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Assessment of indigenous *Bradyrhizobia* spp. population levels in low phosphorus soils of southern Cameroon using cowpea (*Vigna unguiculata* L. Walp) as trap crop

ATEMKENG Maureen Fonji[1,2]* and B. Aime Didier BEGOUDE[3]

[1]Institute of Agricultural Research for Development (IRAD), Leguminous and Vegetable Crops Program, Cameroon.
[2]West African Center for Crop Improvement (WACCI), University of Ghana, Ghana.
[3]Institute of Agricultural Research for Development (IRAD), Regional Biocontrol and Applied Microbiology Laboratory, Cameroon.

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This study used three cowpea (*Vigna unguiculata* L. Walp) varieties, (DschMMBr, Vyuniebe and 58-77) as trap crops to estimate the population of indigenous *Bradyrhizobia* spp. Soil samples were collected in two sites (Nkoemvone and Nkometou) of Southern Cameroon known to accommodate acid soils with low phosphorus (P) levels, and the population of the indigenous rhizobia was determined using the most probable number (MPN) plant infection technique. The results of the MPN counts indicated that the total *Bradyrhizobia* population in Nkoemvone was between 1.0 and 5.8 × 10^5 cells per gram of soil sample while in Nkometou, it was between 5.8 x10^3 and 1.0 x10^4 cells per gram of soil sample. Using the cowpea variety DschMMBr as trap crop, the *Bradyrhizobium* spp. population estimate was 5.8 x10^3 and 1.0 x 10^5 cells per gram of soil sample, in Nkometou and Nkoemvone, respectively. When the trap crop was changed to Vyuniebe, the population estimate remained the same in Nkometou but substantially increased (3.1 x 10^5 cells per gram of soil sample) in Nkoemvone. Using the variety 58-77 in Nkometou, a *Bradyrhizobium* spp. population size of 1.0 x10^4 cells per gram of soil sample was estimated whereas this population was 5.8 x 10^5 in Nkoemvone. Overall, population sizes of rhizobia using the three varieties were higher in Nkoemvone soil than in Nkometou soil. The cowpea variety 58-77 seemed to have high nitrogen fixation potentials as it formed nodules more than the two other varieties in both soils. The levels of *Bradyrhizobia* populations observed in the two sites were adequate to give satisfactory results on nodulation and nitrogen fixation. This suggests that cowpea production in southern Cameroon does not require inoculation.

Key words: *Bradyrhizobia* spp., *Vigna unguiculata*, nodulation, most probable number, low P soils, Southern Cameroon.

INTRODUCTION

In the soil, microbes constitute a large portion of the bio-diversity (Fortin et al., 2008) and their activity can influence a number of important ecosystem processes, including nitrogen and carbon cycling (Fortin et al., 2008; van der Heijden et al., 2008), soil formation (Rillig and Mummey, 2006), plants nutrient acquisition and productivity (Dommergues et al., 1999; Sene et al., 2010). Interactions between plants and microbes are particularly important

since plants represent the main pathway through which element that severely limits microbial growth, enters the soil (Kahindi et al., 1997; van der Heijden et al., 2006; van der Heijden et al., 2008).

Among plant-microbe interactions, the legume-rhizobia symbiosis that converts nitrogen gas (N_2) into ammonia is probably the best studied (Kahindi et al., 1997; Mwend et al., 2011). Legume-nodulating rhizobia play a great role in maintaining soil fertility (Kahindi et al., 1997). However, effective nitrogen fixation in legumes depends on many factors (Voisin et al., 2003, 2007) including the presence of effective and abundant rhizobia in the soil (Giller, 2001; FAO, 1984). These rhizobia can either be indigenous or applied as inoculum.

Legume hosts differ in the range of partners with which they form symbioses. Some legumes nodulate with a restricted number of rhizobial strains or species while others nodulate with a wide range of fast- and slow-growing rhizobia (Maingi et al., 2006). In addition, factors such as high soil temperature (Giller, 2001), nutrient deficiencies (Beck and Munns, 1984; Watkin et al., 1997; O'Hara, 2001), low levels of soil moisture (Boonkerd and Weaver, 1982), low pH (<5.5), low clay and organic matter (Dudeja and Khurana, 1989; De Mallaro and Izaguirre, 1994) adversely affect rhizobial survival. Consequently, soils varying in their fertility status will respond differently to rhizobial inoculation.

