

Symbiotic efficiency of commercial and native rhizobia of cowpea (*Vigna unguiculata* L.) in South Western Kenya

ABSTRACT

Aims: To determine the effects of rhizobia inoculation on nodulation, growth, yield, nitrogen (N) fixation and nodule occupancy of cowpea at two acidic soils in South Western Kenya.

Study design: Randomized complete block design in a 4 x 4 factorial arrangement.

Place and Duration of Study: Field experiments: Kericho East (0° 24' S, 37° 1' E) and Bomet central (0° 47' S, 35° 21' E) between July 2012 and March 2014.

Methodology: Four cowpea varieties (KVU 27-1, K80, M66 and Ngor) received each of the following treatments: inoculation with *Bradyrhizobium* sp. USDA 3456, 20 kg N ha⁻¹, 40 kg N ha⁻¹, or untreated (absolute control). N-fixed by cowpea plants in inoculated and untreated plots was determined using ¹⁵N natural abundance technique. Nodule occupancy was done by sequence analyses of 16S rRNA gene in bacterial isolates from cowpea nodules. Data collected were: nodule number and weight, leaf area index, shoot dry weight, shoot and grain nitrogen content, nitrogen fixed and grain yield.

Results: Rhizobial inoculation significantly ($P \leq 0.05$) increased cowpea nodulation twice out of five sampling times in moderately acidic soils of Bomet central, but increased cowpea nodules only once in strongly acidic soils of Kericho East. *Bradyrhizobium* inoculation had no significant ($P \leq 0.05$) effects on growth, tissue N or on amount of N fixed at the experimental sites. The quantity N-fixed by the four cowpea varieties in the acid soils was between 9.8 - 19.8 mg N plant⁻¹, which was less than 2 kg N ha⁻¹. None of the bacterial isolates from cowpea nodules had similarity to inoculated *Bradyrhizobium* sp. or any species in *Rhizobiaceae* family, possibly due to antagonistic effects of nodule endophytes on rhizobia. Nodules were dominated by two species of endophytic plant growth promoting bacteria (PGPB): *Bacillus megaterium* and *Bacillus aryabhattai*.

Conclusion: Under the prevailing soil conditions in South Western Kenya, cowpea plants do not respond to *Bradyrhizobium* inoculation, and amount of N-fixed by both indigenous and commercial rhizobia is low. Two species of endophytic PGPB are predominant in the acid soils and their role in cowpea production need to be determined.

Keywords: Cowpea, plant growth promoting bacteria, symbiotic efficiency, rhizobia, 16S rRNA gene.

1. INTRODUCTION

Cowpea (*Vigna unguiculata* L.) is the second most important legume crop after common bean in Kenya (1). It is predominantly grown as an indigenous vegetable in regions located west of Rift valley (2). Drought conditions and unpredictable rainfall patterns caused by climate change are associated with yield loss and crop failure in most areas of South western Kenya, which include Kericho (3,4). One of the mitigation strategies against climate change induced drought is growing of tolerant crops such as cowpea (5). However, crop production in Western Kenya is limited by decline in soil fertility probably due to continuous cropping without replenishment of soil nutrients (6,7) Consequently, there has been deficiency of nitrogen (N) and phosphorous in these soils (8).

Cowpea is a leguminous crop which forms symbiotic association with nitrogen fixing bacteria; hence its N nutrition could be optimized by inoculation with efficient strains of rhizobia (9). Currently, the only available commercial rhizobial inoculum of cowpea in Kenya is *Bradyrhizobium* sp. USDA 3456, marketed by MEA limited as Biofix. However, its efficacy in N₂ fixation in SW Kenya has not been well established. Previous research findings however show that this strain is not efficient in N-fixation (10),

though the observations were only based on nodulation and growth data. Nodule occupancy tests based on restriction fragment length polymorphism (RFLP) of the 16S-23S rDNA region of rhizobial DNA, confirmed that *Bradyrhizobium* sp. strain USDA 3456 was inefficient in nodulation of cowpea in Kenyan soils (11). However, none of these studies characterised cowpea nodulating rhizobia to the species and strain level, which could be useful in selection of strains for inoculation. The study objectives were to determine the effects of *Bradyrhizobium* inoculation on nodulation, growth, yield and N-fixation in cowpea, and to characterise the species of cowpea nodulating rhizobia in inoculated and un-inoculated plots at two acidic soils in South Western Kenya.

2. MATERIAL AND METHODS

2.1 Experimental sites and soil analyses

A field experiment was conducted at two sites (Farmers training center at Bomet central- 0° 47' S, 35° 21' E and Kericho Kericho East- 0° 24' S, 37° 1' E) located in South Western Kenya. ATC farm is located at an altitude of 1920 m above the sea level. It receives an average annual rainfall of 1302 mm and its agro-ecological zone is LH2 (12). Kericho farm is located at an altitude of 2182 m above the sea level with an average annual rainfall of 2090 mm and mean annual temperature of 17.2°C. It is found in agro-ecological zone LH1 (12). Prior to field experiments, soil samples were collected from field experimental sites at a depth of 20 cm following procedures described by (13). The samples were analyzed for pH (H₂O), organic carbon (%), total N (%), available P (mg kg⁻¹), available K (cmol kg⁻¹) and also exchangeable Al (cmol kg⁻¹) using the protocols described by previous authors (14). Population of cowpea rhizobia was also determined in the soil samples using the most probable number (MPN) plant infection technique in germination pouches (15), under glasshouse conditions at the University of Nairobi, and cowpea variety K80 was used as trap plant for rhizobia in soils. Isolation of bacteria in cowpea nodules and molecular studies were done at the School of Biological Sciences, University of Reading (U.K).

2.2 Treatments and field experimental design

The cowpea genotypes used in the study were M66, K80, KVVU 27-1 and Ngor, chosen based on their ecological requirements: Variety M66 is grown in medium to higher altitudes, KVVU 27-1 is grown in medium altitudes, K80 is a dry land variety (<http://www.infonet-biovision.org/default/ct/120/crops>), and Ngor is a local variety commonly grown by farmers in S.W Kenya. The four varieties are dual purpose (grown for grain and leaves). Each of the four cowpea genotypes received the following treatments: rhizobia inoculation, 20 kg N ha⁻¹, 40 kg N ha⁻¹ or untreated (neither rhizobia inoculation nor nitrogen fertilizer was supplied). The experimental design used was randomized complete block design in factorial arrangement, and treatments were replicated three times. Nitrogen fertilizer was supplied in form of calcium ammonium nitrate, and peat based cowpea rhizobial inoculant (*Bradyrhizobium* sp. USDA 3456, Mea Ltd Kenya) was applied as seed dress, at the rate of 0.2 kg ha⁻¹. The size of experimental plots was 2.5 m x 2.5 m. Prior to planting, all plots received 25 kg ha⁻¹ of P fertilizer (in form of triple superphosphate). Seed rate was 25 kg ha⁻¹, and plant spacing was 50 cm x 20 cm. Two seeds were placed per hill, but one plant was retained per hill after emergence. As a precaution to avoid cross contamination, plots that were inoculated with rhizobia received the treatment last.

