Ahmad, 1981; Ahmad and Mclaughling, 1985). Slattery *et al*. (2001) reported that naturally occurring rhizobia in Australian soils in many instances are found in their large numbers in the soil, but they are poor in fixing nitrogen and they have a strong ability to compete with the introduced rhizobial strains.

Rhizobial population varies from 10 to more than 106 rhizobium bacteria g^{-1} soil in Australian soils (Slattery and Conventry, 1999). While Slattery *et al.* (2001) opined that the variation in the soil bacteria population reported was due to historical use of the field, location of sampling, soil characteristics and presence of host plant.

Legumes are grown for different purposes such as for pulse, for grain, as green manure, as pasture or as a component of the agro-forestry system. Nevertheless, the ultimate benefit lies in their ability to fix atmospheric N_2 to reduce the cost of manufactured N-fertilizer (Hardarson and Atkins, 2003). Therefore, BNF of an effectively nodulated legume is a vital and indispensable aspect of sustainable agriculture (Graham and Vance, 2000; Sessitch *et al*., 2001). However, before a legume can be successfully established, an understanding of the factors affecting their symbiotic interaction is needed (Howieson and Ballard, 2004).

Characteristics of a superior plant are early nodule formation, a large number of nodules and large nodules (Nutman, 1984). Phillips *et al.* (1985) reported that rhizobia – legume symbiosis for nitrogen fixation is greatly influenced by both the bacteria and the plant genotypes. Rhizobial strains differ in their effectiveness, time to nodulate and are host plant-specific (Kremer and Peterson, 1982). Results of the study conducted by Ranga-Rao *et al.* (1984) revealed that the response of the Indonesian soybean cultivar (Orba) varied with the indigenous rhizobial strains of Western Nigerian soil. Some of these strains were reported to have significantly improved nodulation, acetylene reduction activities and shoot growth but only one out of them all significantly increased seed yield. Sanginga *et al.* (1988) also reported a wide range of effectiveness and competitiveness among the indigenous rhizobia isolates obtained from International Institute of Tropical Agriculture (IITA) and Fashola soils in Nigeria. . Further research work carried out by Okereke and Onochie (1996) confirmed that there is variability in nitrogen-fixing potential of *Bradyrhizobium japonicum* in the Nigerian environment. Gault *et al.* (1994) however reported a wide range of effectiveness among the fifteen rhizobial strains isolated, and substantial symbiotic diversity within the collection was also observed in twenty-one rhizobial strains associated with five host species which indicates the high nodulating capacity of the isolates. However, no highly effective strain was discovered in the collection. On the other hand, a single strain of rhizobium can nodulate a diverse range of legume genera while some rhizobial strains can nodulate with different legume genera as in the case of *Arachis hypogea*. *Arachis hypogea* formed nodules with a wide range of slow and fast-growing rhizobial strains, as reported by Zhang *et al.* (1999). Symbiotic nitrogen fixation depends mainly on the host plant genotypes, the rhizobial strains, interactions of these symbionts with environmental conditions and pedo-climatic factors, (Bordeleau and Prevost, 1994). Wilson *et al.* (1995), also reported that the two major factors determining the extent of nitrogen fixation are (i) the availability of effective, compatible and competitive *Rhizobium* (indigenous or exotic) in the soil, and (ii) plant genotype. Ballard *et al.* (2002) associated the variation observed in rhizobial ability in fixing N_2 to different characteristics demonstrated by each strain in nodulation and or how efficient the rhizobial strains are in converting N_2 to NH_3. In the rain forest Nigeria Little or at best scanty work has been done on cowpea nodulation and N_2 fixation There is,, the need to extend the evaluation of cowpea for nodulation and N_2 fixation of cowpea to rain forest of Nigeria. The study was conducted to investigate the nodulation capability and nitrogen-fixing efficiency of two cowpea varieties in response to rhizobial strain inoculation.