On the other hand, legume hosts also differ in their response to inoculation. For instance, studies have shown that cowpea (*Vigna unguiculata* L.Walp) is a very promiscuous legume host as it has rarely been found to respond to inoculation unless when grown in a soil where the conditions are not conducive for the survival of rhizobia (Ahmad et al., 1981; RangaRao et al., 1985; Giller, 2001). Moreover, from a practical point of view, the use of inoculants is also cumbersome and difficult to exploit by farmers (Hornetz et al., 2000; Kaleem, 2002) who face problems in acquiring and storing inoculants because cooling facilities are not readily available. As a result, cowpea crops as are grown by farmers in Africa and receive no inoculants and little or no commercial nitrogen fertilizer.

To reduce the need for inoculation, legume varieties that can be nodulated by indigenous rhizobia have to be selected for farmer use. As a prerequisite of this selection process, an assessment of the indigenous rhizobial population levels in low phosphorus soils of southern Cameroon using cowpea as trap crop was undertaken: (1) to measure the soil richness of cowpea-nodulating rhizobia in the humid forest zone of Cameroon and (2) to determine the impact of pH and soil P levels on the abundance of this B*radyrhizobia* population and (3) to identify the best variety to use as a trap crop.

MATERIALS AND METHODS

Study site description

Soil samples used in this experiment originated from two sites. The first site is located at the Institute of Agricultural Research for Development (IRAD) experimental station in Nkoemvone, in the South region of Cameroon. The second site belonging to a farmer is located at Nkometou, one of the benchmark villages of the International Institute of Tropical Agricultural (IITA), the central region of Cameroon. Both regions are part of the humid forest zone of Cameroon.

The agro-ecological characteristics of the two locations are presented in Table 1. The IRAD experimental field at Nkoemvone has a past history of trials involving maize (*Zea mays* L.), cowpea and mucuna (*Mucuna pruriens* L.) rotation. The rotatory trails ended in 2004 and since then the field has been cultivated with maize or left un fallow. The dominant species while on fallow was the elephant grass (*Pennisetum purpureum* Schumach). The farmer's field at Nkometou was left unfallowed for 6 years with siam weed (*Chromolena odorata* (L.) R. King & H. Robinson) as domi-nate species; then cassava (*Manihot esculenta* Crantz) and groun-dnuts (*Arachis hypogaea*Linn.) cultivation followed for 2 years (March 2010- December 2011); then maize for two seasons (April - July and September – December) in 2012. Soil samples were collected in December 2012 after the maize was harvested.

Soil sampling

Each study site was divided into 10 sub-sections based on the vegetation, topography and cropping and tillage practices (compact and non compact soils). Ten soil sub-samples were collected from each sub-section in the two study sites, Nkoemvone in the south and Nkometou in the center region. Prior to sampling, surface debris were removed. After clearance of debris from the surface, the soil core was removed at 30 cm depth with a soil auger (Abaidoo et al., 2002). The 10 samples from each sub-section were then bulked, homogenized, sieved (<2 mm), and divided into two parts. Two composite samples were then produced from bulked samples from all the 10 subsections. One composite sample was air-dried for chemical analyses in the biochemical laboratory in the International Institute of tropical Agriculture (IITA) and the second was stored at 4°C in clean paper bags for microbiological analyses.

Soil analysis

Soil pH (Table 2) was determined in water on 1:1 soil/water ratio (IITA, 1982). Organic carbon was determined by chromic acid digestion (Heanes, 1984) and total N was determined using auto-analyser (Bremner and Mulvaney, 1982). Available P was deter-mined using Mehlich-3 extraction method (Mehlich, 1984). Cation exchange capacity was determined by saturation with 1 N ammo-nium acetate and extraction of ammonium with 2 M potassium chloride (TSBF, 1993). Exchangeable acidity was determined by titration method after extraction with 1 N KCl (Anderson and Ingram, 1993) while ECEC was determined by summation of exchangeable cations and exchangeable acidity. Soil particle size (Table 3) analysis was done by the hydrometer method (Bouyoucos, 1951).

*Corresponding author. E-mail: atemaureen@yahoo.com. Tel: +237 78828148

Table 1. The summary of agro-ecological characteristics of the two locations.