2.3 Crop Field data collection and analysis of biological nitrogen fixation

The data collected were: number of active nodules, nodule dry weight (mg plant⁻¹), leaf area index (LAI), shoot dry weight (g plant⁻¹), shoot and grain nitrogen content, proportion of nitrogen derived from atmosphere (Ndfa), nitrogen fixed (mg plant⁻¹) and grain yield tons ha⁻¹. Data collection was done at 50% flowering stage (10 weeks after emergence) and pod filling stage (16 weeks after crop emergence). In Kericho East, data was collected beginning from the 6th week after emergence in short rains season of 2012, due to predicted crop loss as a result of supernormal rains. During the data collection date, six plants were selected at random from the inner rows of each plot and then shoots were separated from roots and oven dried at 60°C till constant in weight, and then shoot dry matter was determined. Immediately after harvesting the shoots, cowpea root cores (6.5 cm in diameter and 15 cm deep) (16) were taken using a soil corer. A total of six cores were taken per plot, and then transported to the laboratory, where the soil was carefully removed with flowing water, and then nodules were removed. Active nodules with pink colour were counted, and then oven dried at 60°C to constant weight and dry weight determined. Leaf area index (LAI) was determined at 50% flowering stage of plants, using the cork borer method (17), where leaf discs were punched using a cork borer,

and the relationship between area and dry weight of the disc was used to determine the leaf area. The leaf area was divided by the ground area occupied by a plant in the field. The oven dried plant shoots harvested at the flowering stage (18) were analysed for tissue N using Kjeldahl method as described (19). Grain samples were analysed for total N using the same procedure. All the pods from the three inner rows were harvested at the end of the season, shelled and then oven dried at 60°C till constant in weight, before weighing to obtain grain yield.

During the early pod filling stage in the short rains season of 2013, three plant shoots were sampled at random from three inner rows in plots that were inoculated with *Bradyrhizobium* sp. and control plots that did not receive any treatment, at both sites. *Zea mays* grown at adjacent plots were also sampled for use as reference plants for ^{15}N isotope analysis. Shoot samples were oven dried at temperature of 60°C until constant in weight, and then weighed and finely ground. Half a gram of ground shoots of each sample were weighed into 5 ml Eppendorf tube and sent to the University of California Davis for determination of isotope ratios of $^{15}\text{N}/^{14}\text{N}$ and nitrogen content, using PDZ Europa ANCA-GSL elemental analyzer connected to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd, Cheshire, UK). ^{15}N abundance ($\delta^{15}\text{N}$) was expressed in per mil (‰) relative to international standard (atmospheric nitrogen) as shown (20): $\delta^{15}\text{N} (\text{‰}) = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$, where R is $^{15}\text{N}/^{14}\text{N}$. The proportion of nitrogen derived from atmospheric nitrogen fixation in cowpea shoots was calculated using the equation: $\text{Ndfa} (\%) = 100 (\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{fixing plant}}) / (\delta^{15}\text{N}_{\text{ref}} - B)$ (21). $\delta^{15}\text{N}_{\text{ref}}$ is the ^{15}N natural abundance of the reference plant, $\delta^{15}\text{N}_{\text{fixing plant}}$ is the ^{15}N natural abundance of the N_2 fixing legume and B value is ^{15}N natural abundance of cowpea plant depending solely on N_2 fixation for nitrogen nutrition. B value used in this study was -1.759 (22). The amount of nitrogen fixed in cowpea shoots was calculated as (23): $\text{N-fixed} = \% \text{Ndfa} \times \text{shoot N content}$.

2.4 Nodule harvesting and isolation of symbiotic and endophytic bacteria from cowpea nodules

In the short rains season of 2013, nodules were harvested from the four cowpea varieties (K80, M66, KVVU27-1 and Ngor) in field experiment plots at Kericho East and Bomet central, when plants had attained 50% flowering stage. Nodule harvesting, sterilising and storage were done as described in previous studies (11, 24, 25). Nodule samples were collected randomly from three plants within inner rows of plots that were inoculated with *Bradyrhizobium* sp. and also control plots with no treatment applied. Harvested nodules were washed and then put in a cool box, and immediately transferred to the Tea Research Institute of Kenya laboratory, where they were surface sterilized by immersion in 70% ethanol for 1 minute, then immersed in 3% sodium hypochlorite for 3 minutes and rinsed 6 times in sterile distilled water. Nodules from each of the four varieties in inoculated and untreated plots that had been replicated thrice at experimental sites were pooled and five representative samples were selected at random and then stored in universal bottles with 40% glycerol at temperature of -20°C. A total of 40 nodules were stored from each of the two experimental sites. Nodule samples were then taken for isolation and DNA extraction of symbiotic and endophytic bacteria at the University of Reading (UK). During isolation, nodules were sterilized in 70% ethanol for 2 minutes and finally rinsed in 3 changes of nanopure water. Each nodule was crushed using a plastic pestle in an Eppendorf tube containing 100 μl of 40% glycerol and 20 μl of the resulting cell suspension was streaked onto yeast extract mannitol agar (YEMA) containing 0.025% Congo red dye (15, 26), and incubated at 28°C for 10 days. Bacterial colonies were not obtained from nodule samples from Kericho East. Twelve distinct colonies (based on colour, size and shape) isolated from Bomet central were re-isolated on tryptone yeast (TY) agar (27) and incubated at 28°C for 2-4 days. Pure overnight cultures were then made by aseptically transferring single bacterial colonies with a loop from TY agar plates into 10 ml TY broth and incubating them at 27°C on a rotary shaker at 200 rpm until they turned turbid (about 24-48 hours). The overnight cultures were used for DNA extraction.

