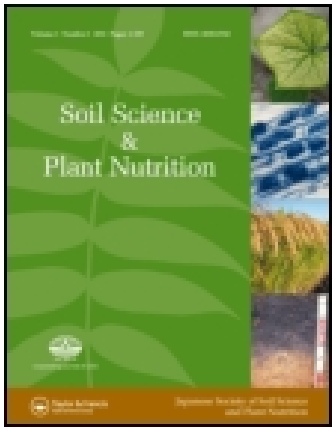

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Potential of indigenous bradyrhizobia versus commercial inoculants to improve cowpea (Vigna unguiculata L. walp.) and green gram (Vigna radiata L. wilczek.) yields in Kenya

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ORIGINAL ARTICLE

Potential of indigenous bradyrhizobia versus commercial inoculants to improve cowpea (*Vigna unguiculata* L. walp.) and green gram (*Vigna radiata* L. wilczek.) yields in Kenya

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Abstract

Limited information is available on reduced cowpea (*Vigna unguiculata* L. Walp.) and green gram (*Vigna radiata* L.Wilczek.) yields in Kenya. Declining soil fertility and absence or presence of ineffective indigenous rhizobia in soils are assumptions that have been formulated but still require to be demonstrated. In this study, soils were collected from legume growing areas of Western (Bungoma), Nyanza (Bondo), Eastern (Isiolo), Central (Meru) and Coast (Kilifi) provinces in Kenya to assess indigenous rhizobia in soils nodulating cowpea and green gram under greenhouse conditions. Our results showed that highest nodule fresh weights of 4.63 and 3.32 g plant^{-1} for cowpea and green gram were observed in one soil from Isiolo and another from Kilifi, respectively, suggesting the presence of significant infective indigenous strains in both soils. On the other hand, the lowest nodule fresh weights of 2.17 and 0.72 g plant^{-1} were observed in one soil from Bungoma for cowpea and green gram, respectively. Symbiotic nitrogen (N) fixation by cowpea and green gram was highest in Kilifi soil with values of 98% and 97%, respectively. A second greenhouse experiment was undertaken to evaluate the performance of commercial rhizobial inoculants with both legumes in Chonyi soil (also from Coast province) containing significant indigenous rhizobia [$>13.5 \times 10^3$ Colony Forming Units (CFU) g^{-1}]. Rhizobial inoculation did not significantly ($P < 0.05$) affect nodulation, biomass yield and shoot N content in cowpea and green gram compared with controls. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the 16S-23S rDNA intergenic spacer (IGS) region analysis of nodules revealed six groups of which only IGS Group IV corresponded with those from commercial inoculants applied, indicating a lower competitiveness of inoculated strains. In cowpea, IGS III was dominant in nodules of plants inoculated with Biofix and Rizoliq commercial inoculants, and the uninoculated control treatment (63.2, 60 and 52.9%, respectively). Similarly, in green gram, IGS Group III was dominant in nodules of plants inoculated with Biofix 704 and Rizoliq commercial inoculants, and the uninoculated control treatment (75, 73.7 and 61.1%, respectively). Our results suggest that the systematic inoculation of both legumes with current available commercial inoculants to improve biomass yields is not necessary in these regions of Kenya. Also, according to our study, it would make sense to promote the utilization of indigenous strains performing well with both legumes.

Key words: Biological nitrogen fixation, commercial inoculants, competition for nodulation, cowpea, green gram, indigenous rhizobia.

INTRODUCTION

Declining soil fertility, high fertilizer costs and intensification of agriculture coupled with reduction in farm sizes are major limitations to crop production in smallholder farms in Kenya (Maobe *et al.* 2000; Cheruiyot *et al.* 2001; Chemining'wa *et al.* 2004). As a result cheaper

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sources of nitrogen (N) need to be sought if yields are to be sustained and food security attained (Otieno *et al.* 2009). Grain legumes contribute more than 20 million tonnes of fixed N to agriculture each year (Herridge *et al.* 2008). Such fixation of N can only be achieved in the presence of efficient rhizobial strains, which can be native to the soil or introduced in the form of commercial inoculants. Biologically fixed N_2 comes from symbioses involving leguminous plants and species of rhizobia distributed in 13 genera: *Azorhizobium, Bradyrhizobium, Burkholderia, Cupriavidus, Devosia, Ensifer, Herbaspirillum, Mesorhizobium, Methylobacterium, Ochrobacterium, Phyllobacterium, Shinella* and *Rhizobium* (Dudeja and Narula 2008). Symbiotic N_2-fixation by legume-rhizobia associations potentially provides large N inputs for agriculture (Larnier *et al.* 2005). Inoculation of legumes with effective rhizobia can improve grain yields (Giller 2001; Zengeni *et al.* 2006; Nkwiine and Rwakaikara-Silver 2007). However, inoculation is not universal and inoculation does not always elicit positive responses. Inoculation of legumes is necessary in the absence of compatible rhizobia and when rhizobial populations are low or inefficient in fixing N (Brockwell *et al.* 1995; Catroux *et al.* 2001; Fening and Danso 2002; Abaidoo *et al.* 2007). Use of rhizobia inoculants for soybeans (*Glycine max* L. Merr.), green gram (*Vigna radiata* L. Wilczek.), and cowpea (*Vigna unguiculata* L. Walp.) among other legumes in South America, USA, Bangladesh and in many other countries worldwide (Giller 2001; Alves *et al.* 2003; Martins *et al.* 2003; Hungria *et al.* 2006; Bhuiyan *et al.* 2008) has been successful, and is an option that has potential to increase legume production in Kenya (Mungai and Karubiu 2010).

Cowpea (*Vigna unguiculata* L. Walp.) and green gram, also known as mung bean (*Vigna radiata* L. Wilczek.), are important legumes cultivated for their ability to fix N through nodule symbiosis with rhizobia. Their drought tolerance, N-fixation capacity and shade tolerance make these plants important components in intercropping with maize (*Zea mays* L.) or sorghum (*Sorghum angustum* L.) (Zhang *et al.* 2008). Cowpea is the third most important grain legume in Kenya after common beans (*Phaseolus vulgaris* L.) and pigeonpea [*Cajanus cajan* (L.) Millsp] (Kimiti *et al.* 2009) and is grown in semi-arid areas predominantly in the eastern province. The area under cowpea in Kenya is 18,000 ha, with about 85% of this area being in the arid and semi-arid lands of eastern Kenya (Muthamia and Kanampiu 1996). Green gram is native to India (Zhang *et al.* 2008); it is an important crop in the warm, dry parts of eastern Kenya where it is grown for both subsistence and as a cash crop (Shakoor *et al.* 1984). It can escape drought through its early maturing ability (Rowe 1980) and some varieties are perhaps more resistant to drought than cowpea (Waite *et al.* 1984). Limited information is available on reduced cowpea and green gram yields in Kenya, and although biological nitrogen fixation (BNF) through exploitation of the rhizobia legume symbiosis and use of inoculants offers in part a solution, awareness and use of rhizobia inoculants in legume production in Kenya is limited (Woomer *et al.* 1997). The potential for improving BNF through rhizobial inoculation requires knowledge of the abundance and effectiveness of the indigenous rhizobia population in the soil (Fening and Danso 2002).

