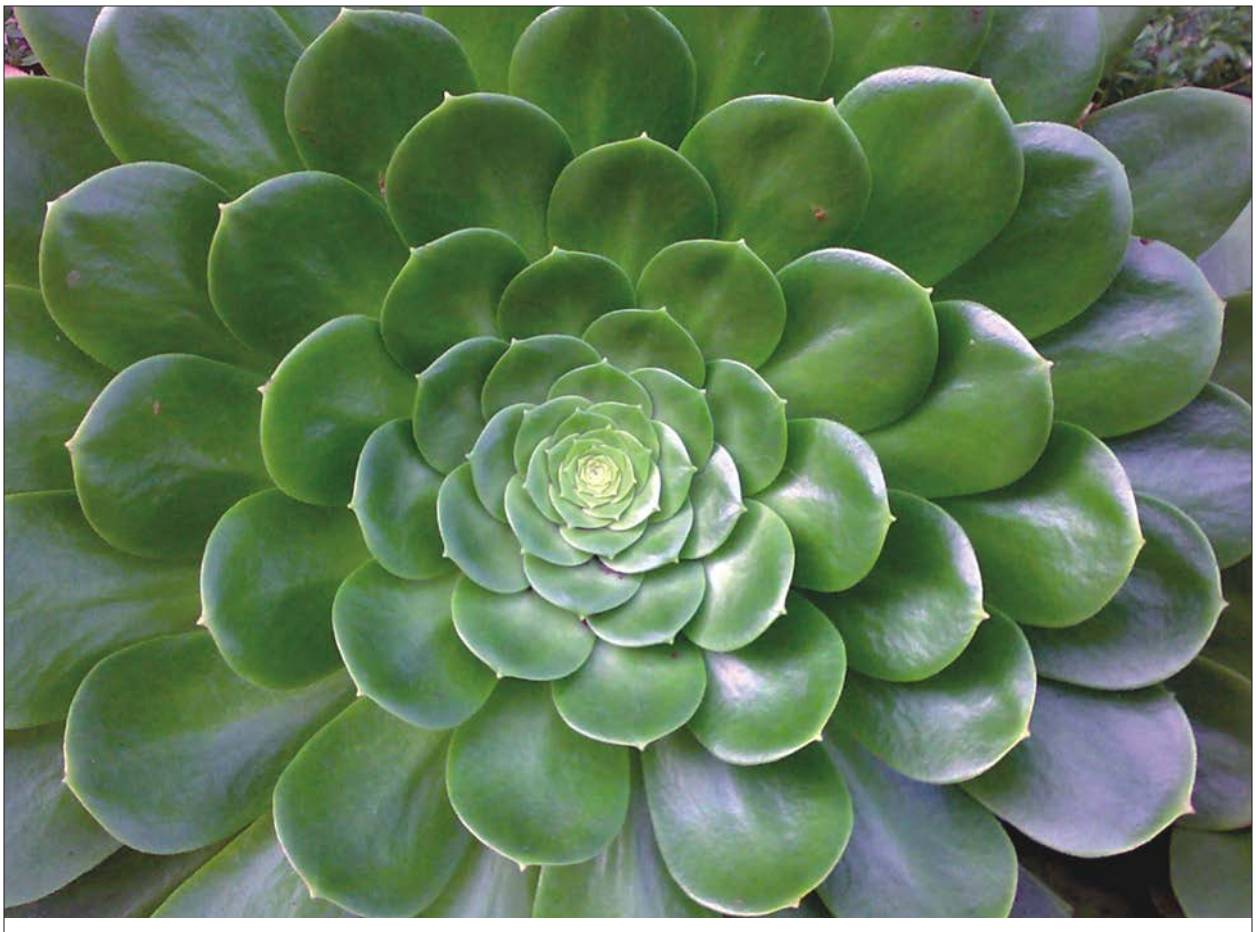

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Research Article
Occurrence of Nodule Occupancy in *Rhizobium*-Cowpea Symbiosis in Adamawa-Cameroon