2.4.1 DNA extraction and polymerase chain reaction

An overnight culture of bacteria was grown in TY broth, and used for DNA extraction using Gene Elute bacterial genomic DNA kit for gram positive bacteria (Sigma Aldrich Ltd). Quality of DNA was measured using nanodrop 1000 spectrophotometer (labtech Ltd, UK) and was within the required 260 nm/280 nm absorbance ratios of 1.7-2.0. Universal primers that target 16S rRNA gene of bacteria were subjected to 50 μl polymerase chain reaction (PCR) reactions that consisted of: 25 μl of 2x

145 PCR BIO Taq Mix (PCR biosystems Ltd); 2 µl of each of the 10 µM forward and reverse primer; 5 µl
146 DNA and 16 µl of nanopure water. Primer sequences and PCR conditions are shown (Table 1). The
147 amplified PCR products (5 µl) were separated on 1% agarose gel stained with GelRed dye (Biotium,
148 USA), run at 90 V for 40 minutes in TE buffer, and then finally visualized on Syngene G: BoxChemi
149 XL Gel documentation system to confirm the success of PCR amplification. A 1 kb hyperladder was
150 used as a molecular weight marker (Bioline, UK). The PCR products were purified using QIAquick kit
151 (Qiagen Ltd) before sequencing.

Table 1: Primers used in this study

Target				
Primer	gene	Sequence (5' - 3')	PCR conditions	Reference
27F	16s rRNA	AGA GTTTGATCCTGGCTCAG	94°C 5mins; 35 cycles (94°C 40s, 65°C 40s, 72°C 1.5mins) and Final extension of 72°C for 7 minutes	(28, 29)
1492R	16s rRNA	GGTTA CCTTGTTACGACTT		

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154 **2.4.2 DNA sequencing, identification of isolates and phylogenetic analyses**

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156 Samples for sequencing were prepared as follows: 15 µl of each pure DNA sample obtained after
157 PCR product purification was pipetted in duplicate into Eppendorf tubes; then DNA was mixed with 2
158 µl of a forward and reverse primer (Table 1) in the separate tubes. Samples were then sent for
159 sequencing in both forward and reverse directions at Eurofins genomics (Germany). Forward and
160 reverse nucleotide sequences of each DNA sample were aligned and edited for similarities using
161 Bioedit software, version 7.2.5 (30). Identification of bacterial isolates was then done by submitting
162 their edited sequences for comparison with National Centre for Biotechnology Information (NCBI)
163 GenBank sequences, using nucleotide Basic Local Alignment Search Tool (BLASTN). Phylogenetic
164 analyses were then done in MEGA 6 software (31), where DNA sequences of bacterial isolates and
165 reference strains were first aligned using MUSCLE (32). Evolutionary history of the isolates were then
166 inferred using maximum likelihood tree based on Kimura 2- parameter model (33). A bootstrap
167 confidence analysis (34) was conducted with 1000 replicates.

168

169 **2.5 Statistical analyses**

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171 Data collected from field were subjected to analysis of variance using Genstat statistical software, 16th
172 edition (VSN International Ltd). Means were compared using Fischer's Protected LSD at 5% level of
173 significance.

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176 **3. RESULTS**

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178 **3.1 Soil chemical conditions and rhizobial population**

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180 Bomet central (agricultural training centre farm) had the following soil chemical properties: pH 5.58,
181 organic carbon 2.57%, total N 0.21%, available P 8.85 mg kg⁻¹, available K 0.80 cmol kg⁻¹ and
182 exchangeable Al 0.30 cmol kg⁻¹ of soil. Rhizobial population was 6 cells g⁻¹ of soil. In Kericho East
183 (Nile heritage farm), pH was 4.85; organic carbon 4.19%, total N 0.42%, available P 8.19 mg kg⁻¹,
184 available K 0.60 cmol kg⁻¹ and exchangeable Al was 0.75 cmol kg⁻¹ of soil. Rhizobial cells were not
185 detected by MPN plant infection method in the experimental site at Kericho East.

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188 **3.2 Effects of rhizobia inoculation and N fertilizer on active nodule numbers and dry 189 weight of four cowpea varieties**

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191 Interactions between treatments and nodulation parameters were significant ($p \leq 0.05$) only at Bomet
 192 Central (Table 2). Application of 20 kg N ha⁻¹ increased number of active nodules in cowpea variety
 193 K80 during the short rains of 2013 (Table 2). Rhizobia inoculation increased nodule dry weights in
 194 varieties K80 and K66 during the long and short rain seasons of 2012 respectively (Table 2).
 195 However, in the short rains season of 2013, untreated plants of the local variety Ngor and K80 had
 196 higher nodule dry weights than other treatments (Table 2). Application of 40 kg N ha⁻¹ depressed
 197 nodule numbers and dry weights in variety Ngor and K66 in all the seasons (Table 2).
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Table 2: Influence of variety and soil amendments on nodule numbers and dry weight in a field experiment conducted in Bomet central over three seasons between 2012 and 2014

Treatment	Parameter, season, year and sampling time			
	active nods			
	plant ⁻¹	Nodule dry weight in mg plant ⁻¹		
				Short rains-
	Short rains [†] - 2013	Long rains - 2012	Short rains -	2013
	(10 WAE [‡])	(10WAE)	2012 (16WAE)	(10WAE)
K80 [§]	2.11 _{bc}	19.0 _{bc}	0.00 _d	24.56 _a
KVU 27-1 [§]	0.39 _d	17.22 _{cd}	0.00 _d	0.44 _c
Ngor [§]	2.22 _{bc}	15.67 _{cde}	1.00 _d	27.89 _a
M66 [§]	0.39 _d	7.78 _{fgh}	2.56 _{cd}	4.89 _c
K80 + 20 kg N ha ⁻¹	6.56 _a	10.56 _{efg}	1.78 _{cd}	5.44 _c
KVU 27-1 + 20 kg N ha ⁻¹	0.11 _d	7.67 _{fgh}	1.22 _{cd}	0.78 _c
Ngor + 20 kg N ha ⁻¹	2.56 _b	3.11 _{hi}	0.00 _d	4.44 _c
M66 + 20 kg N ha ⁻¹	1.09 _{bcd}	8.33 _{fgh}	0.89 _d	3.72 _c
K80 + 40 kg N ha ⁻¹	1.56 _{bcd}	10.44 _{efg}	1.89 _{cd}	1.44 _c
KVU 27-1 + 40 kg N ha ⁻¹	0.89 _{cd}	7.00 _{fghi}	1.11 _{cd}	0.78 _c
Ngor + 40 kg N ha ⁻¹	0.11 _d	1.22 _i	0.00 _d	0.67 _c
M66 + 40 kg N ha ⁻¹	0.33 _d	1.78 _i	1.22 _{cd}	0.67 _c
K80 + Inoculation ^{§§}	2.39 _{bc}	35.00 _a	4.11 _{bc}	7.44 _{bc}
KVU 27-1 + Inoculation	1.44 _{bcd}	23.89 _b	7.11 _b	1.56 _c
Ngor + Inoculation	1.61 _{bcd}	12.78 _{def}	1.44 _{cd}	1.89 _c
M66 + Inoculation	2.28 _{bc}	6.11 _{ghi}	10.22 _a	15.11 _b
Mean	1.63	11.72	2.16	6.36

LSD _{0.05}	1.52	5.80	3.08	8.86
CV (%)	12.20	29.70	27.50	25.60

[†] Short rains season extended into dry season of the following year; [‡] Weeks after crop emergence; [§] neither nitrogen fertilizer nor rhizobia inoculation was supplied; ^{§§} inoculation with *Bradyrhizobium* sp. USDA 3456. Means followed by same letter within a column are not significantly different at $p \leq 0.05$ (Fischer's protected LSD test).