2.0 Materials and Methods

Location, climates and soils of the experimental sites

Multilocational trial was conducted in three different locations namely: Idi-Ayunre (IA), Orile-Ilugun (OI) and the University of Ibadan Teaching and Research Farm (UITRF) within the rainforest-savanna transition zone of Nigeria. The soil characteristics of the location are as described in Table 1. The rhizobial population count of the three locations was determined using the Most Probable Number (MPN) (Somasegaran and Hoben, 1994). Two soybean varieties, TGx1448–2E and TGx 1456-2E, and one cowpea variety IT89KD-288 seeds were sterilized, pre-germinated and transplanted into sterilized growth pouches containing modified Jensen's N-free nutrient solution (Roughley, 1984). A 5-fold dilution series (5^{-1} – 5^{-6}) with four replicates was used to inoculate each plant in the growth pouch one week after planting.

Rhizobial Isolates

Rhizobial strains IDC8, OISa-6e and TRC2 were highly infective indigenous rhizobia isolated from the soils of locations (Ojo et al., 2015). Rhizobial strain R25B and IRj2180A, used as reference strains, were collected from IITA and termed exotic in this study. Rhizobial strain R25B and IRj2180A were isolated in Zaria (Sanginga 1993)

Cowpea varieties Two cowpea varieties: IT97K-568-18 and IT89KD-288 were planted. The two varieties were the creeping type

Table 1. Geographical and soil characteristics of the study sites

Characteristics	Idi Ayunre	Orile Ilugun	UITRF
Latitude and Longitude	7°26′N and 3°54′E	7°13′N and 3°31′E	7°30′N and 3°45′E
Soil Classification[1]	Nitosol	Luvisol	Alfisol
Soil Series[2]	Olorunda	Apomu	Egbeda
pH (KCl)	6.55	6.09	5.76
Total N (g/kg)[3]	0.23	0.17	0.08
Available P (mg/kg)[4]	0.42	0.06	0.13
CEC (cmol/kg)[5]	11.73	10.42	7.77
Sand (g/kg)[5]	645	675	812.5
Clay (g/kg) [5]	185	185	100
Silt (g/kg) [5]	170	140	87.5
Rhizobial count (cell g-1 soil)[6]	3.8	7.81	13.54

[1] USDA 2006 [2] Smyth and Montgomery 1962 [3] Bremmer 1982 [4] Bray and Kurtz 1945[5] Okalebo 1993 [6] Somasegaran and Hoben, 1994

Field trials

The field experiment was set up in a randomized complete block design with three replicates of 4 m × 4 m each. Planting was done at the three locations in early August, being the beginning of the second growing season, and the recommended planting period for cowpea in the rainforest zone of Nigeria. Before planting, inoculation of cowpea seeds was done following the procedure outlined by Somasegaran and Hoben (1994). The YMB cultures of each strain were aseptically injected into different peat package carriers using manually operated syringes at ratio 1:1 (ml/wt in g) broth culture to peat. Inoculated peats were incubated for 2 weeks at 28 ^oC to gain an excess of 10^8 - 10^9 cells g^{-1}. Planting was done at 75 cm x 25 cm, 2 plants / stand which gave a total population of 106,666 plants / hectare. The experiment was laid out in a Randomized Complete Block Design (RCBD). Each treatment was replicated three times.

Weed, insect and pest control: At planting "Dual 960EC" (active ingredient-- S-metolachlor) was applied as pre-emergence herbicide at the rate of 200 ml / 20 L water, Gramozone (active ingredient-- paraquat 276 g/l) was also added and applied at the rate of 100 ml / 20 L water to get rid of the newly emerged weeds on the plots. Subsequent weed control was done manually using a hoe. Insect attack on cowpea was noticed at about 3 WAP and Sherpaplus 280EC (active ingredient—30 g/l Cypermethrin and 250 g/l Dimethoate) was applied at the rate of 80-100 ml / 20 L water depending on the severity of the attack.