Thus, this study aimed to assess the potential of indigenous rhizobia on nodulation and biomass yield responses in several agro-ecological zones (AEZ) where legumes are widely cultivated and to evaluate if commercial inoculants can improve cowpea and green gram biomass and growth in the presence of indigenous rhizobia.

MATERIALS AND METHODS

Greenhouse experiment

Two experiments were established in a greenhouse at the Tropical Soil Biology and Fertility Institute of the International Center for Tropical Agriculture (TSBF-CIAT) located at the World Agroforestry Centre, Nairobi, Kenya. The first experiment was to assess the populations of indigenous rhizobia through the nodulation and biomass yield responses of cowpea and green gram without inoculation. The second experiment was set up to evaluate whether commercial rhizobial inoculants could significantly improve cowpea and green gram biomass yields in the presence of indigenous rhizobia.

In the first experiment two legume species were used as trap hosts: cowpea (*Vigna unguiculata* L. Walp., cv. M66) and green gram (*Vigna radiata* L.Wilczek., cv. N26). A Completely Randomized Design (CRD) was used with three replicates per treatment. The set-up consisted of the two legumes grown in ten soils. Sorghum (*Sorghum angustum*, cv. Seredo) and wheat (*Triticum aestivum*, cv. Njoro BwII) were grown in the ten soils as reference plants for BNF assessment using the ^{15}N isotope natural abundance method. Soil from Bondo, Bungoma, Isiolo, Kilifi and Meru south regions (Fig. 1) in Kenya (two sites in each of the five regions, designated A and B respectively) collected from the 0–20-cm top layer was sun-dried, sieved to pass 2 mm and thoroughly homogenized. Two sites were selected from each region so that the different soil types within each region were represented. Chemical analysis of soils was done before planting for nutrient composition [N, available phosphorus (P), potassium (K), carbon (C), magnesium (Mg), calcium (Ca), and sodium (Na)], Cation Exchange

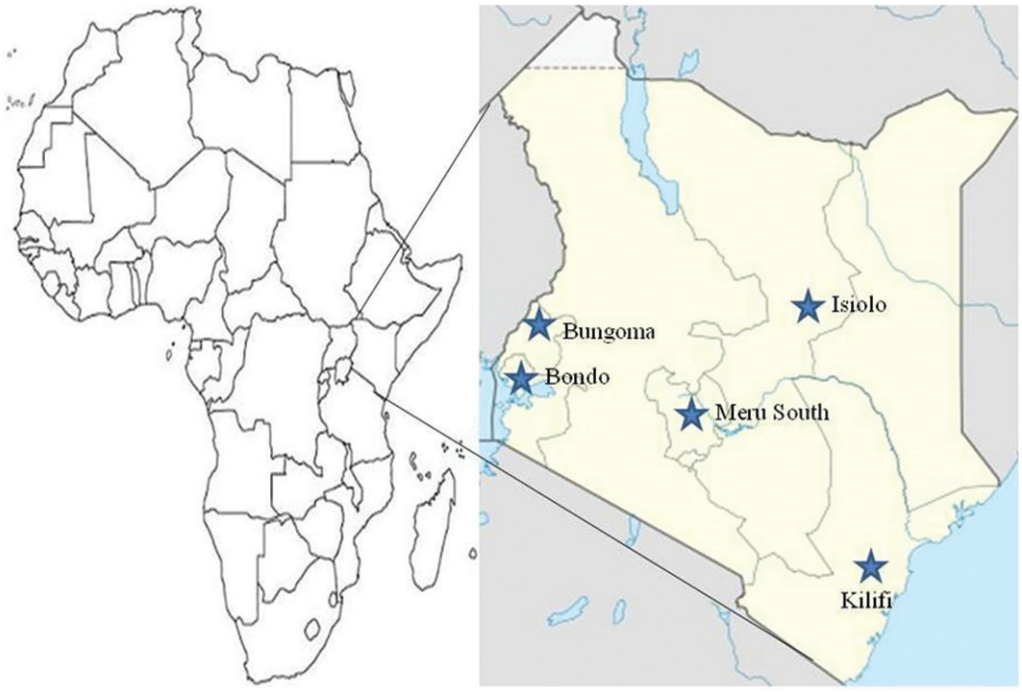

Figure 1 Geographical distribution of regions in Kenya from which soils were sampled for greenhouse experiments.

Table 1 Soil physiochemical properties

Properties	Units	Bondo A	Bondo B	Bungoma A	Bungoma B	Isiolo A	Isiolo B	Kilifi A	Kilifi B	M. south A	M. south B	Coast (Chonyi)
pH (H_2O)	_	6.15	5.24	5.9	5.39	8.54	8.38	7.54	6.61	5.69	6.79	6.06
P(Olsen)	(mg P kg^{-1})	8	2	3	5	1	3	13	3	12	32	6
CEC	($cmol_c$ kg^{-1})	11	8	8	7	124	128	9	19	15	26	33
Exchangeable K	($cmol_c$ kg^{-1})	0.21	0.47	0.39	0.69	0.86	1.31	0.16	1.98	1.14	2.62	1.07
Exchangeable Ca	($cmol_c$ kg^{-1})	6.83	2.81	4.88	2.29	94.49	97.05	7.67	9.96	6.96	16.95	15.75
Exchangeable Mg	($cmol_c$ kg^{-1})	1.69	1.28	0.89	1.19	18.57	22.14	0.70	5.16	2.12	4.01	9.52
Exchangeable Na	($cmol_c$ kg^{-1})	0.08	0.10	0.05	0.03	6.26	3.45	0.07	0.16	0.08	0.08	0.41
Total N	(%)	0.1	0.099	0.098	0.147	0.152	0.129	0.038	0.156	0.235	0.283	0.17
C	(%)	1.163	1.062	1.33	1.784	2.235	2.128	0.52	1.527	2.394	3.326	2.27
Clay	(%)	21	23	19	29	35	25	7	30	24	18	40.3
Sand	(%)	65	65	64	52	56	60	88	56	62	61	40.4
Silt	(%)	15	13	17	19	9	15	5	15	15	21	19.3

CEC, cation exchange capacity; P, phosphorus; K, potassium; Ca, calcium; Mg, magnesium; Na, sodium; N, nitrogen; C, carbon.