199

200 At Bomet Central, it was generally observed that: rhizobia inoculation enhanced cowpea nodule
 201 numbers compared to the control in only two sampling times, both of which were in the short rains
 202 season; in 4 out of 5 sampling times, control and inoculated plots had plants with similar nodule
 203 numbers (Table 3). Rhizobia inoculation also enhanced nodule dry weights only during two sampling
 204 times at the same site (Table 3). At Kericho East, rhizobia inoculation enhanced cowpea nodule
 205 numbers compared to the control only during the short rains season of 2012, but had no significant
 206 effect on nodule dry weight (Table 4). Generally, application of 40 kg N ha⁻¹ reduced nodule numbers
 207 and dry weight in cowpea plants in both sites during most of the sampling times (Table 3 and 4).

Table 3: Effects of rhizobia inoculation and nitrogen fertilizer on cowpea nodule numbers and dry weight in a field experiment conducted at Bomet Central during the 10th and 16th week after emergence (WAE) in three rain seasons between 2012 and 2014

Treatment	Active nodules plant ⁻¹			Active nods		Nodule dry			Nodule dry	
	plant ⁻¹			plant ⁻¹		matter plant ⁻¹			matter plant ⁻¹	
	(10WAE)			(16WAE)		(10WAE)			(16WAE)	
	Long	Short	Short	Long	Short	Long	Short	Short	Short	Short
	rains	rains	rains	rains	rains	rains	rains	rains	rains	rains
	(2012)	(2012)	(2013)	(2012)	(2012)	(2012)	(2012)	(2013)	(2012)	(2012)
Control [†]	11.33 _{ab}	2.69 _{ab}	1.28 _{bc}	8.86 _a	0.61 _b	14.92 _b	4.62	14.11 _a	67.58 _a	0.89 _b
20 kg N ha ⁻¹	8.83 _b	2.47 _b	2.58 _a	5.33 _{ab}	0.89 _b	7.42 _c	4.85	3.60 _{bc}	25.11 _b	0.97 _b
40 kg N ha ⁻¹	7.81 _b	1.81 _b	0.72 _c	3.25 _b	0.75 _b	5.11 _c	1.39	0.89 _c	16.08 _b	1.06 _b
Inoculation [‡]	14.33 _a	4.14 _a	1.93 _{ab}	8.69 _a	2.92 _a	19.44 _a	4.99	6.5 _b	40.17 _b	5.72 _a
Mean	10.56	2.78	1.63	6.53	1.29	11.72	3.96	6.36	37.24	2.16
LSD _{0.05}	3.83	1.54	0.76	3.58	1.34	2.9	Ns	4.43	26.41	1.54
CV (%)	23.10	19.70	12.2	27.40	21.10	29.7	29.2	25.6	27.4	27.5

[†] Neither nitrogen fertilizer nor rhizobia inoculation was supplied; [‡] inoculation with *Bradyrhizobium* sp. USDA 3456. Means followed by same letter within a column are not significantly different at $P \leq 0.05$; Ns – treatment effects not significant at $P \leq 0.05$.

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Table 4: Effects of Rhizobia inoculation and nitrogen fertilizer on nodule numbers and dry weight of four cowpea varieties in a field experiment conducted in Kericho East over three seasons between 2012 and 2014

Treatment	Parameter, season, year and sampling time					
	Active nodules plant ⁻¹			Nod dry weight plant ⁻¹		
	Short [†]		Short	Long	Short	Short
	Long rains	rains	rains	rains	rains	rains
	2012	2012	2013	2012	2012	2013
	(10WAE [†])	(6WAE)	(10WAE)	(10WAE)	(6WAE)	(10WAE)
Control	4.19	3.08 _b ⁵	2.08 _{ab}	15.17 _a	6.78 _{ab}	3.72 _{ab}
20 kg N ha ⁻¹	2.14	1.36 _c	1.56 _{ab}	3.56 _b	2.03 _b	2.53 _{bc}
40 kg N ha ⁻¹	3.28	0.53 _c	0.75 _b	4.42 _b	3.90 _{ab}	1.06 _c
Inoculation	5.31	4.69 _a	2.81 _a	18.11 _a	8.69 _a	5.31 _a
Mean	3.73	2.42	1.80	10.32	5.35	3.15
LSD	NS	1.35	1.34	7.1	5.43	2.49
CV (%)	26.7	17.60	19.80	28.9	33.3	25.80

Means followed by same letter within a column are not significantly different at $P \leq 0.05$

(Fischer's protected LSD test).[†] Weeks after crop emergence

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210 3.3 Nodulation characteristics of four cowpea varieties in a field experiment 211 conducted in Bomet central and Kericho East over three seasons between 2012 and 212 2014

213 In Bomet central, cowpea variety K80 had significantly ($P < 0.05$) higher nodule numbers than other
214 varieties during the long and short rains season of 2012 and 2013 respectively (Fig. 1). During the
215 late pod filling stage (16 WAE) of the long rains season of 2012, all the varieties except M66 had
216 similar number of active nodules. Cowpea variety M80 also had higher nodule dry weight than the
217 other three varieties during the 10th WAE in the long rains season of 2012 (Fig. 2). However, the local
218 variety (Ngor) had higher nodule dry weight than the other varieties during the 16th WAE of the same

season (Fig. 2). In Kericho East, there were no significant varietal differences in the number of active nodules over all the seasons (Fig. 3). The nodule dry weight of variety K80 was significantly lower than for other varieties during the long rains season of 2012 (Fig. 3). In the short rains season of 2012, cowpea variety KVVU 27-1 had significantly higher nodule dry weight than K80; variety Ngor had the least nodule weights in the short rains season of 2013 (Fig. 3). In general, cowpea varieties K80 and KVVU 27-1 had better nodulation in Bomet Central and Kericho East respectively. Nodulation was generally depressed during the short rains compared to the long rains season.