Parameter	Nkometou	Nkoemvone
*AEZ	Zone V	Zone V
Longitude	11°15' E	11º20' E
Latitude	3°62'N	2º90'N
Altitude	700(masl)	560(masl)
Climate	Equatorial	Equatorial
Annual Rainfal1	1600 mm	1692,2 mm
Rainfall pattern	Bimodal	Bimodal
Temperature:		
Mean Minimum	19.2°C	19.7°C
Mean Maximum	26.6°C	29.1°C
Dry months:		
Long	Mid DEC-Mid MAR	Mid DEC-Mid MAR
Short	Mid JUN-Mid AUG	Mid JUN-Mid AUG
Rainy season:		
First	Late March –Early June	Late March –Early June
Second	Early Sept – Early Dec	Early Sept – Early Dec
Vegetation	Degraded Tropical Rainforest	Tropical RainForest
Soil type:		
FAO	Ferric Acrisols	Ferric Acrisols
USDA	Rhodic Kandiudult	RhodicKandiudult

*AEZ = Agro-ecological zones of Cameroon; Zone V = humid forest with bimodal rainfall; masl = meters above sea level. Adapted from ICRAF, 1993

Table 2. Soil chemical properties of the study sites.

Characteristic	Site	
	Nkoemvone	Nkometou
Soil depth	0-20cm	0-20cm
pH (water)	4.52	5.49
Ca cmol(+) /kg	0.58	1.92
Mg cmol(+) /kg	0.32	1.30
K cmol(+) /kg	0.18	0.13
Na cmol(+) /kg	0.28	0.29
Al cmol(+) /kg	2.33	0.40
CEC cmol(+) /kg	8.07	7.27
P ppm or ug/g	7.51	3.07
Mn ppm or ug/g	1.26	123.20
Fe ppm or ug/g	106.00	65.70
Org C (%)	1.73	1.69
Total N (%)	0.12	0.12
C/N	13.92	14.32
Oxalate extractable Fe (ppm or ug/g)	1976.60	1418.91

Sterilization and pre-germination of seeds

Seeds of cowpea varieties, DschMMBr, Vyuniebe and 58-77 were used. DschMMBr and Vyuniebe are local varieties grown in the western and northern regions of Cameroon, respectively, while the variety 58-77 was provided by the Senegalese Agricultural

Table 3. Soil physical properties of the study sites.

Site	Sand	Clay	Silt	textural class	bulk density	*Field capacity*
	%	%	%		Mg/m^3	cm³ water/cm³ soil
Nkoemvone	42.25	46.38	11.37	Clay	1.29	0.360
Nkometou	53.32	34.26	12.42	Sandy clay loam	1.35	0.290

Research Institute *(*ISRA*)*. These varieties were chosen because they nodulated well with soils from both sites in a previous experiment (Atemkeng, unpublished data). Cowpea seeds were collected, pre-screened and purified in pots. Seeds of good viability (with a germination percentage higher than 80%), undamaged and of uniform colour and size were selected (Maingi et al., 1999). One hundred seeds of each variety were surface sterilized by immersing them into a 3% solution of sodium hypochlorite for 5-10 min. The solution of sodium hypochlorite was prepared by adding 10 parts of commercial bleach (5.25% sodium hypochlorite) to 7.5 parts of water. The seeds were rinsed 8 times with sterile distilled water after surface sterilization. They were then soaked in clean sterile distilled water and allowed to imbibe it for one hour. They were transferred aseptically to 2% water agar plates with a spoon-shaped spatula. Twenty five seeds were placed in each plate. The plates with the seeds were incubated upside down at 2°C to enable the radicles to grow away from the water agar. The incubation period was four days. Seedlings whose radicles attained a length of 1-2 cm after the incubation period were considered ready for transferring to glass tubes.

Plant growth medium and inoculation

The growth medium, used in this study was a mixture of sterile black soil and sand (3:1) sterilized in an autoclave at 121°C for 15 min. Five nitrogen-free stock solutions was prepared as described by Beck et al. (1993). For each litre of full-strength plant growth solution, 0.5 ml was added from each of the five stock solutions. The pH of the solution was adjusted to 6.8 using NaOH (1.0 M) or HCL (1.0 M). All solutions were sterilized by autoclaving at 121°C for 15 min. To prepare the inocula, whole soil inocula were diluted to 10^{-1} by suspending 10 g of each soil sample in 90 ml of sterile water and then shaking for 15 to 20 min with a wrist shaker. Serial dilutions were made to give from 10^{-1} to 10^{-10}. An aliquot of 1 ml of diluent was used to inoculate pre-germinated, surface-disinfected cowpea seedlings grown in glass tubes. Four tubes were inoculated with each dilution. Uninoculated control plants were included for each dilution to determine if cross contamination of tubes occurred. The growth tubes in rackholders were transferred to a growth chamber in the Regional Biocontrol Laboratory at IRAD Yaounde, Cameroon where the temperature was maintained at 30°C with a 14-h photoperiod provided by fluorescent lighting. Sterile water was added as required through sterile straws. The racks were positioned 60 cm apart in the growth chamber in a completely randomized design. Application and regular checking of levels of nitrogen-free nutrient solution was done on daily basis to ensure that the seedlings were adequately moistened.