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Abstract

Background and Objective: Cowpea (*Vigna unguiculata* L. Walp.) is an important food grain legume in adamawa region of Cameroon and its production can be enhanced using efficient and competitive strains of *Rhizobium* inoculum. Furthermore, the success of inoculation practices depends on the competitive capacity of strains for nodule occupancy. Hence, the aim of this study was to assess the nodules occupancy by the indigenous rhizobia in 2 cowpea varieties (FEKEM and BRI) and their effectiveness in cowpea nodulation. **Materials and Methods:** *Rhizobium* strains were trapped from nodules of BRI and FEKEM cowpea varieties, cultivated in pots filled with in 2:1 (soil:sand) ratio. Collected nodules were segregated in morphotypes from which *Rhizobium* were isolated. From the morphocultural characteristics, individual isolates were determined and the occupancy rate was calculated. **Results:** Three nodules morphotypes were obtained namely spherical (M1), amorphous (M2) and agglomerated (M3). A pool of 27 isolates (11 from BRI and 16 from FEKEM) was obtained on YEMA medium. All isolated strains had phenotypical, morphological and cultural characteristics of the genus *Rhizobium*. Although some strains were from all nodules morphotypes (FRS1, FRS4, FRS6, BRS1, BRS4 and BRS7), others were only present in nodule morphotype M1 (FRS2, FRS7, FRS12, BRS3 and BRS5), M2 (FRS14, BRS8 and BRS9) and M3 (FRS7, FRS11, FRS15, FRS16, BRS10 and BRS11). Nodule occupancy varied from 11.11 to 100%. **Conclusion:** FRS1 and BRS4 strains were the most competitive strains with an occupancy rate of 100%. This study is a step forward for the selection of the competitive and efficient strains to boost the biological nitrogen fixation by cowpea plants.

Key words: *Rhizobium* strains, nodule occupancy, nodule morphotypes, cowpea varieties, cameroonian soils

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nitrogen is an important nutrient for plant and is a limiting factor for agriculture because of its limited quantity in the soil[1]. In some cases, 75% of the observed yields improvement can be attributed to nitrogen nutrition[2]. Averagely, 150-200 million t of synthetic fertilizers are used each year for global agricultural production. Whereas only 30-50% of widespread inorganic nitrogen fertilizers are used by crops, the rest is lost through volatilization, denitrification or leaching of nitrates into groundwater[2].

Crop legumes play a critical role in natural ecosystems, agriculture and agro-forestry[3], since they are equipped with the facility to acquire a major portion of nitrogen directly from the atmospheric N_2 pool through bacterial fixation. This ability to fix nitrogen in association with bacteria and to colonize low-nitrogen environments makes them an obvious choice for use in agriculture within semi-arid or arid areas[3]. Thus, biological nitrogen fixation has been recommended for the sustainable traditional agriculture[4,5] and can help poor farmers, who are unable to afford the expensive synthetic fertilizer[6]. Inoculation with the most efficient N-fixing bacteria specific to crop legumes is necessary to help maximizing nodulation and N-fixing ability[7] as well as to avoid the inoculation failure. In fact, rhizobia are randomly distributed in growing soils where they compete with other indigenous strains[8], which often form less efficient nodules[9]. Thus the number of strains in nodules may fluctuate according to soil and crop legume species[6]. Inoculant strains of rhizobia selected or engineered for the improvement nitrogen fixation ability usually do not achieve nodule occupancy in soils that already contain active indigenous populations of rhizobia[10]. Therefore, it is a necessity to identify the competitive and effective strains that are involved in process. The Determination of nodule occupancy is a key approach to the study of the nodulation competitiveness of rhizobia strains and the associated quality of commercial bacterial preparations[11]. Therefore, the main goal of this study was to determine the nodules occupancy in cowpea and the impact of nodules shapes on the biological nitrogen fixation.