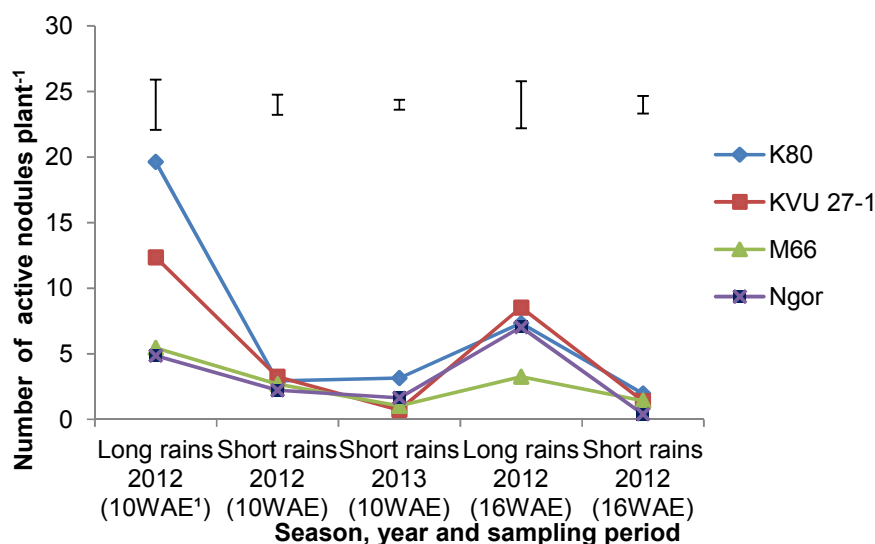


Figure 1: Number of active nodules of four cowpea varieties in a field experiment conducted in Bomet central over three seasons between 2012 and 2014. Error bars shows differences within varietal means at $P \leq 0.05$, according to Fischer's protected least significance difference (LSD) test. ¹Weeks after crop emergence

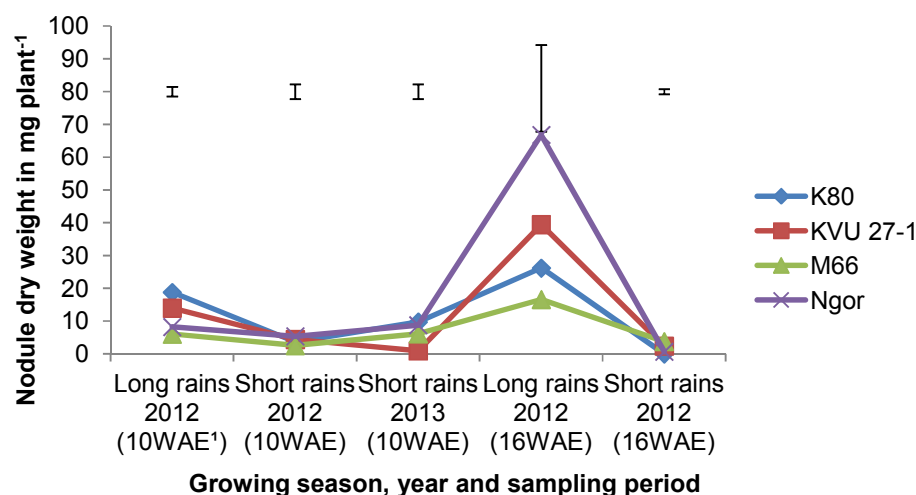


Figure 2: Nodule dry weights of four cowpea varieties in a field experiment conducted in Bomet central over three seasons between 2012 and 2014. Error bars shows differences within varietal means at $P \leq 0.05$ (Fischer's protected LSD test). ¹Weeks after crop emergence

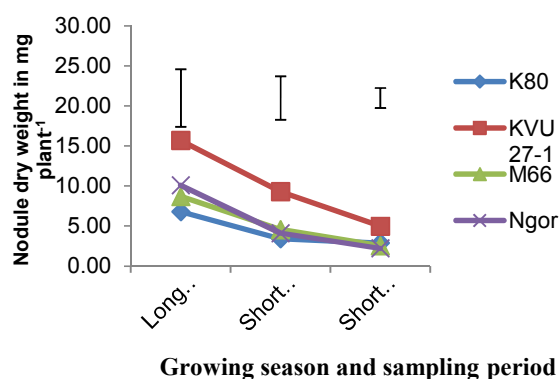
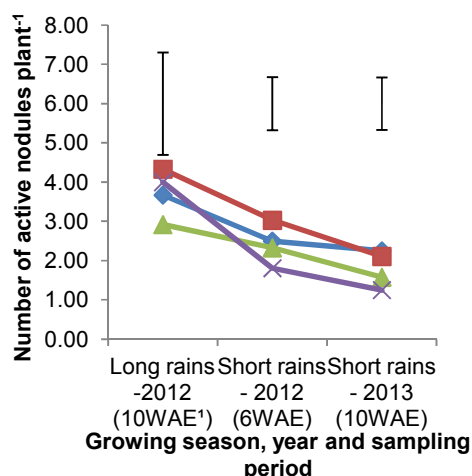


Figure 3: Number of active nodules and nodule dry weights of four cowpea varieties in a field experiment conducted in Kericho East over three seasons between 2012 and 2014. Error bars show differences within varietal means at $P \leq 0.05$ (Fischer's protected LSD test). ¹Weeks after crop emergence

3.4 Effects of rhizobia inoculation and nitrogen fertilizer on growth, grain yield and tissue nitrogen of four cowpea varieties in Kericho East and Bomet central

Rhizobia inoculation and nitrogen fertilizer had no significant ($P \leq 0.05$) effects on shoot dry matter of cowpea in both sites (Table 5). Most of the data on cowpea were not obtained in Kericho East, due to crop damage by hailstorms during the long rains season.

Rhizobia inoculation and nitrogen fertilizer had no significant effects on leaf area indices (LAI) and grain yield of cowpea in all seasons and in both sites (Table 6). There were no significant ($P \leq 0.05$) treatment effects on shoot and grain nitrogen content of cowpea plants in both sites (Table 7 and 8).