Data collection

Harvesting at 50% podding and xylem sap collection for ureide- N analysis

At 50% podding, 5 plants were randomly sampled; at three points per plot using a 30 cm × 30 cm quadrant. the shoots were cut below the first node at about 3 cm above the ground with the aid of secateurs. A latex rubber-tubing sleeve of about 2 cm long about the stem diameter or slightly smaller than the stem was placed over the cut stump. With the aid of a clean and dry syringe, xylem sap was collected from the tubing sleeve reservoir and injected into a labelled vacutainer containing 95% ethanol of equal volume with the sap (i.e 1 ml of sap randomly sampled from five plants was added to 1 ml of ethanol). The ethanol and the sap were shaken together to avoid sagging and kept chilled for Ureide – N and NO_3 – N analyses in the laboratory.

Nodule collection and root sampling for AM colonization: The remaining part of the plants were uprooted, the nodules were detached, counted and weighed to get the fresh nodule weight and oven-dried at 78^{o}C to a constant dry weight. Approximately 5 g of the fine root of the 5 sampled plants were taken fresh in the field, washed and preserved in 50% ethanol for AM colonization. The AM colonization was done in IITA Soil Microbiology Laboratory using the Grid-line Intersection method (Giovanetti and Mosse, 1980).

Sampling for stem + petioles tissue extraction for ureide - N analysis

The leaves of the five sampled plants per plot were detached fresh from stem + petioles. The stem + petioles were weighed fresh, air-dried for 72 hours, oven-dried at 78^{o}C to constant dry weights and ground. Sub-samples were taken for tissue extraction (Hot water extract) for Ureide – N and NO_3 – N analyses in the laboratory. The procedure followed was as outlined by Herridge (1982). Sub-samples were also taken from leaves, stem + petioles for total crop N and P analyses (% N and % P in the shoots) in the laboratory using the method of Herridge (1982).

Harvesting at physiological maturity: At physiological maturity, cowpea pods were picked and weighed to get fresh pod weight Dry pods were threshed, winnowed and sun-dried to obtain field grain yield.

Soil and grains sampling: Five core soil samples were taken from each plot after harvesting, while fifty grains of each cowpea variety planted were taken for % N and % P analyses in the laboratory.

Statistical analysis*:* Data were analyzed using analysis of variance (ANOVA) PROC GLM of Statistical Analysis System (SAS, 2003), least significant differences and Duncan Multiple Range Test (DMRT). Means were separated using Least Significance Difference (LSD) at P<0.05. Standard Errors of Mean was reported for the interaction effect.

3.0 Results and Discussion

Analysis of variance: The analysis of variance showed that inoculation significantly influenced the number of nodules/ plots in each of the study sites (Table 2). More nodules were formed by cowpea variety IT97K568-18 than cowpea variety IT89KD-288 (Fig 1). When similar inoculation treatments were compared between cowpea varieties IT97K568-18 and IT89KD-288, significantly (P<0.05) higher numbers of nodules were formed by cowpea variety IT97K568-18 treatments. The number of nodules formed by inoculated cowpea variety IT97K568-18 was more than 100% higher compared to that of cowpea variety IT89KD-288 except under non-inoculated treatment. In both varieties, isolate IDC8 formed a significantly (p<0.05) higher number of nodules than other strains (Table 2).