MATERIALS AND METHODS

Description of the experimental site: The field trial was carried out during the 2018 cropping season at Dang (Ngaoundere 3), which stretches from latitude 7°31′ N to longitude 13°37' E. The area belongs to the Guinea Savannah zone of Cameroon. The rains last for 6-7 months starting from April-October, whereas the dry periods last for 5-6 months starting from mid-November. The annual rainfall, temperature, relative humidity are respectively 1600 mm, 7-35°C and 37.7-81%. The soils of ferralitic type developed on the basalt layer. The laboratory work was carried out at the Microbiology section of IRAD-Wakwa.

Fig. 1(a-b): Cowpea seeds varieties used (a) FEKEM "black eyes" and (b) BRI "brown eyes"

Trapping of rhizobia: Rhizobia were trapped in nodules using 2 cowpea varieties (Fig. 1) with a life-cycle varying from 80-85 days (FEKEM) to 75-80 days (BRI)obtained from IRAD-Garoua. FEKEM variety is characterized by semi-erected stem, while BRI variety is erected. They were cropped in pots using soil collected from fields where cowpea were cultivated[12] soils were sampled at different points and mixed in a composite sample, before immediate transportation to the experimental site. Soil samples were taken from the top 0-20 cm soil depth. To these soils, sterile sand was mixed in the ratio 2:1 (w/w) to obtain the sowing substrate. Sand was added to avoid substrate compaction, facilitate roots penetration and oxygen circulation as well as increased porosity. The substrate was packaged into 2 kg black plastic bags[6,13]. The experimental setup was a complete block

design with 2 treatments (cowpea varieties FEKEM and BRI). The size of the blocks was respectively 1×4.5 m area for FEKEM variety and 1.5×4.5 m area for BRI variety. Each block consisted of 30 pots placed on 75 cm between the lines and 50 cm between the pots for the variety FEKEM and 50cm between the lines and 50 cm between the pots for the variety BRI. Three seeds were sown using a stick in 3 different holes of 3 cm depth/pot in which substrate were previously watered with drilling water. Two weeks after germination, seedlings were thinned to one/pot before their growth development[14].

Assessment of nodulation, plant size and biomass: Data were collected on all the 60 plants at 50 days after sowing. Plants of each cowpea variety were labeled separately and according to the position in the blocs. The size of the primary stem of each plant was taken with an ordinary ruler (30 cm). As far as nodulation is concerned, all the plants were harvested one by one, to carefully remove nodules. Hence, pots were carefully turned over without touching the substrate, then, plastics were destroyed to clear nodules that were collected separately and counted. All nodules from the same plant were stored together. The root system of each plant was washed with tap water and the entire plant was kept into transparent plastics to avoid the dispersion and mixture plants leaves.

Plants and nodules were dried separately. Labeled envelopes were used for nodules, while ‹‹baba Ghana›› bags were used to store plants. Both were exposed under sunlight for 7 days. Then the drying process was completed in a hot air oven for 2 h at 100°C. Dried nodules/plant and dried plants were weighed separately using an analytical balance with 0.001 sensibility.

Nodules were segregated into 3 morphotypes (M1, M2 and M3) according to the criterium shapes: M1 for spherical nodules, M2 for amorphous nodule and M3 for agglomerated nodules.

Evaluation of nodule efficiency: Sterilized nodules were cut opened with a sterile razor blade, to observe the inner coloration. The red or pink were considered as efficient, while whitish were considered as not efficient[6,14]. The efficiency of nodules was calculated using formula, (1) expressing the efficient nodules (%) within a morphotype, whereas formula (2) assesses the efficient nodules (%) of a morphotype within the total nodules of a given plant variety.

$$\text{Efficiency of nodules in a morphotype (\%)} = \frac{\text{Number of efficient nodules in a morphotype}}{\text{Total number of nodules of this morphotype}} \times 100 \quad (1)$$

$$\text{Total efficiency of nodules morphotype (\%)} = \frac{\text{Number of efficient nodules in a morphotype}}{\text{Total number of efficient nodules in plant variety}} \times 100 \quad (2)$$