Table 5: Effects of rhizobia inoculation and nitrogen fertilizer on shoot dry weight of four cowpea varieties in a field experiment conducted in Bomet central and Kericho East over three seasons between 2012 and 2014

	Site, sampling period and growing season						
	Bomet central					Kericho East	
	10 WAE†			16 WAE		10 WAE	
	Long	Short	Short	Long	Short		Short
	rains	rains	rains	rains	rains	Long rains	rains
Treatment	(2012)	(2012)	(2013)	(2012)	(2012)	(2012)	(2013)
Control	15.69	10.57	5.13	47.50	13.48	0.92	2.14
20 kg N ha ⁻¹	16.79	10.51	7.92	48.00	13.87	0.91	2.87
40 kg N ha ⁻¹	15.00	13.46	5.28	39.10	17.42	0.91	2.18
Inoculation	17.07	8.93	5.96	36.10	14.15	1.11	2.70
Mean	16.14	10.87	6.07	42.68	14.73	0.96	2.47
LSD _{0.05}	Ns	Ns	Ns	Ns	Ns	Ns	Ns
CV (%)	13.5	13.6	16.3	9.6	13.6	14.2	16.3

Ns – treatment effects were not significant, † Weeks after crop emergence

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Table 6: Effects of rhizobia inoculation and nitrogen fertilizer on leaf area index and grain yield of four cowpea varieties in a field experiment conducted in Bomet central and Kericho East over three seasons between 2012 and 2014

	Site, parameter, season and year						
	Bomet central					Kericho East	
	LAI†			Grain yield			Grain yield
				(tons ha ⁻¹)		LAI	(tons ha ⁻¹)
	Long	Short	Short	Long	Short	Short	
	rains	rains	rains	rains	rains	rains	Short rains
Treatment	(2012)	(2012)	(2013)	(2012)	(2013)	(2013)	(2013)
Control	0.72	0.14	0.53	0.85	1.17	0.21	0.27
20 kg N ha ⁻¹	0.69	0.23	0.7	0.99	1.37	0.25	0.31

40 kg N ha ⁻¹	0.57	0.16	0.46	1.02	1.48	0.18	0.23
Inoculation	0.60	0.15	0.68	1.16	1.47	0.32	0.28
Mean	0.65	0.17	0.59	1.01	1.37	0.24	0.27
LSD	Ns	Ns	Ns	Ns	Ns	Ns	Ns
CV (%)	9.1	6.8	10.9	14.3	9.9	7.4	11

[†] Leaf area index

Table 7: Effects of rhizobia inoculation and nitrogen fertilizer on shoot and grain nitrogen content of four cowpea varieties in a field experiment conducted at Bomet central during the short rains season of 2013

Shoot nitrogen content						Grain nitrogen content				
N treatment	Variety (V)				Mean (N)	Variety (V)				Mean (N)
	K80	KVU	Ngor	M66		K80	KVU	Ngor	M66	
		27-1					27-1			
Control	0.80	0.77	0.82	0.82	0.80	1.87	1.69	2.08	1.59	1.81
20 kg N ha ⁻¹	0.79	0.81	0.77	0.84	0.80	1.89	1.79	1.59	1.66	1.73
40 kg N ha ⁻¹	0.86	0.76	0.79	0.79	0.80	1.96	1.79	2.00	1.86	1.90
Inoculation	0.79	0.84	0.78	0.86	0.82	1.96	1.81	1.62	2.11	1.87
Mean (V)	0.81	0.79	0.79	0.83		1.92	1.77	1.82	1.81	
LSD _{0.05} (N)		Ns				Ns				
LSD _{0.05} (V)		Ns				Ns				
LSD _{0.05} (NxV)		Ns				Ns				
CV (%)		5.80				13.00				

Ns: Treatment effects not significant

Table 8: Effects of rhizobia inoculation and nitrogen fertilizer on shoot and grain nitrogen content of four cowpea varieties in a field experiment conducted in Kericho East during the short rains season of 2013

N source	Shoot nitrogen content (%)					Grain nitrogen content (%)				
	Variety (V)					Variety (V)				
	KVU				Mean	KVU				Mean
	K80	27-1	Ngor	M66		K80	27-1	Ngor	M66	
Control	0.85	0.80	0.86	0.85	0.84	1.72	1.88	1.86	1.46	1.73

20 kg N ha ⁻¹	0.79	0.87	0.86	0.85	0.85	1.94	1.93	1.79	1.58	1.81
40 kg N ha ⁻¹	0.81	0.77	0.86	0.85	0.82	1.65	1.85	1.93	1.83	1.81
Inoculation	0.88	0.87	0.84	0.86	0.86	2.01	1.74	1.73	1.71	1.80
Mean (V)	0.83	0.83	0.86	0.85		1.83	1.85	1.83	1.64	
LSD _{0.05} (N)		Ns				Ns				
LSD _{0.05} (V)		Ns				Ns				
LSD _{0.05} (NxV)		Ns				Ns				
CV (%)		5.2				11.6				

Ns – Treatment effects not significant

3.5 Effects of rhizobia inoculation on biological nitrogen fixation in four cowpea varieties at Bomet central and Kericho East

Rhizobia inoculation did not significantly ($P \leq 0.05$) increase the proportion of nitrogen derived from the atmosphere (Ndfa) or the amount of nitrogen fixed (N-fixed) in four cowpea varieties at both experimental sites (Table 9). It was however observed that there was no nitrogen fixation by rhizobia in inoculated plots with local variety of cowpea (Ngor) at Kericho East (Table 9). It was further observed that cowpea plants in Bomet central had more Ndfa and N-fixed than those in Kericho East (Table 9).

Table 9: Influence of rhizobia inoculation on nitrogen derived from atmosphere (Ndfa) and nitrogen (N) fixed of four cowpea varieties in a field experiment conducted in Bomet central and Kericho East during the short rains season of 2013

Treatment	Bomet central		Kericho East	
	Ndfa (%)	N-fixed (mg plant ⁻¹)	Ndfa (%)	N-fixed (mg plant ⁻¹)
K80 - inoculation†	4.99	19.89	3.67	9.06
KVU 27-1- inoculation	2.84	6.84	2.05	3.84
M66 – inoculation	8.36	23.70	4.15	11.86
Ngor – inoculation	2.79	21.55	2.04	5.61
K80 + inoculation‡	5.93	29.92	4.18	16.32
KVU 27-1 + inoculation	2.78	14.23	8.23	22.93
M66 + inoculation	6.35	19.66	6.48	8.36
Ngor + inoculation	13.12	22.71	0.00	0.00

Mean	5.90	19.81	3.85	9.75
LSD _{0.05} (variety x inoculation)	Ns	Ns	Ns	Ns

†- inoculation: plants were not inoculated with a commercial strain of rhizobia,

‡+ inoculation: plants inoculated with *Bradyrhizobium* sp. USDA 3456, Ns: treatment effects not significant at $P \leq 0.05$.