Capacity (CEC), pH and physical characterization of soils for soil texture composition (% clay, % sand and % silt) as described by Okalebo *et al.* 2002. The soil physico-chemical characteristics are presented in Table 1. Soils were obtained from fields in which legumes had previously been cultivated (see Table 2) to increase the likelihood of occurrence of indigenous rhizobia. Soil was weighed (see Table 2) and added to polyvinyl chloride (PVC) tubes (16.5 cm in diameter and 16.3 cm in length). Essential nutrients with the exception of N were added per pot for optimal crop growth [600 mg P, 340 mg K, 40 mg Ca, 40 mg Mg, 20 mg sulfur (S), 0.4 mg manganese (Mn), 0.4 mg zinc (Zn), 0.1 mg copper (Cu), 0.1 mg boron (B), 0.01 mg molybdenum (Mo) and 0.01 mg cobalt (Co)], nodulation and BNF in both legumes. Nutrient rates were calculated using optimal tissue (shoot) concentrations and an expected maximal biomass production of 20 g dry matter (DM) per plant. Nutrients were applied as solutions of potassium chloride (KCl), potassium phosphate (KH_2PO_4), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), magnesium sulphate

Table 2 Weight, textural class and legume history of soils used and the altitude and agro-ecological zone (AEZ) of sites from which they were obtained

Soil	Trial in which soil was used	Dry soil in pot (kg)	Textural class	Previous legume history	Altitude (m above sea level)	Agro-ecological zone (AEZ)
Bondo A	Expt 1	3.1	Sandy clay loam	Common bean (*Phaseolus vulgaris* L.)	1135–1500	Lower midland (LM)
Bondo B	Expt 1	2.9	Sandy clay loam	Common bean (*Phaseolus vulgaris* L.)		
Bungoma A	Expt 1	2.9	Sandy loam	Ground nut (*Arachis hypogaea* L.)	1200–1500	Lower midland (LM)
Bungoma B	Expt 1	2.8	Clay loam	Ground nut (*Arachis hypogaea* L.)		
Isiolo A	Expt 1	2.5	Clay loam	Soy bean (*Glycine max* L. Merr.)	180–1140	Low land (L)
Isiolo B	Expt 1	2.5	Sandy clay loam	Soy bean (*Glycine max* L. Merr.)		
Kilifi A	Expt 1	3.5	Sand	Cowpea (*Vigna unguiculata* L. Walp)	90–300	Coastal lowland (CL)
Kilifi B	Expt 1	3.0	Sandy clay loam	Cowpea (*Vigna unguiculata* L. Walp)		
Meru South A	Expt 1	2.1	Sandy clay loam	Cowpea (*Vigna unguiculata* L. Walp.)	1830–2200	Lower highland (LH)
Meru South B	Expt 1	2.3	Sandy loam	Common bean (*Phaseolus vulgaris* L.)		
Coast (Chonyi)	Expt 2	2.3	Clay loam	Cowpea (*Vigna unguiculata* L. Walp.)	90–300	Coastal lowland (CL)

Expt, experiment.

Table 3 Commercial inoculant products used in the greenhouse evaluation

Product	Producer	Microorganism strains and minimum concentration according to producers	Formulation	Rate of inoculant application
Biofix cowpea	Mea Ltd Kenya (MIRCEN)	*Bradyrhizobium* spp. (Not stated)	Peat	1 g 25 seeds^{-1}
Biofix green gram704	Mea Ltd Kenya (MIRCEN)	*Bradyrhizobium* spp. (Not stated)	Peat	1 g 25 seeds^{-1}
Biofix green gram2447	Mea Ltd Kenya (MIRCEN)	*Bradyrhizobium* spp. (Not stated)	Peat	1 g 25 seeds^{-1}
Cowpea peat inoculant	Becker underwood USA	*Bradyrhizobium* spp. (1×10^9 CFU mL^{-1})	Peat	1 g 25 seeds^{-1}
Green gram peat inoculant	Becker underwood USA	*Bradyrhizobium* spp. (1×10^9 CFU mL^{-1})	Peat	1 g 25 seeds^{-1}
Rhizoliq cowpea	Rizobacter Argentina S.A.	*Bradyrhizobium* spp. (1×10^9 CFU mL^{-1})	Liquid	1 mL plant^{-1}
Rhizoliq green gram	Rizobacter Argentina S.A.	*Bradyrhizobium* spp. (1×10^9 CFU mL^{-1})	Liquid	1 mL plant^{-1}

CFU, colony forming units; MIRCEN, microbial resources centres.

($MgSO_4$), manganese chloride ($MnCl_2$), zinc chloride ($ZnCl_2$), copper chloride ($CuCl_2$), cobalt chloride ($CoCl_2$), sodium borate ($Na_2B_4O_7$) and sodium molybdate (Na_2MoO_4) at planting and at three and six weeks after planting.

Cowpea and green gram seeds were surface-sterilized by soaking in 3.3% calcium hypochlorite [$Ca(ClO)_2$] solution for 5 min, thoroughly washed in sterile water and pre-germinated on moist filter paper for 36 h at 28°C. Three pre-selected healthy seeds of uniform size were then planted per pot and thinned to one plant per pot of comparable height and vigor at 14 d after planting (DAP). Green gram and cowpea plants were harvested at 8 and 9 weeks after planting respectively. Shoots were cut using a clean, sharp knife at 1 cm above the soil surface. The pots were emptied onto a 2-mm sieve and soil washed to isolate the roots. Nodulation (fresh weight) and fresh biomass weights were recorded. Nodules for occupancy assessment were immediately stored in glycerol at −20°C until analysis. Roots and shoots were oven-dried at 60°C to assess dry matter (DM) yields.

A second experiment was set up to evaluate the effects of various rhizobial inoculants on cowpea and green gram in soil from Chonyi. Results of the first experiment on nodule fresh weight, biomass and % Ndfa in Coast (Kilifi) convinced us to select soil from Chonyi, also located in the Kenyan Coast province. The soil was collected, prepared and packed in pots as described for Experiment 1; the physio-chemical characteristics are also presented in Table 1. A Most Probable Number (MPN) experiment in sterile sand using dilutions of soil from Coast (Chonyi) was carried out according to Brockwell (1963) to estimate the indigenous rhizobial population with the two legume species in this soil. Serial dilutions of soil were done to obtain a fourfold serial dilution of 1:50, 1:250, 1:1250, and 1:6250, with four replicates per dilution for both legumes. Sand was used as the growth medium and was washed thoroughly, dried and autoclaved at 121°C for 1 hr and placed in sterile PVC planting pots (14 cm in diameter and 11.5 cm in length).

Commercial inoculants were obtained from Becker Underwood (USA), MEA Ltd. (Kenya) and Rizobacter (Argentina) as shown in Table 3. This experiment was composed of 11 treatments: seven with application of rhizobial inoculants, a negative control for each legume (without inoculation) and a reference treatment for each legume [with N applied as ammonium nitrate (NH_4NO_3) at a rate of 400 mg N pot^{-1}]. A Completely Randomized

Design (CRD) was used with four replicates per treatment. Liquid-based inoculum (Rizoliq) was poured at the base of the stem at 4 days after emergence to ensure that the bacteria reached the roots. Peat-based product was mixed with seeds at planting. Essential nutrients with the exception of N were added at optimal rates per pot as described for Experiment 1. Seed sterilization, time of harvest, harvesting procedures and data recording were done as described for Experiment 1.