Isolation of *Rhizobium* from different nodules morphotypes: Sun-dried nodules were washed separately under tap water to remove soil, transferred into sterile water and kept in the refrigerator at 4°C overnight to absorb water[15]. The following day, nodules were removed from the refrigerator, soaked for 1 h into distilled water at room temperature[15]. Rehydrated nodules were sterilized using the method described by Ngakou *et al.*[6] (modified), in which 0.1-0.2% $HgCl_2$ was replaced by the commercial sodium hypochlorite[15,16]. All nodule morphotypes were submitted to 95% alcohol for 30 sec, rinsed with 6 changes of sterilized distilled water to remove all traces of alcohol then in sodium hypochlorite for 5 min, followed by 6 rinses with several changes of distilled water to remove all traces of sodium hypochlorite.

Three efficient nodules per morphotype with 3 assays (3×3) were selected and crushed in a sterile petri dish using a sterile forceps, then the content was streaked onto YEMA solid medium (10 g mannitol, 0.2 g $MgSO_4$, $7H_2O$, 1 g yeast extract, 0.5 g, KH_2PO_4/K_2HPO_4, 0.1 g NaCl, 1 L of distilled water, 15 agar pH = 6.8)[6,16]. Each labeled culture had 3 essays and each assay was repeated 3 times. Petri dishes streaked with nodule preparation were incubated at room temperature for 3 days in an inverted position and observed[6]. After this incubation period, separated colonies with distinct phenotypic characteristics were purified by picking up and aseptically sub-culturing in YEMA solid medium before incubation at room temperature for 3 days.

Characterization of isolated strains: Three days after incubation, all streaked petri dishes were observed and all the formed colonies were counted. Differences between colonies were assessed based on their color, aspect, edges and shape[6,15]:

- **Congo red absorption:** All the isolates were streaked on YEMA+0.25% Congo-red (diphenyl diazo-bis-oL-naphthylamine sulfonate) and incubated in the dark[15], at room temperature for 3 days. The strains that did not absorb Congo-red appeared whitish, whereas those that slightly absorbed were pinkish, while strains that absorbed were red[17,18]
- **Growth at pH 11:** The culture medium was adjusted with 0.1 N NaOH to pH 11 before autoclaving. All the isolates were submitted to growth at this pH and incubated at 28°C for 3 days[19]

Table 1: Differential growth and nodulation between FEKEM and BRI cowpea varieties

Variables	Number of nodules/plant	Nodules dry weight/plant (g)	Efficiency of nodules (%)	Plant size (cm)	Plant dry weight (g)
FEKEM	23.86±1.69[a]	0.156±0.012[a]	77.71±4.79[a]	10.89±0.28[a]	2.93±0.14[a]
BRI	33.93±3.15[b]	0.154±0.013[a]	45.79±3.57[b]	18.98±0.88[b]	5.05±0.23[b]
p-value	0.006	0.9	0.0001	0.0001	0.0001
LSD	10.06	0.002	31.92	8.08	2.11

For each parameter values on the column affected by the same letter are not significantly different at the indicated level of probability

Gram staining: This test carried out to confirm the Gram status of isolated rhizobia. According the method described by Somasegaran and Hoben[20], a fresh drop sample of colonies was taken and spread over on a slide in a drop of sterile water and allowed to air drying. After drying, the Gentian violet solution was applied and let to act for 1 mn. Gentian was eliminated with a solution of lugol for 30 sec and rinsed with sterile water, then decolored with alcohol (90°), rinsed against with sterile water. Fuchsine was added for 30 sec, rinsed and allowed to dry on the slides. Slides were observed under a light microscope within immersion oil. Under these conditions, all the bacteria stained by the first dye (Gentian violet) are known to be Gram positive, while those stained by the second dye (Fuchsine) are Gram-negative.