277 3.6 Phylogenetic affiliation of isolates of bacteria from cowpea nodules

278 Twelve isolates that had close sequence similarity to 16 S rRNA sequences of plant growth promoting
 279 bacteria were obtained from nodules of three cowpea varieties, which were either inoculated or un-
 280 inoculated in Bomet central. None of the isolates was obtained from nodules of cowpea variety K80
 281 (Table 10, Fig. 4). There were no successful cultures of bacterial isolates from nodules harvested in
 282 Kericho East. Isolates obtained from nodules in Bomet central had 99-100% sequence similarity to
 283 strains of *Bacillus megaterium*, *Bacillus aryabhattai* or *Bacillus altitudinis* (Table 10). None of the
 284 isolates had similarity to the *Bradyrhizobium* sp. inoculated to plants in the field experiment (Table
 285 10). In a phylogenetic tree, six isolates grouped at 69% bootstrap support with four type strains of *B.*
 286 *megaterium*

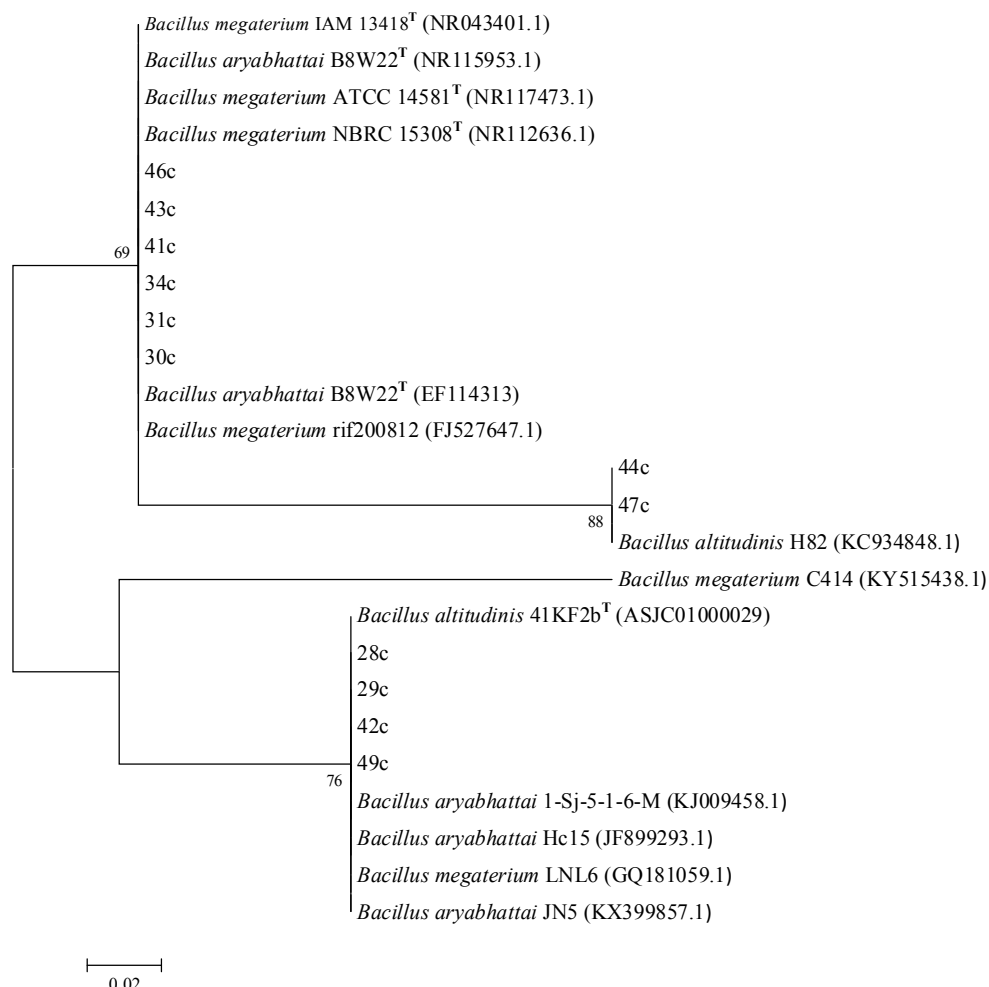
287 and *B. aryabhattai*; four isolates grouped at 76% bootstrap value to one type strain of *Bacillus*
 288 *altitudinis* and other reference strains of *B. megaterium* and *B. aryabhattai* obtained from NCBI
 289 GenBank; two isolates grouped with *Bacillus altitudinis* strain H82 at 88% bootstrap value (Fig. 4).

Table 10: Phylogenetic affiliation of bacterial isolates from nodules of three cowpea cultivars that received two treatments in a field experiment conducted at Bomet central in Kenya during short rains season of 2013

Isolate name	Host cowpea variety	Treatment	Closest species and strain on NCBI nucleotide BLAST ¹	GenBank accession number	Similarity (%)
28c	M66	Inoculated†	<i>Bacillus megaterium</i> strain 66-Y143	KU647259.1	99
29c	M66	Inoculated	<i>Bacillus aryabhattai</i> strain JN5	KX399857.1	100
30c	M66	Inoculated	<i>Bacillus aryabhattai</i> strain Hc15	JF899293.1	100
31c	Ngor	Inoculated	<i>Bacillus megaterium</i> strain C414 <i>Bacillus aryabhattai</i> strain 1-Sj-5-1-6-M	KY515438.1	100
34c	Ngor	Inoculated		KJ009458.1	100
41c	KVU 27-1	Inoculated	<i>Bacillus aryabhattai</i> strain Hc15	JF899293.1	100
42c	M66	Control‡	<i>Bacillus megaterium</i> strain LNL6	GQ181059.1	100
43c	M66	Control	<i>Bacillus megaterium</i> strain MBFF6	HQ840732.1	99
44c	M66	Control	<i>Bacillus altitudinis</i> strain H82	KC934848.1	100
46c	Ngor	Control	<i>Bacillus aryabhattai</i> strain LSR8.3	KT718049.1	99
47c	Ngor	Control	<i>Bacillus altitudinis</i> strain CORSS02 <i>Bacillus megaterium</i> strain rif200812	MF425586.1	100
49c	KVU 27-1	Control		FJ527647.1	99

¹Nucleotide blast (BLAST N) was done using 16s RNA gene sequences of bacterial isolates; † plants inoculated with *Bradyrhizobium* sp. USDA 3456; ‡ plants were not inoculated with commercial rhizobia.