Plant infection counts

The rhizobial populations in each soil were estimated using the most probable number technique as described by Somasegaran and Hoben (1994). Cowpea was used as the trap host to check for the abundance of indigenous cross nodulating *Bradyrhizobium* spp. Data were collected 8 weeks after planting. At harvest, the stems of the plants were cut at the level of the growth medium. The plants in the glass tubes were scored for the presence or absence of nodules. The roots were carefully washed using a gentle stream of water to remove sand, taking care not to destroy the roots and nodules. Nodulation was observed (+, for nodulation or - for no nodulation) and the number of nodulated (+) plants units was recorded beside each dilution. The presence of a single nodule in a tube was considered a positive score. The total number of nodulated units was obtained by summing up the nodulated units at each dilution level. Uninoculated controls were used to check for sterile conditions.

The MPN was calculated from the most likely number (m) obtained from the MPN tables according to the formula:

$$MPN = (m \times d)/v$$

where: m is the most likely number from MPN tables, d is the lowest dilution in the series and v is the aliquot used for inoculation (Somasegaran and Hoben, 1994).

RESULTS AND DISCUSSION

The most probable number technique based on plant infection count is commonly used to estimate numbers of rhizobia in soil or to determine the quality of inoculants produced in sterile conditions (Beck et al., 1993; Somasegaran and Hoben, 1994). Empirical models have been used to describe the response to inoculation of legumes (Thies et al., 1991). These models indicate that population density as estimated by the MPN- plant infection assay is one of the primary factors determining the magnitude of legume response to indigenous soil rhizobia. This is one of the main reasons why the *Bradyrhizobia* populations had to be determined in the two field sites before evaluating cowpea for nitrogen fixation related traits.

In this study, the cowpea varieties used formed nodules following inoculation with serial dilutions of soils from the two study sites. However, there were variations in the number of nodulated units per variety and per study site. The estimated total *Bradyrhizobium* spp. population in Nkoemvone (Table 4) soil ranged between 1.0 and 5.8× 10^5 cells per gram of soil sample while the population size at Nkometou (Table 5) was between 5.8 x $10^3$and 1.0 x10^4 cells per gram of soil sample. Using the cowpea variety DschMMBr as trap crop, the *Bradyrhizobium* spp population estimated was 5.8 x10^3 and 1.0 x10^5 cells per gram of soil sample, respectively, in Nkometou and Nkoemvone soils. The population estimate was the same in Nkometou but increased in Nkoemvone when the trap

Table 4. Nodulated units planted with three cowpea varieties with inocula from Nkoemvone soil.

Serial dilution	Number of nodulated units by each variety		
	Dsch MMBr	Vyu niebe	58-77
10^{-1}	4	4	4
10^{-2}	4	4	4
10^{-3}	3	4	4
10^{-4}	3	3	3
10^{-5}	2	2	3
10^{-6}	2	2	2
10^{-7}	1	1	1
10^{-8}	0	1	1
10^{-9}	0	0	0
10^{-10}	0	0	0
control	0	0	0
Total	19	21	22
MPN (cells per gram of soil sample)	1.0×10^5	3.1×10^5	5.8×10^5

Number of replications, n = 4; dilution steps, s = 10; number of nodulated units, (+) = 19; 21; and 22. Lowest dilution in the series, d = 10^{-1}; v = 1 ml.

Table 5. Nodulated units planted with three cowpea varieties with inocula from Nkometou soil.