Determination of nodule occupancy and nodulation capacity: The morpho-cultural characteristics were used to count and distinguish individual strains. The average number of colony/nodule was calculated by formula, (3) while the nodule occupancy was estimated with formula, (4) which is similar to the formula used by Khan *et al.*[21]. The total number of tested nodules per cowpea variety was 9 and the nodule occupancy was calculated for each cowpea variety:

$$\text{Colony number/nodule} = \frac{\sum(\text{Colony number/repetition})}{\text{Total number of repetition}} \quad (3)$$

$$\text{Nodule occupancy (\%)} = \frac{\text{Number of nodule occupied by a strain}}{\text{Total number of tested nodule}} \times 100 \quad (4)$$

Statistical analysis: Data was ranged on Excel Microsoft office program and subjected to analysis of variance (one-way-ANOVA) and correlation test using a Statgraphic Plus, version 5.0 (SIGMA PLUS) computer program. ANOVA was used to find out the means of nodules and nodule dry weight. Means were compared between treatments using the Duncan Multiple range test at 5% level.

RESULTS AND DISCUSSION

Differential nodulation between the two cowpea varieties: All plants from the 2 cowpea varieties developed nodules in the soils. Irrespective to their size and shape nodules were harvested and counted manually. In general, nodulation of the BRI variety was the best, while FEKEM produced very few nodules (Table 1). The average number of nodules was 23.86±1.69 and 33.93±3.15 for FEKEM and BRI varieties, respectively. The one-way ANOVA indicates that the nodule number varies significantly among both varieties ($p = 0.0067$). These results indicate that, although native cowpea varieties have a great nodulation capacity, the nodulation potential of different varieties may vary greatly[22].

Nodules dry weight varied from 0-0.26 g (FEKEM) and from 0.02-0.29 g (BRI). There was not significance difference ($p>0.05$) between the nodule weight of these two cowpea varieties, although nodule number from BRI was higher than that of FEKEM variety (Table 1). This could be explained by the fact that FEKEM nodules were bigger than BRI nodules.

Despite the poor number, nodules from FEKEM cowpea variety were more efficient in nitrogen fixation than those from BRI variety (Table 1). There was statistically significant difference ($p<0.0001$) between the number of efficient nodules of the 2 cultivars. This finding is similar to the reported production of highest number of efficient nodules/plants of one plant variety, which was able to fix the highest amount of nitrogen[22].

As far as morphotype distribution is concerned, 3 shape types were recorded (Fig. 2): spherical (M1), amorphous (M2) and agglomerate (M3). The number of nodule with M1, M2 and M3 shapes statistically differed in FEKEM and BRI varieties (Table 2). The nodule dry weight was significantly different between morphotypes of FEKEM variety, although the dry weight of nodules between morphotypes M2 and M3 were similar. Nodules morphotypes M2 and M3 were more efficient than those of M1, although the number of nodules morphotype M1 was greater than those of M2 and M3 of both cowpea varieties (Table 2).

Rhizobia strains isolated from FEKEM and BRI nodules: All colonies formed on YEMA were detected and displayed in three nodule morphotype groups from which they were isolated. Figure 3 presents the number of different *Rhizobium* strains isolated per morphotypes. In morphotypes M1 and M2, the number of *Rhizobium* strains was statistically

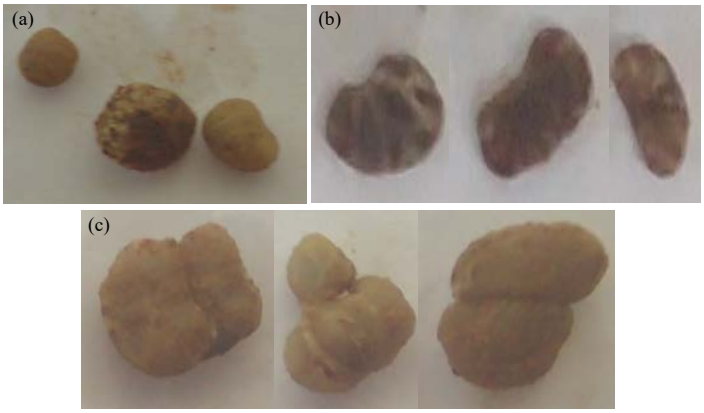

Fig. 2(a-c): Cowpea nodules shapes, (a) M1 (spherical), (b) M2 (amorphous) and (c) M3 (agglomerate)