BNF assessment using ^{15}N isotope natural abundance method

The ^{15}N natural abundance (NA) technique relies on the slight natural enrichment of ^{15}N that is observed in many agricultural soils, relative to atmospheric N (Unkovich *et al.* 2008). The ^{15}N natural abundance and total N content of the shoot biomass were measured at the University of California, Davis (USA) using a PDZ Europa 20-20 isotope ratio mass spectrometer in line with a PDZ Europa ANCA sample combustion system. The precision (as determined from the standard deviation of the check samples) was <0.2‰. The percentage of N derived from atmospheric N_2 (Ndfa%) was calculated according to the following equation (Bardin *et al.* 1977):

$$\text{Ndfa} = \left(\delta^{15}\text{Nnf} - \delta^{15}\text{Nf}/\delta^{15}\text{Nnf} - \delta^{15}\text{Na}\right) \times 100 \quad (1)$$

where δ^{15}Nnf is the natural isotopic abundance value of reference plants (value of N from sources other than atmospheric N_2); δ^{15}Nf is the isotopic abundance of the N_2-fixing legume grown under conditions in which atmospheric N_2 and N from other sources are available, and δ^{15}Na is the δ^{15}N measured in the N_2-fixing legume depending solely on fixed N growing in an N-free medium, also known as the B value.

For cowpea and green gram B values of −1.75‰ (Pule-Meulenberg *et al.* 2010) and −2.50‰ (Unkovich *et al.* 2008) were used, respectively.

Nodule occupancy analysis by RFLP

Twenty nodules per treatment (Experiment 2) were analyzed. Nodules were surface disinfected with 70% ethanol and 3.3% $Ca(ClO)_2$ solution then rinsed thrice with sterile distilled water. Each nodule was crushed in a sterile 1.5 mL Eppendorf tube, after addition of 150 µL of sterile micropure water, using sterile plastic pestles. The nodule suspension was then used for DNA extraction according to the protocol described by Krasova-Wade *et al.* (2003).

A fragment of the intergenic region between the 16S and 23S rDNA [930–1050 base pairs (bp)] was amplified by polymerase chain reaction (PCR) using two primers: FGPS 1490-72; 5′-TGCGGCTGGATCCCCTCCTT-3′ (Normand *et al.* 1996), and FGPL 132-38; 5' CCG GGTTTCCCCATTCGG-3' (Ponsonnet and Nesme 1994). PCR amplification was performed in a Biorad PCR system thermal cycler adjusted to the following program: initial denaturation for 5 min at 94°C, 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 58°C, extension for 30 sec at 72°C, and final extension for 7 min at 72°C. PCR amplification was carried out in a 25 µL reaction volume containing 2 µL of total DNA extract, 1.0 µM of each primer and one freeze-dried bead (Ready-to-Go PCR beads, Pharmacia Biotech) containing 2.5 U of *Taq* DNA polymerase, 10 mM Tris-hydrochloric acid (HCl), (pH 9 at RT), 50 mM potassium chloride (KCl), 1.5 mM magnesium chloride ($MgCl_2$) and 200 µM of each deoxyribonucleotidetriphosphate (dNTP). PCR amplified DNA was visualized after electrophoresis on a 2% agarose gel (w/v) pre-stained with ethidium bromide (0.117 µg mL^{-1}). Aliquots (10 µL) of PCR products were digested with 5 U of *Msp* I restriction endonuclease in a total volume of 15 µL for 2 h at 37°C. The restriction fragment length polymorphism (RFLP) profiles were then visualised by gel electrophoresis on a 3% agarose gel (w/v) pre-stained with ethidium bromide (0.117 µg mL^{-1}). The gels were run at 100 V for 3 h then visualized under UV trans-illumination and photographed using gel documentation system. Strains with identical restriction fragment profiles (in individual fragment size and number) were classified into the same intergenic spacer (IGS) group. *Msp* I was the only restriction enzyme used and it provided the required resolution for this study.

Statistical analysis

Data on nodule fresh weight, shoot dry weight and N fixation from both experiments was subjected to analysis of variance to assess the effects of (and interactions between) treatments using the mixed procedure of the SAS System (SAS 2006). Least square means and standard errors of the difference (SED) were calculated using the lsmeans (least square means) and pdiff (test of significance for differences) options of the mixed procedure. Significance of difference was evaluated at $P < 0.05$.

RESULTS

Experiment 1

Nodulation

There were significant differences observed in nodule fresh weight between the different soils for both cowpea and green gram (Fig. 2). In cowpea, plants cultivated in soils from Isiolo A, Bondo B, and Kilifi A had the highest

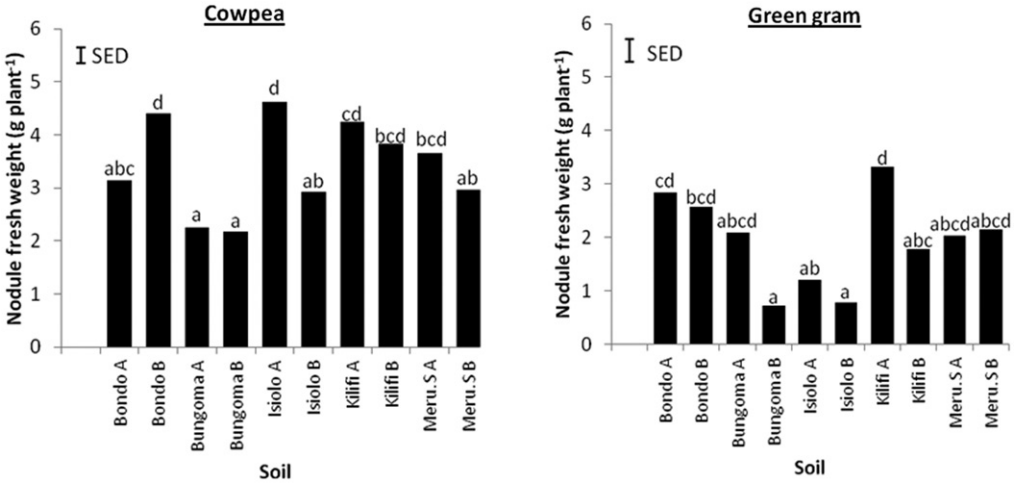

Figure 2 Nodule fresh weight of cowpea (*Vigna unguiculata* L. Walp.) and green gram (*Vigna radiata* L. Wilczek.) grown in 10 different soils. The error bars represent the standard error of the difference (SED) for the soil × legume interaction. Means followed by different letters are significantly different at $P < 0.05$. Means separated by Tukey's test.