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294 **Figure 4:** Maximum Likelihood tree of the 16s rRNA sequences showing phylogenetic relationships of
 295 isolates of plant growth promoting bacteria isolated from root nodules of cowpea at Bomet central in
 296 Kenya, and type/reference strains. NCBI Genbank and EZbiocloud accession numbers of type and
 297 other reference strains are shown in parentheses; type strains are shown by letter T. The tree with the
 298 highest log likelihood (-49.9647) is shown. The percentage of trees in which the associated taxa
 299 clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths
 300 measured in the number of substitutions per site. The analysis involved 25 nucleotide sequences. All
 301 positions containing gaps and missing data were eliminated.

302 3. DISCUSSION

303 Inoculation of cowpea plants with commercial *Bradyrhizobium* enhanced nodule numbers and dry
 304 weights in cowpea plants, though not consistently in all sampling times during the research period.
 305 Increase in nodule numbers and dry weights in cowpea are in agreement with the work of other
 306 researchers (9, 35). In Bomet central, rhizobia inoculation enhanced nodule dry weights in two
 307 genotypes, K80 and M66. Varietal difference in legume nodulation has been reported previously (36),
 308 and it is a trait that can be exploited in breeding programs for improving N₂ fixation. It was further
 309 observed that untreated cowpea local variety Ngor and improved variety K80 had high nodule dry
 310 weights during periods of low soil moisture (short rains season) in Bomet central. This suggests that
 311 native rhizobia may have nodulation preference for the two varieties under moisture stress conditions.

Although moisture stress is known to limit biological nitrogen fixation (37), there are strains of cowpea rhizobia that have high nodulation efficiency under water limited conditions (38). Nodulation efficiency in drought conditions could be attributed to high antioxidant and acid phosphatase activities in nodules (39). Varietal differences in cowpea nodulation were observed in the two experimental sites; K80 and KVVU 27-1 appeared to nodulate better in Bomet central and Kericho East respectively. Variation in nodulation of cowpea under different ecological conditions was also reported in a study involving nine cowpea genotypes in diverse regions of Ghana and South Africa (36). Bomet central receives less rainfall and has higher mean annual temperature than Kericho East (12), and that could explain why K80, which is a dry land variety can nodulate better in this site. Application of 40 kg N ha⁻¹ reduced nodule numbers and weight. High soil N, especially NO₃⁻ N is known to reduce nodulation and nitrogen fixation in cowpea and other legumes (40-42). Some of the possible reasons for reduced nodulation due to high N concentration could be inhibition of cell division in root cortex during initial stages of nodule development (43) and reduction in activity of nitrogenase enzyme in root nodules (44). It was however observed that 20 kg N ha⁻¹ enhanced the number of active nodules during the short rains of 2013 in variety K80 at Bomet central. Most researchers have reported that a small concentration of starter nitrogen fertilizer may enhance nodulation and legume plant growth (42, 45, 46). This is because legume plants may not meet their N requirements from biological nitrogen fixation alone (47). However, response of legume plants to starter nitrogen may be attained in soils with NO₃⁻ N levels below 20 kg N ha⁻¹ (48).

Rhizobia inoculation and application of nitrogen fertilizer had no significant effects on shoot dry matter, leaf area index, grain yield and tissue N of cowpea plants in this study. Similar findings were reported in earlier studies done in seven geographic regions of Kenya (10, 11). It was further observed that rhizobia inoculation had no significant effects on Ndfa and N-fixed in this study, which may erroneously suggest that indigenous rhizobia could have been efficient in nitrogen fixation. However, the mean Ndfa value attained in the experimental sites was below 6%, which is very low compared to 83-98% reported in other agro-ecological zones of Kenya (11). The amount of nitrogen fixed was on average less than 20 mg plant⁻¹, which was also low compared to 401-934 mg plant⁻¹ obtained in other regions of Africa (36). These findings confirm that neither indigenous nor commercial cowpea rhizobia in the study area are efficient in nitrogen fixation. There is a possibility that soil chemical conditions could have limited biological nitrogen fixation and plant growth in the study area as reported in a similar study (49). Although nitrogen could have been sufficient for cowpea production, the site with higher total N (0.42%) had plants with very low shoot dry matter and marginal grain yield. It is hypothesised therefore that low available P in both sites, and low pH coupled with higher levels of exchangeable Al³⁺ in Kericho East, could be responsible for low symbiotic efficiency as it is in agreement with findings from a similar study (50). In previous research work where cowpea showed significant increase in nodulation, growth and grain yield in Kenya, soil pH was higher than 5.5 and soil was amended with P fertilizer (51, 52). Phosphorous is fixed by Al³⁺ in acid soils hence unavailable for plant use (13), yet its deficiency is known to reduce nodule numbers, nodule weight and nitrogen fixation (53, 54). Phosphorous has been reported to enhance cell division in early stages of nodule development (43), which may explain why nodulation declines with its deficiency. Phosphorous deficiency may also be associated with decline in activity of nitrogenase enzyme (55). The most important role of P in N₂ fixation may be ATP synthesis, which generates chemical energy required for the physiological process (13).

None of the cowpea nodules harvested from the acid soils was occupied by any known species of rhizobia. Since isotopic analysis showed evidence of nitrogen fixation in the two sites, it is hypothesised that growth of N₂ fixing rhizobia on culture media may have been suppressed by nodule endophytes, which are known to produce antagonistic compounds that may negatively affect rhizobia (56). Three species of plant growth promoting bacteria (PGPB) were isolated from cowpea nodules, which is consistent with findings from a similar study (57). Nitrogen fixing genes (NifH) have been isolated from PGPB that include *Bacillus marisflavi*, *Paenibacillus massiliensis* and *Bacillus megaterium* in China (58). It is however unclear whether the isolates of PGPB in this study were involved in N₂ fixation. PGPB isolated in this study serve several functions in crop production. *Bacillus megaterium* is known to solubilise phosphorous in soil (59). Apart from P solubilisation, *Bacillus altitudinis* produces indole acetic acid (IAA) which is a growth hormone, and siderophore (60). The later chelates iron and makes it available for microbial and plant use (61). *Bacillus aryabhattai* promotes plant growth through production of growth hormones (IAA, gibberellic acid and cytokinin) and also enhances heat stress tolerance in plants (62). In general, rhizobia and PGPB play synergistic roles in growth and development of legumes (63).

4. CONCLUSION

Application of *Bradyrhizobium* inoculants and nitrogen fertilizer has no significant effects on growth, grain yield, tissue N or nitrogen fixation of cowpea in soils with similar physiochemical conditions with the two sites in South Western Kenya. Two bacterial species of cowpea nodule endophytes (*Bacillus megaterium* and *Bacillus aryabhattai*) are dominant in acid soils of South Western Kenya. There is need to establish whether PGPB plays a role in plant growth and nitrogen fixation of cowpea.

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