Table 2: Variation of the distribution of nodules number, nodules dry weight and efficiency within nodule morphotypes of each cowpea variety

Cultivars	Morphotypes	Nod number/ Morphotype	Nod dry weight/ Morphotype	Efficient Nod/ Morphotype	Efficiency/ Morphotype (%)	Total efficiency (%)
FEKEM	Spherical	16.26±1.5^{c}	0.093±0.009^{c}	13.03±1.4^{c}	80.13	64.34
	Amorphous	5.96±0.75^{b}	0.062±0.009^{b}	5.66±0.78^{b}	94.96	27.95
	Agglomerate	1.63±0.23^{a}	0.021±0.003^{a}	1.56±0.23^{a}	95.70	7.70
	p-value	<0.0001	<0.0001	<0.0001		
BRI	Spherical	28.66±1.5^{b}	0.143±0.012^{b}	12.93±1.3^{b}	45.11	86.20
	Amorphous	2.66±0.40^{a}	0.031±0.09^{a}	1.80±0.19^{a}	67.66	11.69
	Agglomerate	1.33±0.20^{a}	0.016±0.027^{a}	0.66±0.84^{a}	49.62	4.28
	p-value	<0.0001	<0.0001	<0.0001		

Nod: Nodule, for each cowpea variety and for each parameter values on the same column affected by the same letter are not significantly different between nodule morphotypes

more elevated ($p<0.001$) in FEKEM than in BRI variety. In morphotype M2, there were more *Rhizobium* strains from BRI than FEKEM variety. The greatest number of *Rhizobium* strains from FEKEM variety was 11 compared to 8 for BRI variety. The lowest number of *Rhizobium* were 6 and 5/nodule morphotype from FEKEM and BRI variety, respectively. These results indicates that the number of *Rhizobium* isolates might be influenced by the nodule shape but no correlation was found between the prototypical classification of bacteria and nodule shape[23].

On the basis of identical morphocultural characters, *Rhizobium* strains of the same cowpea variety were grouped. Sixteen strains were obtained from FEKEM and 11 from BRI (Table 3). These characteristics line with those of indigenous rhizobia previously reported to be isolated from Ngaoundere soil[6].

Cultural characteristics of rhizobia isolates: All *Rhizobium* strains from BRI and FEKEM plants varieties were negative or weakly positive to Congo-red staining. *Rhizobium* strains were reported to absorb very little not or not at all Congo-red, when grown on YEMA+Congo-red medium[15,17,18]. *Rhizobium* strains isolated from both cowpea varieties had no or very weak growth on YEMA at pH11. *Agrobacteria* was shown to grow at higher pH values than rhizobia and therefore their growth in YEM broth with elevated pH of 11.0 is considered as a useful means to distinguish between the 2 allied genera[19]. Isolated strains were all negative to Gram staining, confirming another character of genus *Rhizobium* (Table 4).