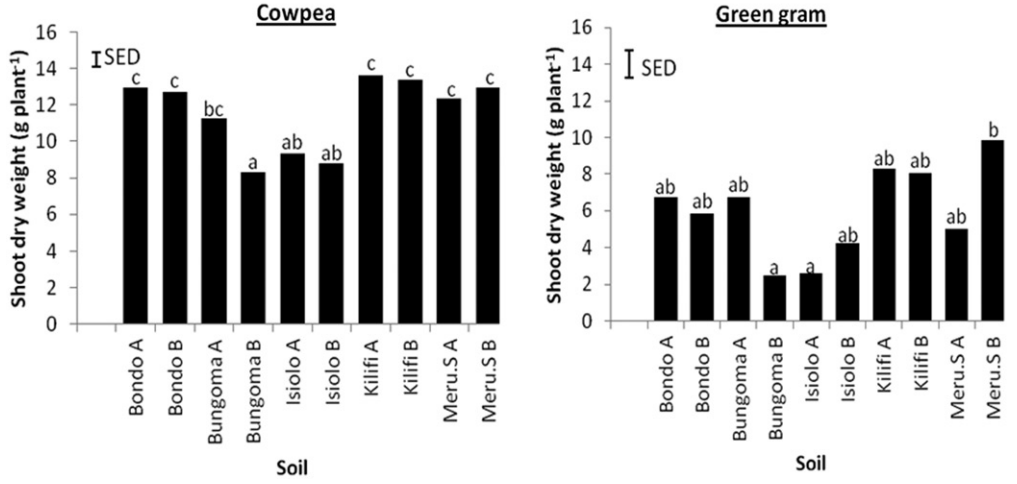

Figure 3 Biomass yields of cowpea (*Vigna unguiculata* L. Walp.) and green gram (*Vigna radiata* L. Wilczek.) grown in 10 different soils. The error bars represent the standard error of the difference (SED) for the soil × legume interaction. Means followed by different letters are significantly different at $P < 0.05$. Means separated by Tukey's test.

nodule fresh weight (4.63, 4.40 and 4.25 g plant^{-1}, respectively). The lowest nodule fresh weight was observed in plants cultivated in soils from Bungoma B and A (2.17 and 2.24 g plant^{-1}, respectively). No significant differences were observed between plants cultivated in soils from Bondo A, Isiolo B and Meru south B (Fig. 2).

In green gram, the highest nodule fresh weight was observed in plants cultivated in soils from Kilifi A and Bondo A (3.32 and 2.84 g plant^{-1}, respectively). No significant effects on nodule fresh weight were observed between plants cultivated in soils from Bungoma A, and both sites in Meru South (Fig. 2). The lowest nodule fresh weight was observed in plants cultivated in soils from Bungoma B, Isiolo B and A (1.22, 0.79 and 0.72 g plant^{-1}, respectively).

Biomass

There were significant differences observed between the different soils in biomass yield in both cowpea and green gram (Fig. 3). In cowpea, plants cultivated in soils from Kilifi A and B, Meru South B, and Bondo A had the highest shoot dry weight (13.60, 13.37, 12.93 and 12.93 g plant^{-1}, respectively), while plants cultivated in soils from Bungoma B, Isiolo B and Isiolo A had

Table 4 δ^{15} N values (‰) of cowpea (*Vigna unguiculata* L. Walp.), green gram (*Vigna radiata* L. Wilczek.), sorghum (*Sorghum angustum* L.) and wheat (*Triticum aestivum* L.) cultivated in 10 soils from Experiment 1

Soil	Cowpea (*Vigna unguiculata* L. Walp.)	Green gram (*Vigna radiata* L. Wilczek.)	Sorghum (*Sorghum angustum* L.)	Wheat (*Triticum aestivum* L.)
Bondo A	−0.61 bc	−1.16 ab	6.50 abc	3.02 ab
Bondo B	−0.99 abc	−0.72 ab	4.90 ab	1.72 ab
Bungoma A	−2.00 a	−2.20 a	2.54 a	2.67 ab
Bungoma B	1.03 d	4.05 bc	13.13 d	4.75 ab
Isiolo A	−1.93 a	0.73 abc	7.95 bc	1.94 ab
Isiolo B	−0.88 abc	2.87 abc	6.18 abc	3.49 ab
Kilifi A	−1.69 ab	−2.28 a	5.56 abc	0.72 a
Kilifi B	−0.45 bc	5.48 c	9.52 cd	7.10 b
Meru.S A	−1.10 abc	−0.78 ab	4.69 ab	0.63 a
Meru.S B	−0.14 cd	0.93 abc	8.22 bc	3.65 ab
(SE)	(0.25)	(1.22)	(0.90)	(1.15)

†Values indicate the means (SE). Means followed by the same letter in a row are not significantly different from each other at $P < 0.05$. Means separated by Tukey's test.

the lowest shoot dry weight (8.30, 8.76 and 9.36 g plant^{-1}, respectively). No significant differences were observed between plants cultivated in soils from both sites in Bondo, Kilifi and Meru South (Fig. 3).

In green gram, the highest shoot dry weight was observed in plants cultivated in soil from Meru South B (9.82 g plant^{-1}). No significant differences were observed in plants cultivated in soils from Bondo A and B, Bungoma A, Isiolo B, Kilifi A and B, and Meru South A. The lowest shoot dry weights were observed in plants cultivated in soils from Bungoma B and Isiolo A (2.51 and 2.58 g plant^{-1}, respectively). For both legumes there was no relationship between nodule fresh weight and biomass yield since high nodule fresh weight did not lead to high shoot dry weight in any of the soils for both legumes.

Biological N fixation

For correct estimation of BNF in cowpea and green gram using the natural abundance method, the δ^{15}N values in sorghum were preferred for use in % Ndfa calculation as shown in Table 4.

In cowpea, plants cultivated in soil from Kilifi A resulted in the highest Ndfa (98%) while plants cultivated in soil from Bungoma A had the lowest Ndfa (83%) (Fig. 4). No significant differences were observed in % Ndfa values of plants cultivated in soils from Bondo A and B, Bungoma B, Isiolo A and B, Kilifi B and Meru South B, with Ndfa values ranging from 86 to 94% (Fig. 4).

In green gram, the highest % Ndfa was observed in plants cultivated in soil from Kilifi A (97%) and Bungoma A (94%), respectively. No significant effects on % Ndfa were observed between plants cultivated in soils from sites in Bondo B, Bungoma B, Isiolo A and both sites in Meru South (Fig. 4). The lowest % Ndfa was observed in plants cultivated in soils from Kilifi B and Isiolo B (34 and 38%, respectively).

Experiment 2

MPN assessment

MPN assessment showed an estimate of $>13.5 \times 10^3$ CFU rhizobia g^{-1} of the initial soil suspension for each legume in soil from Chonyi.

Nodulation

Rhizobial inoculation did not significantly ($P < 0.05$) affect nodulation in cowpea (Fig. 5). In green gram, the highest nodule fresh weight was observed in green gram peat inoculation treatment (2.99 g plant^{-1}), but this was not significantly different from Biofix 2447 (2.65 g plant^{-1}), the control (2.16 g plant^{-1}) and mineral N treatment (1.95 g plant^{-1}). The lowest nodule fresh weight was observed in Biofix 704 and Rizoliq treatments (1.75 and 1.63 g plant^{-1}), respectively.