Serial dilution	Number of nodulated units by each variety		
	Dsch MMBr	Vyu niebe	58-77
10^{-1}	4	4	4
10^{-2}	3	4	4
10^{-3}	2	2	3
10^{-4}	2	2	2
10^{-5}	2	1	1
10^{-6}	1	1	1
10^{-7}	0	0	0
10^{-8}	0	0	0
10^{-9}	0	0	0
10^{-10}	0	0	0
control	0	0	0
Total	14	14	15
MPN (cells per gram of soil sample)	5.8×10^3	5.8×10^3	1.0×10^4

Number of replications, n = 4; dilution steps, s = 10; number of nodulated units, (+) = 14; 14; 15. Lowest dilution in the series, d = 10^{-1}; v = 1 ml.

crop was changed to Vyuniebe. A population of 5.8×10^3 and 3.1×10^5cells per gram of soil sample was recorded in Nkometou and Nkoemvone, respectively. The cowpea variety, 58-77 seemed to have higher nitrogen fixation potentials as it formed nodules more than the two other varieties in both soils. Indeed, using this variety, a *Bradyrhizobium* spp population size of 1.0×10^4 cells per gram of soil sample was estimated in Nkometou as compared to 5.8×10^5 in Nkoemvone. Similar high rhizobia populations of 4.9×10^2, 3.5×10^3 and 4.3×10^4 cells g-1 of soil were recorded from three soils in West Africa (Ahmad et al., 1981). However, the cropping history of the soils was not provided.

Generally, population sizes of rhizobia using the three varieties were higher in Nkoemvone soil than in Nkometou soil. These results showed that the levels of *Bradyrhizobia* populations recorded in the two study sites were adequate to give satisfactory outputs on nodulation and nitrogen fixation without inoculation. This is in agreement with earlier findings by Nambiar et al.(1983)

where it was shown that most cultivated tropical soils have a rhizobial population of more than 100 rhizobia cells per gram of soil capable of nodulating the legumes grown on such soils. More so, in soils where naturalized rhizobial populations are high (>10^3 rhizobium bacteria per g soil), introduction of new strains can be difficult and often unsuccessful (Thies et al., 1991; Brockwell et al., 1995).

The Nkoemvone site had higher *Bradyrhizobia* populations in all the categories than the Nkometou site. This difference could be linked to the cropping history of both sites as well as the soil physico-chemical properties especially the pH and soil P level. Both soils had been cultivated with legumes in the past. However, while cowpea had been cultivated at Nkoemvone, the site at Nkometou had only been cultivated with groundnut that is known to be nodulated only by a subgroup of *Bradyrhizobium* spp. (Yousef et al., 1987). Legumes serve to maintain rhizobia in the soil through rhizosphere effects and senescence of nodules. Studies by Woomer et al. (1988) and Yousef et al. (1987) highlighted the effects of legumes on rhizobial populations. They reported that groundnut rhizobia which prefer a pH ranging from 7.6 to 8.1, are not favored by increasing soil organic carbon over 1%. In our study, soil samples collected in Nkometou displayed low pH (5.49) and organic C content above 1% (1.69%). This implies that the survival of *Bradyrhizobium* spp. that nodulate groundnut in Nkometou was hampered as is evident by the lower soil rhizobia population estimated. Soil P in Nkometou was also very low (3.07 ppm) as compared to Nkoemvone (7.51 ppm). Since cowpea is more tolerant to phosphorus deficiency than are most grain legumes (Alkama et al., 2008), the growth, nodulation and rhizobia survival in previous experiments with cowpea in Nkoemvone may have been less affected as was groundnuts in Nkometou soil with very low P. The fact that cowpea could be nodulated by soil dilutions from both sites corroborated the idea that cowpea is a promiscuous legume, and rhizobia are facultative symbionts which in the sapro-phytic state, are independent of their host legumes (Woomer et al., 1988).

Conclusion

Bradyrhizobium spp. also referred to as cowpea miscellany was detected in the soils from the two study sites. However, the numbers varied per site and depended on the trap crop used. Good infectivity was observed using the cowpea variety 58-77 in this study. This implies that this variety may have high nitrogen fixation potential and can be cultivated in southern Cameroon to improve soil fertility. From this study, the rhizobia population from both sites was above 10^3 cells per gram of soil. This strongly suggests that cowpea production in southern Cameroon does not require inoculation. The low soil pH and P status can even aggravate the negative effects of inoculating cowpea in this zone. This knowledge of indigenous bradyrhizobia populations in Southern Cameroon will be very valuable for developing strategies to improve biological nitrogen fixation (BNF) for increasing cowpea yields at low costs since inoculation is proven not to be necessary.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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