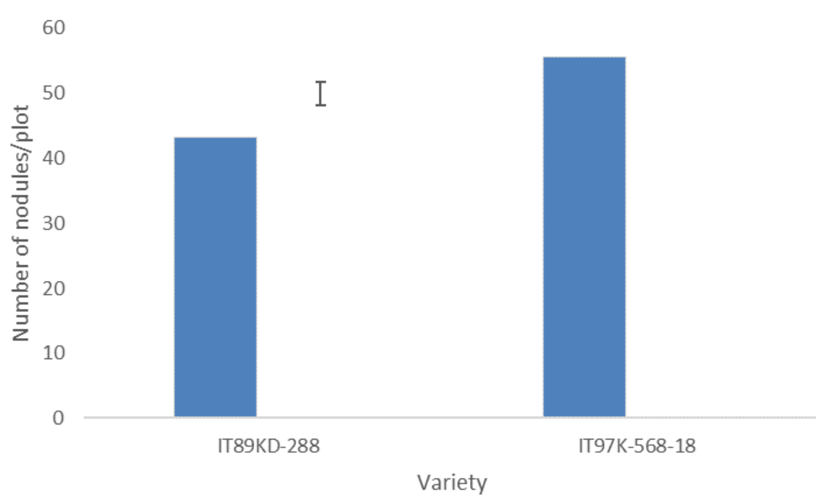

Fig 1: Number of nodules per plot (9 m^2) in two varieties of cowpea. Bar represents SE

Table 2: Effect of rhizobial inoculations on cowpea nodulation, N fixed (hot water) (kg/ha), biomass and grain yields (t/ha) in Idi-Ayunre field

Variety	Strain	Number of nodules/plot
IT89KD-288	OIa6(c3a)	29.7
	IDC8	89.3
	TRC2	41.0
	R25B+IRj2180A	19.3
	control	13.7
IT97K 568-18	OIa6(c3a)	67.0
	IDC8	122.3
	TRC2	71.3
	R25B+IRj2180A	38.3
	control	21.0
LSD		
Variety (V)		17.7
Strain (S)		28.0
V×S		39.4
ANOVA		
Variety (V)		**
Strain (S)		***
V×S		Ns

ns = not significant; ***, ** and * = $p<0.001$, 0.01 and 0.05 respectively

Table 3 . Number of nodules in cowpea as affected by rhizobial inoculation in three locations

	Location			
Rhizobial Inoculation	ID	OI	TR	Mean
OIa6(c3a)	48.33	73.00	59.83	60.39
IDC8	105.83	34.33	67.33	69.17
O	17.33	22.67	22.83	20.94
R25B	28.83	51.33	43.00	41.06
TRC2	56.17	66.00	44.00	55.39
Mean	51.30	49.47	47.40	
SE	9.94			

Multiply SE by 2.78 to separate the means for comparing interactions. It should not be used for comparing main effect means

Table 4: Effect of rhizobial inoculation on nodulation, nutrient uptake and N fixation

Rhizobial Inoculation	Total N Fixed (kg/ha)	Total N Uptake (kg/ha)	Total P Uptake (kg/ha)	Number of nodules/plot
OIa6(c3a)	72.76a	124.45a	11.23a	60.39ab
IDC8	60.26b	103.37bc	9.50b	69.17a
O	49.14c	89.33d	8.07c	20.94d
R25B	57.74b	98.70cd	9.33bc	41.06c
TRC2	68.43a	112.03b	10.01ab	55.39b

Table 5: Analysis of variance showing level of significance of the main and interaction effects

Source of Variation	Total N Fixed	% ndfa1sap	Total P uptake	Total N uptake	Grain P uptake	Grain N uptake	Shoot P uptake	grain yield	No of nodules	Shoot N uptake	shoot dry wt
Location	***	***	***	***	***	***	***	***	ns	***	***
Variety	***	ns	**	***	***	***	Ns	***	*	ns	ns
Strain	**	ns	*	*	*	ns	Ns	Ns	***	ns	ns
Location ✻ Variety	***	ns	**	***	***	***	Ns	***	ns	ns	ns
Location ✻ strain	*	ns	Ns	ns	ns	ns	Ns	Ns	***	ns	ns
Variety ✻ strain	Ns	ns	Ns	ns	ns	ns	Ns	Ns	ns	ns	ns
Location ✻ Variety ✻ strain	*	ns	Ns	ns	ns	ns	Ns	Ns	ns	ns	ns

The numbers of nodules formed by isolate IDC8 in cowpea varieties IT89KD-288 and IT97K568-18 were approximately 117% and 72% higher than any other strain. The least number of nodules was recorded in both varieties when there was no inoculation (Table 2). With IDC8 inoculation more nodules were formed in IA and OI locations. And across the locations, IDC8 formed a significantly higher ($p<0.05$) number of nodules compared to R25B +IRj2180A, TRC2 and the control (Table 3 & 4).