Biomass

Inoculation did not significantly affect shoot dry weight in cowpea (Fig. 6). In green gram, the highest shoot dry weight was observed in mineral N applied treatment (12.41 g plant^{-1}); while inoculation with Rizoliq had the lowest effect on shoot dry weight (11.85 g plant^{-1}). There was no significant difference among treatments (Fig. 6).

Shoot N content

Rhizobial inoculation did not significantly ($P < 0.05$) affect shoot N content in green gram relative to the control. In cowpea, the highest total N content was

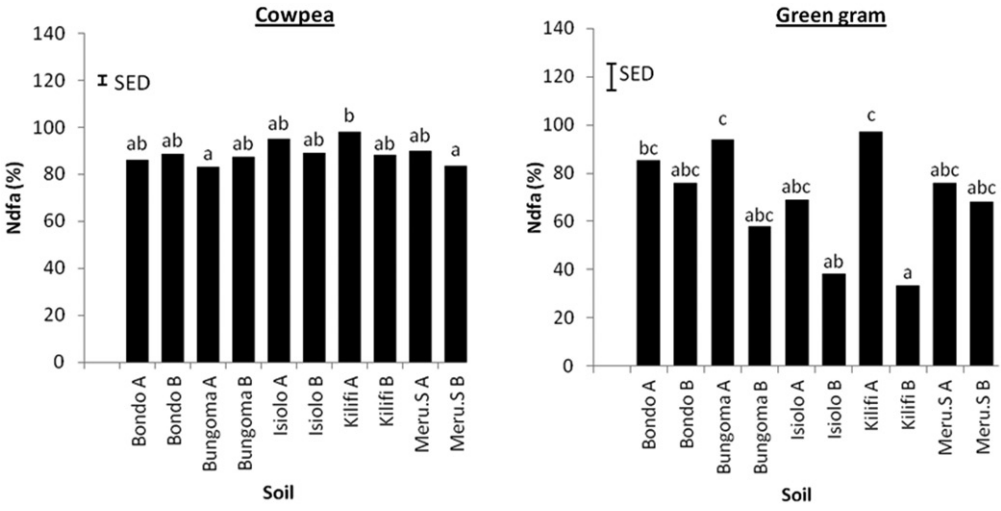

Figure 4 Proportion of nitrogen (N) derived from the atmosphere (% Ndfa) by cowpea (*Vigna unguiculata* L. Walp.) and green gram (*Vigna radiata* L. Wilczek.) in 10 different soils containing indigenous rhizobia. The error bars represent the standard error of the difference (SED) for the soil × legume interaction. Means followed by different letters are significantly different at $P < 0.05$. Means separated by Tukey's test.

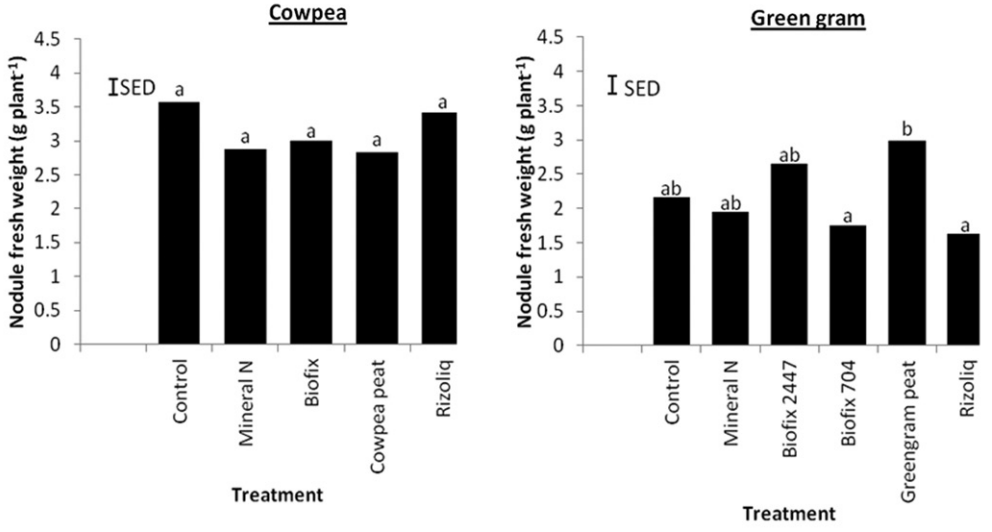

Figure 5 Nodule fresh weight of cowpea (*Vigna unguiculata* L. Walp) and green gram (*Vigna radiata* L. Wilczek.) plants inoculated with various inoculants. Error bars represent standard error of the difference (SED) for the inoculant effect. Means followed by different letters are significantly different at $P < 0.05$. Means separated by Tukey's test.

observed in the cowpea peat inoculation treatment (402.08 mg plant^{-1}), but this was not significantly different from Biofix, Rizoliq and mineral N treatments. The lowest total N content was observed in the control (291.56 mg plant^{-1}) (Fig. 7).

Nodule occupancy

In total, seven IGS profiles were obtained from PCR-RFLP analysis of pure cultures isolated from the commercial products as follows: three from Biofix cowpea, two from Rizoliq green gram and one each from green gram peat and Rizoliq cowpea (Fig. 8). No profiles were obtained from cowpea peat, Biofix green gram 704 and Biofix green gram 2447 inoculants due to failure of restriction of their respective PCR products.

A total of five IGS groups were obtained from the PCR-RFLP analysis of 71 nodules from cowpea plants inoculated with Biofix, Cowpea peat, Rizoliq inoculants, and uninoculated control treatments. IGS III was present in all the treatments and predominant in Biofix, Rizoliq and the uninoculated control (63.2, 60 and 52.9%

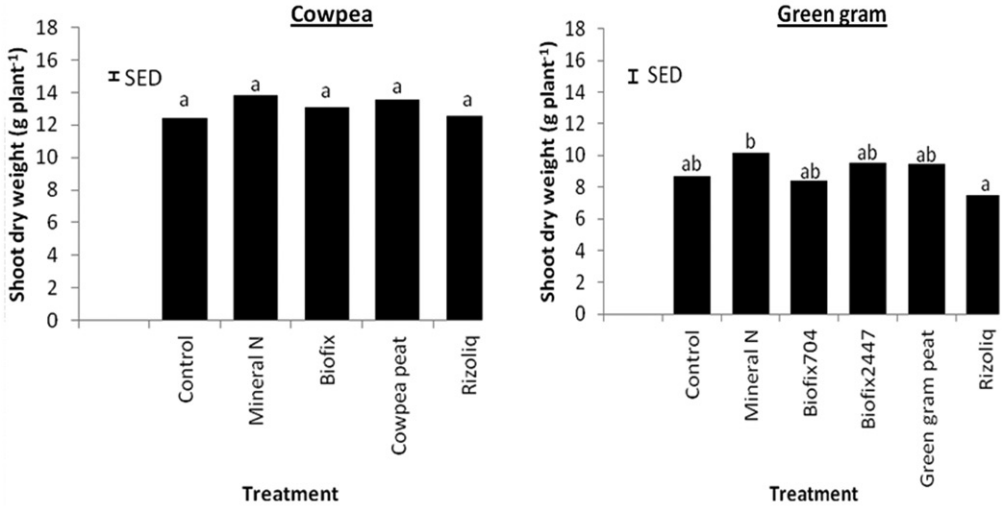

Figure 6 Biomass yield of cowpea (*Vigna unguiculata* L. Walp.) and green gram (*Vigna radiata* L. Wilczek.) plants inoculated with various inoculants. Error bars represent standard error of the difference (SED) for the inoculant effect. Means followed by different letters are significantly different at $P < 0.05$. Means separated by Tukey's test.