Determination of nodule occupancy: The average number of *Rhizobium* strains/nodule irrespective to the morphotype was 3±1. Table 5 presents the nodules occupancy rate. Nodule occupancy by these native strains of rhizobia varied between 11.11 and 100%. *Rhizobium* strains FRS1 and BRS4 showed a greater occupancy (100%) flowed by FRS4 and BRS1 (77.77%) and BRS7 (66.66%). FRS1 and BRS4 strains were the most competitive, therefore possess a better potential for use as inoculant for increased cowpea production as recently pointed out[24]. The native rhizobia are more persistent and well adapted to local conditions, this gives them added advantage of competing successfully at the expense of introduced strains for nodule occupancy[25]. Moreover, while testing 2 *Bradyrhibium japonicum* strains,

Table 3: Phenotypical characteristics of isolated strains and their distribution/cowpea variety

Varieties	Strain	Strain number	Shapes	Aspect	Colour	Edges
FEKEM	FRS1	16	Spherical	Translucent		Regular
	FRS2		Spherical joint	Humid/milky	White	Regular
	FRS3		Spherical	Humid/pasty/central mark	White	Regular
	FRS4		Spherical	Humid/milky	Whitish	Regular
	FRS5		Spherical	Humid/pasty	Whitish	Regular
	FRS6		Spherical	Humid/central mark	Whitish	Regular
	FRS7		Spherical	Humid/milky/domed	Whitish	Regular
	FRS8		Spherical	Humid/central mark/pasty	Greenish	Regular
	FRS9		Spherical	Humid/central mark	Yellow	Regular
	FRS10		Spherical	Humid	Yellowish	Regular
	FRS11		Oval	Humid/pasty	Whitish	Regular
	FRS12		Spherical	Humid	Yellow	Regular
	FRS13		Oval	Humid/translucent		Regular
	FRS14		Spherical	Humid/milky	Whitish	Irregular
	FRS15		Deformed	Humid	Yellow	
	FRS16		Spherical joint	Humid	White	Regular
BRI	BRS1	11	Spherical	Humid/milky	Whitish	Regular
	BRS2		Spherical	Humid/milky	Yellowish	Regular
	BRS3		Spherical	Humid	Whitish	Irregular
	BRS4		Spherical	Humid/translucent		Regular
	BRS5		Deformed	Humid/translucent		
	BRS6		Oval	Humid/milky	Whitish	
	BRS7		Spherical	Humid/milky/central mark	Whitish	Regular
	BRS8		Spherical	Humid/central mark	Yellow	Regular
	BRS9		Spherical	Humid	Pink	Regular
	BRS10		Spherical	Humid/translucent/central mark		Regular
	BRS11		Spherical joint	Humid/translucent		Regular

Table 4: Cultural characteristics of rhizobia isolated from FEKEM and BRI cowpea varieties

	Rhizobia strains isolated from nodules of FEKEM variety															
Characteristics	FRS1	FRS2	FRS3	FRS4	FRS5	FRS6	FRS7	FRS8	FRS9	FRS10	FRS11	FRS12	FRS13	FRS14	FRS15	FRS16
FEKEM cowpea variety																
Congo-red	-	±	±	-	±	±	±	-	-	±	±	-	±	±	±	±
pH 11	±	-	-	±	±	±	-	±	-	±	±	-	-	-	±	-
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BRI cowpea variety	BRS1		BRS2		BRS3		BRS4		BRS5		BRS6		BRS7	BRS8	BRS9	BRS10	BRS11
Congo-red	-		-		-		-		-		-		-	-	-	-	-
pH 11	±		±		±		±		-		-		±	±	-	±	±
Gram stain	-		-		-		-		-		-		-	-	-	-	-

±: Very weak absorption of Congo-red or very weak growth at pH11, -: Gram negative/no absorption of Congo-red/or no growth at pH11

it was found that when efficient and inefficient strain were used alone, nodule occupancy was respectively 100 and 0%, whereas when they were mixed together, nodule occupancy ranged between 0 and 100% according to the host plant[26]. A previous reported research has shown that when inoculated at the equal proportion, some fast-growing strains could produce 95% of nodules than slow-growing strains[27]. In a related study, revealed that 73-93% of nodules were occupied by the inoculated strains, against 7-27% occupancy of nodule by the non-competitive indigenous strains[21]. This result is in agreement with those of the present study and indicates that all the strains with nodule occupancy rate lower than 50% were non-competitive indigenous strains.