The total N fixed by OIa6(c3a) and TRC2 were not significantly different but were significantly higher ($p<0.05$) compared to IDC8 which formed the highest significant number of nodules (Table 3) The % nitrogen derived from the atmosphere (% NDFA) ranged between 50 – 74 %. However, the total N fixed (kg/ha) was significantly higher in rhizobial strains O1a6 (c3a) and TRC2, 72.8 kg N/ha and 68.4 kg N/ha, respectively, compared to the control (49 kg N/ha). The total N uptake in cowpea inoculated with OIa6(c3a) was significantly higher ($p<0.05$) compared to all other strains inoculated, N uptake of R25B+IRj2180A and that of the control were not significantly different from one another (Table 4). Across the locations, the total N uptake by the two varieties was significantly higher ($p<0.05$) when inoculated with OIa6(c3a) compared to other strains were different (Table 4). Like the N uptake, OIa6(C3a) inoculated plants was higher than other inoculated treatments when the P uptake of both varieties was considered (Table 4.). Under IT97K568-18, OIa6(C3a) inoculated plant was significantly ($p<0.05$) higher than other strains in P uptake except that of TRC2. Analysis of variance showing the level of significance of the main and interaction effects (Table 5).

Symbiotic effectiveness of a strain is often associated with its infectivity which is the ability of the strain to formed nodules. It is expected that the higher the number of nodules formed by a rhizobial strain, the higher the N_2 fixed. But from this study, it was observed that in some cases infectivity of the introduced strains or the numbers of nodules formed was not the true representation of symbiotic effectiveness of the strains as it happened with IDC8 which formed a significant higher number of nodules compared to OIa6(C3a), whereas OIa6(C3a) and TRC2 happened to fix N significantly higher than IDC8. Inoculated strains with a large number of nodules and those with few numbers of nodules were not different in terms of N-fixed. Where the strains that nodulate a legume are not efficient, the presence of nodules on plant roots may not necessarily mean that a sufficient quantity of N_2 is being fixed for the host plant's benefit (Cardoso and Kuyper, 2006). And this could be due to mispairing of the legumes and the rhizobia strains (Bernal and Graham, 2001). This implies that the number of nodules formed by the strain on the legumes may lead to an erroneous assessment of the effectiveness of the strains.

Nitrogen-fixing efficiencies of the indigenous isolates and the exotic strains differ with locations, in that the percentage of ureide N_2 fixed in all the legume cultivars planted were significantly higher in Idi-ayunre than in Orile-Ilugun and the University of Ibadan Teaching and Research farm. This is an indication that the symbiotic properties of each strain from each location also differ. Orile-Ilugun and University of Ibadan Teaching and Research farm were similar in the percentage N fixed. This similarity was also observed in the rhizobia population of the two locations. However, in Idi-ayunre where the rhizobial population was found to be lower compared to those of the other locations, inoculated strains performed excellently better. This is likely to be a result of the difference in soil properties (which did or did not support nodulation and N_2 fixation) and the rhizobia population of each location. Slattery *et al. (*2001) reported that soil environmental constraints and edaphic factors such as soil temperature do affect nodulation. Giller and Wilson (1991) reported that the most significant environmental factor limiting nodule occupancy of an introduced strain is the size of the indigenous rhizobial population in the soil. The percentage ureide N_2 fixed by the strains (strain effectiveness) also differs with different legume cultivars.

4.0 Conclusion

The indigenous rhizobial isolates OIa6(c3a) and TRC2 had potentials for higher N fixation for cowpea production in the rainforest of Nigeria. While indigenous rhizobial isolates IDC8 is a good nodulator of cowpea, Co-inoculation of R25B and IRj2180A in field trial was not efficient at increasing nodulation and grain yield of the two cowpea varieties. Further studies on the performance of co-inoculation of the indigenous isolates in soil with the low and high rhizobial count are suggested.

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