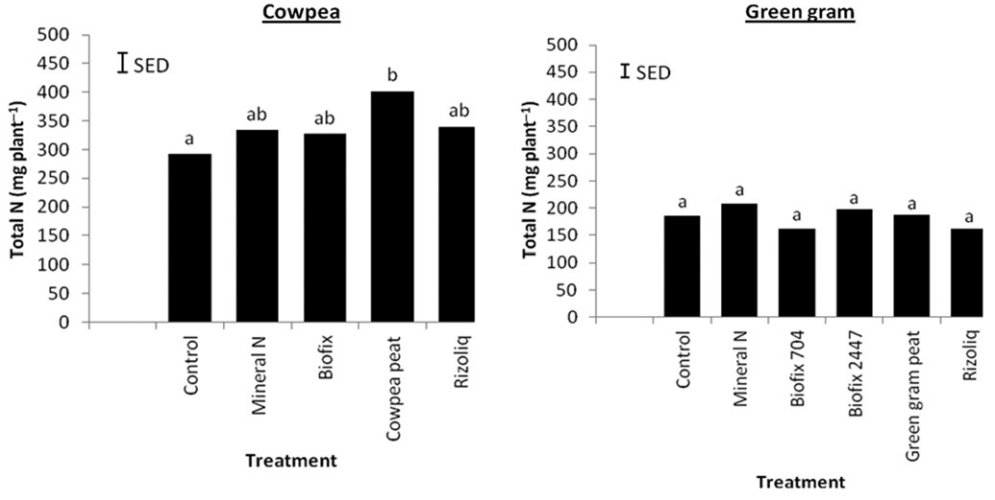

Figure 7 Total nitrogen (N) content of cowpea (*Vigna unguiculata* L. Walp.) and green (*Vigna radiata* L. Wilczek.) gram plants inoculated with various inoculants. Error bars represent standard error of the difference (SED) for the inoculant effect. Means separated by Tukey's test.

occupancy, respectively). IGS V was only present in cowpea peat treatment and had low occupancy (5.9%). Nodules from cowpea peat treatment were the only ones represented in all the five IGS profiles (Table 5). On the other hand, six IGS groups were obtained from analysis of 97 nodules from green gram plants inoculated with Biofix 704, Biofix 2447, Green gram peat, Rizoliq inoculants and uninoculated control treatments. IGS III was present in all treatments and predominant in Biofix 704, Rizoliq and in the uninoculated control treatments (75, 73.7 and 61.1%, respectively). IGS VI was only present in green gram peat treatment and had low occupancy (5%). IGS II was also present in all treatments albeit with low occupancy. The IGS profiles were common in both legumes except IGS VI, which was unique in green gram nodules as shown in Table 5. IGS III was the most predominant in both species.

DISCUSSION

Cowpea and green gram plants grown in 10 soils from five regions in Kenya were all nodulated indicating presence of indigenous rhizobial populations in these

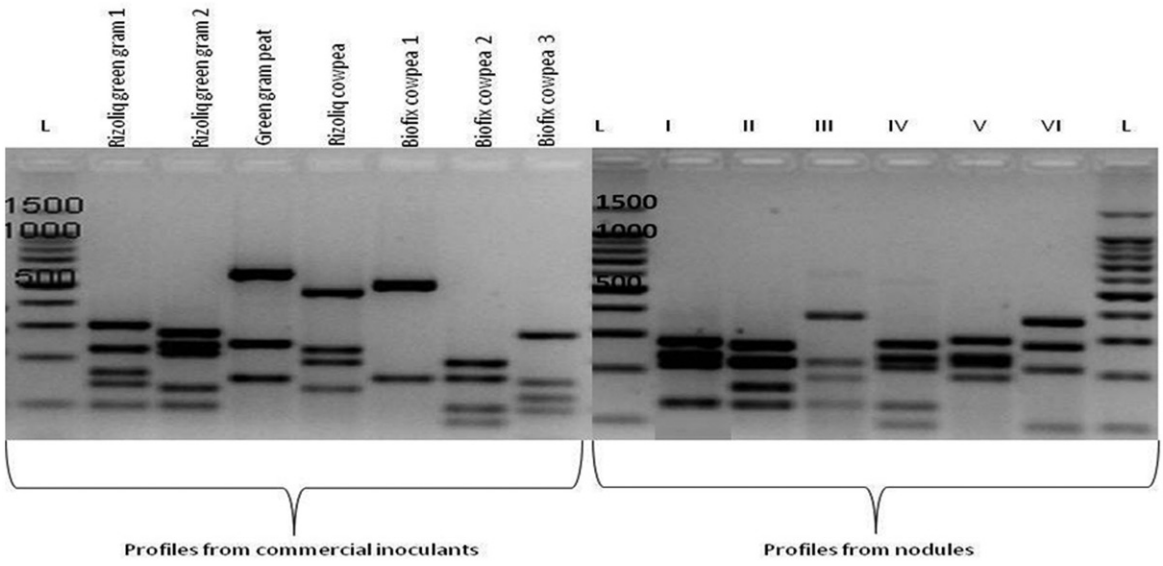

Figure 8 Intergenic spacer region (IGS) profiles obtained from *Msp* I restricted products of bradyrhizobia isolated from commercial inoculants and nodules of cowpea (*Vigna unguiculata* L. Walp.) and green gram (*Vigna radiata* L. Wilczek.) in the inoculation Experiment 2.

Table 5 Summary of intergenic spacer region (IGS) groups from the nodule occupancy in cowpea (*Vigna unguiculata* L. Walp) and green gram (*Vigna radiata* L. Wilczek.) (Experiment 2)

Treatment	Nodule occupancy in IGS groups (%)						No. of nodules analyzed
	I	II	III	IV	V	VI	
Cowpea (*Vigna unguiculata* L. Walp.)							
Control	47.1	0	52.9	0	0	0	17
Cowpea peat	35.3	17.6	35.3	5.9	5.9	0	17
Biofix	0	10.5	63.2	26.3	0	0	19
Rizoliq	0	15	60	25	0	0	20
Green gram (*Vigna radiata* L. Wilczek.)							
Control	33.3	5.6	61.1	0	0	0	18
Green gram peat	35	20	25	5	10	5	20
Biofix 704	15	5	75	0	5	0	20
Biofix 2447	0	15	35	50	0	0	20
Rizoliq	0	5.3	73.7	21	0	0	19

soils. These results are in agreement with previous studies which reported the presence of indigenous rhizobia nodulating diverse legumes in Kenyan soils (Wasike *et al.* 2009; Chemining'wa *et al.* 2011; Mwangi *et al.* 2011; Mwenda *et al.* 2011). The presence of these indigenous rhizobia can be attributed to widespread integration of the legumes in the cropping systems in Kenya (Gethi *et al.* 1997).