From Table 5, *Rhizobium* strains were distributed according to nodule morphotypes from which they were isolated. Some strains were common to all the 3 nodule morphotypes (FRS1, FRS4, FRS6, BRS1, BRS4 and BRS7), while others were only present in a single nodule morphotype M1(FRS2, FRS7, FRS12, BRS3 and BRS5), M2 (FRS14, BRS8 and BRS9) and M3 (FRS7, FRS11, FRS15, FRS16, BRS10 and BRS11).

Table 5: Nodule occupancy and strains number of *Rhizobium*/morphotype

Strains	Morphotypes			Nodule occupancy by native rhizobia strains (%)
	Spherical	Amorphous	Agglomerate	
FRS1	8	7	8	100.00
FRS2	2	0	0	44.44
FRS3	1	0	1	44.44
FRS4	7	2	2	77.77
FRS5	0	0	7	44.44
FRS6	1	1	1	33.33
FRS7	1	0	0	11.11
FRS8	1	2	0	33.33
FRS9	1	0	0	11.11
FRS10	1	1	0	22.22
FRS11	0	0	1	11.11
FRS12	1	0	0	11.11
FRS13	0	1	2	33.33
FRS14	0	1	0	11.11
FRS15	0	0	1	11.11
FRS16	0	0	2	22.22
Total	24	15	25	
BRS1	9	7	2	77.77
BRS2	2	1	0	33.33
BRS3	1	0	0	11.11
BRS4	6	6	9	100.00
BRS5	1	0	0	11.11
BRS6	1	1	0	22.22
BRS7	3	4	2	66.66
BRS8	0	2	0	11.11
BRS9	0	1	0	22.22
BRS10	0	0	6	33.33
BRS11	0	0	1	11.11
Total	23	22	20	

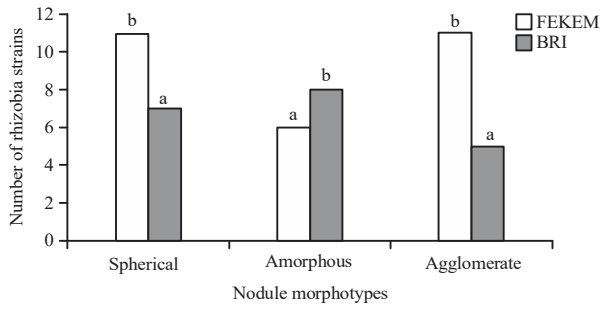

Fig. 3: Rhizobia strains/nodule morphotypes from the 2 cowpea varieties

For each nodule morphotype, bars affected with the same letter are not significantly different between the 2 cowpea varieties

CONCLUSION

The outcome of this study is that cowpea in Adamawa region of Cameroon has the ability to nodulate with a high diversity of rhizobia that grow within different nodules morphotypes. *Rhizobium* isolates from different nodule morphotypes of cowpea were phenotypically different. Isolates obtained may be useful to increase the symbiotic nitrogen fixation in crop legumes. The obtained results provides the basis for further research on the phylogeny of *Rhizobium* strains nodulating cowpea as well as their use as inoculants to improve growth and nitrogen fixation of this crop in Adamawa region. However, molecular studies are needed to confirm the identity of isolates, while ecological studies are necessary to determine the relationship between *Rhizobium* strains inhabiting the same nodule.

SIGNIFICANCE STATEMENT

This study has discovered the diversity of rhizobia in different cowpea nodule morphotypes referring to as nodule occupancy. Hence, when producing Rhizobium biofertilizer, researchers should isolate strains from different morphotypes prior to their characterization. Such Rhizobium biofertilizers could compete the market of biofertilizers, so as to boost the *Rhizobium*-crop legumes symbiosis, thus the biological nitrogen fixation and the improvement of soil fertility in nitrogen, that could benefit the succeeding cereal crops in the context of research sustainability.

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