Effective nodules were present in both legumes as estimation of Ndfa in the different soils showed a high level of dependence on symbiotic N fixation. Ndfa values of 83–98% for cowpea are in line with findings of Naab *et al.* (2009) where Ndfa values, ranging from 70.6–99.7% were obtained, indicating that cowpea meets a large proportion of its N requirements from symbiotic fixation. In green gram, lower Ndfa values were observed than those from cowpea across plants cultivated in soils from the different regions. This variation in N_2 fixation by both legumes is in agreement with the results of Dayathilake *et al.* 2001, who observed differences in N_2 fixation (Ndfa) among cowpea (49–76%) and mungbean (45–70%), and Tien *et al.* 2002 who obtained Ndfa ranges of 56–89% for soybean and 45–76% for mungbean. Influence of the host plant

on symbiotic effectiveness, nodulation, N_2 fixation and biomass yield has been reported in other studies (Hafeez *et al.* 2000; Thrall *et al.* 2011). The variability observed in nodulation, shoot dry weight and BNF for both legume species in the 10 different soils can be attributed to the differences in competitive ability and effectiveness of the indigenous rhizobial populations in these soils. According to Martins *et al.* (2003), the rhizobial population in the soil can be extremely variable, both in the composition and the symbiotic characteristics of a species. Giller (2001) suggests that this can be due to differences in levels of soil pH, plant nutrients, soil type, soil moisture, temperature and crop or soil management, and previous cropping history among other factors.

In the second experiment, failure of inoculation to elicit response in cowpea and green gram could be attributed to the presence of effective indigenous rhizobia or highly competitive but ineffective indigenous strains that lock out the inoculant strains from occupying the nodules (Theuri *et al.* 2006). Yield and nodulation are seldom increased by inoculation partly due to high population size of indigenous rhizobia in tropical soils (Kang 1977; Danso and Owiredu 1988; Thies *et al.* 1995). This was confirmed by the MPN trial on the soil from Chonyi indicating rhizobial populations of $>13.5 \times 10^3$ CFU g^{-1} of soil for each legume. Indigenous populations of *Bradyrhizobium* can range from a few to $>10^5$ cells g^{-1} soil, depending on soil moisture, soil fertility and the extent of the homologous legume component of the vegetation (Woomer *et al.* 1988). According to Brockwell *et al.* (1995) naturally occurring populations of rhizobia are significant factors determining the establishment of inoculants strains in the field. Where inoculants are added to soil harboring large background populations of rhizobia of variable effectiveness, a low proportion of nodule occupancy by inoculant strains is the major barrier to increased N input by fixation (McInnes and Haq 2003). However, there is evidence that a significant response to inoculation is possible in soils containing large numbers of established rhizobia when strains with both superior N_2 fixation efficiency and nodulation competitiveness are inoculated (Bradley *et al.* 1991; Hungria *et al.* 1998). Another factor that may have resulted in no inoculation response is the quality of inoculants used; it was not possible to obtain any PCR product of the expected size (930–1050 bp) from Biofix 704 and 2447 inoculants for green gram, while Biofix from cowpea contained three different strains. The primer set for PCR selected in this study was specific for amplification of the 16S and 23S rDNA IGS region which is present in cowpea and green gram rhizobia. This suggested the presence of other microorganisms in the commercial inoculants. Given that none of the strains in Biofix was competitive enough against indigenous rhizobia strain, competitiveness may be influenced by the presence of other strains in the same inoculant (Raposeiras *et al.* 2006). Partial 16S rRNA sequencing of strains contained in Biofix inoculants revealed presence of *Bacillus* spp., *Pseudomonas* spp. and *Stenotrophomonas* spp. (M. Atieno, personal communication). The presence of multiple strains, some of which may not be rhizobia, raises concern about the quality.

Five out of the six groups obtained by the PCR-RFLP analysis did not correspond to any of the profiles from the commercial inoculants applied. This suggests that the strains in the commercial inoculants were less competitive than indigenous strains in the soil tested and that competition between applied and indigenous rhizobia was strongly influenced by the size of indigenous rhizobial populations. According to Leite *et al.* (2009) ineffective indigenous rhizobia can compete with introduced inoculant strains for sites of infection on roots of the host plant. Reports by Dudman and Brockwell (1968), Ham (1980), and Carter *et al.* (1995) indicate that when soils contain indigenous rhizobia, the inoculant rhizobia have to compete with these indigenous rhizobia for the formation of nodules. Very often they are not successful and cannot be recovered from the plant nodules.

Both legumes belong to the *Vigna* spp. and are cross-nodulated by *Bradyrhizobium* spp. and have been shown to share common bradyrhizobial genotypes (Zhang *et al.* 2008; Appunu *et al.* 2009) as seen for IGS profiles I–V in our study. IGS III was predominant in both legumes and was distributed in all the treatments applied to cowpea and green gram. The dominance of indigenous strains can be attributed to a number of factors; the adaptability of indigenous rhizobia to their environment results in high levels of saprophytic competence (Zengeni *et al.* 2006); build up of background strains in the presence of the host rhizosphere (Dowling and Broughton 1986; Bushby 1993; Mendes and Bottomley 1998) and the competitive ability of background strains for sites of infection on the host plant roots and nodulation (Laguerre *et al.* 2003; Leite *et al.* 2009). It is therefore imperative to consider how rhizobial populations in the soil can be manipulated to influence, with or without effectual inoculation, legume nodulation, N_2 fixation and plant productivity (Brockwell *et al.* 1995; McInnes and Haq 2003). Hence, screening is required for the selection of effective indigenous strains that are competitive for nodulation that have potential use as inoculants to improve cowpea and green gram yields.

CONCLUSIONS

Our results suggest that the systematic inoculation of cowpea and green gram with commercial inoculants to

improve biomass yields is not necessary in the regions of Kenya reported in this study. However, the study reveals that it would make sense to promote the utilization of effective indigenous strains. The six IGS profiles obtained in our study from Chonyi soil indicates that there is diversity of indigenous cowpea and green gram rhizobia in Kenyan soils and further exploration in different AEZ is required. It would therefore be useful to select competitive and well adapted strains from diverse ecological regions that are capable of dominating nodule occupancy in mixed background populations for production of effective inoculants in order to optimize BNF and thus increase cowpea and green gram yields at low cost.

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