



Symbiotically effective native rhizobia obviate significant response to inoculation with introduced exotic strains

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Abstract: Rhizobia inoculation is an ecological option for enhancing grain yield of legumes in soils with low nitrogen and native rhizobia. The contribution of *Bradyrhizobium japonicum* strain 532c in nitrogen fixation and grain yields of soybean and cowpea was evaluated in a field experiment in the Upper East region of Ghana. The effectiveness of isolated indigenous rhizobia from the soils at the study location was evaluated in sterile medium at the greenhouse to ascertain non-response in the field data. *Bradyrhizobium japonicum* strain 532c produced a significantly lower grain yield compared to the nitrogen-fertilized plants and the uninoculated control for soybean. Strain 532c produced the highest grain yield on cowpea. Strain 532c did not increase N fixation in soybean and cowpea. Sixteen and eleven percent of the isolates were effective on cowpea and soybean respectively. Our results imply that a small number of effective strains among indigenous rhizobia population can obviate significant response to introduced strains. This gives an indication that effective indigenous rhizobia can be harnessed for increased soybean and cowpea production in Ghana.

Keywords: rhizobia, grain yield, biological nitrogen fixation, inoculation

1. Introduction

Leguminous crops need considerable amounts of nitrogen for enhanced growth [11]. However, in most smallholder farms in Africa, nitrogen is often deficient in the soils. The nutrient deficiency problem is further compounded by continuous cropping on the same piece of land year after year with minimal or no fertilizer inputs, thus depleting the nutrient reserves in the soils. This deficiency could largely be corrected using inorganic fertilizers and rhizobia inoculants. Though inorganic fertilizers are very productive and efficient, the excessive cost is disincentive to smallholder farmers, and the negative impacts on the environment when misused are major concerns [17]. More so, farmers rarely apply inorganic fertilizers to their legume crops as compared with cereals and other crops. Rhizobia inoculants contain bacteria which possess unique features that enable them to form symbiotic association with legumes, which results in the production of ammonium from atmospheric nitrogen [23]. Rhizobium inoculant is said to offer a better option for improving the yields of legumes by enhancing soil fertility through nitrogen fixation [19]. The use of inoculant is environmentally friendly, whereby it serves the dual purpose of increasing yield and protecting the soil.

Soybean and cowpea are among the predominant grain legumes grown in sub-Saharan Africa (SSA) and are becoming increasingly relevant due to their varied usage. This makes them ideal legume crops for this experiment. Soybean and cowpea can form symbiosis with native rhizobia, but cowpea is highly promiscuous and can form symbiosis with a wide range of rhizobia. Until recently, *Bradyrhizobium japonicum* hardly existed in Ghanaian soils, since soybean is an introduced crop from America [18]. To fully exploit the benefits of soybean and cowpea production in SSA, promiscuous soybean varieties have been introduced to address the rhizobia specificity traits of the American soybean varieties and to allow the plant to form symbiosis with a wide range of native rhizobia [18]. However, native rhizobia are not able to meet the nitrogen demand of promiscuous cowpea and soybean. Consequently, superior strains must be introduced into the rhizosphere through inoculation for an adequate infection and effective symbiosis, which will lead to higher grain yields [22]. Introducing exotic strains into an area is not a means to an end, as the genetic potential of the exotic strains is limited by several factors, including competition from indigenous rhizobia. The indigenous rhizobia are more persistent [9] and well adapted to local conditions, which gives them competitive advantage over exotic strains for nodule occupancy. Exotic strains of rhizobia must be assessed in the presence of indigenous rhizobia strains for their superior symbiosis. We hypothesised that soybean and cowpea will not respond to exotic rhizobia strains in the presence of low and effective indigenous rhizobia populations. The study was conducted to evaluate the effect of rhizobia inoculation on the growth and grain yield of soybean and cowpea.

2. Materials and methods

Field evaluation of rhizobia inoculant

Study location

Field work was conducted at Tono in Kassena Nankana District, Upper East region, Ghana. The study was conducted between June and November 2021. The study location was situated at latitude 10° 49' N and longitude 1° 05' W. The agroecological zone of the study location is the Sudan Savannah. The study location has a unimodal rainfall pattern that lasts for five to six months. The average annual rainfall is 885 mm.

Soil sampling, sample preparation, and laboratory analysis

Prior to planting, seven core soil samples were collected on cowpea and soybean fields at 0–20 cm depth with the help of an auger. The soil samples were bulked and thoroughly mixed. A composite sample was used for the physical and chemical analyses as well as enumeration of rhizobia. The samples were passed through a 2-mm mesh sieve to remove debris before the analyses. The standard procedures of [3] were used for the physical and chemical analysis. The indigenous rhizobia population was estimated using the most probable number count technique [31]. Five-fold, six-step serial dilution was performed for the soil samples. One millilitre aliquot of each dilution level for the soil sample was inoculated onto cowpea and soybean seedlings in growth pouches and monitored for 28 days. Root nodule scoring was done after 28 days. The rhizobia population was estimated using the MPNES software [32]. The number of cells in the inoculant (*Bradyrhizobium japonicum* strain 532c) obtained from the Microbiology Laboratory of KNUST was assessed using the Miles and Misra Method [16]. Petri dishes were incubated for 5 days at 28 °C. Petri dishes with colonies between 5 and 50 for each dilution level were counted and used for the estimation based on the below formula:

$$\text{CFU/ml} = \text{number of colonies} \times 50 \times \text{dilution factor}, \quad (1)$$

for drop plate technique

Land preparation, planting, and inoculant application

The experimental field was harrowed and ridged at 75 cm apart after ploughing. The plot sizes for soybean and cowpea fields were 3 m x 5 m. The seeds were planted at 75 cm x 5 cm (soybean) and at 75 cm x 20 cm (cowpea) using the dibbling method. Three seeds were planted per hill and then thinned to two seedlings per

hill after germination. On the other hand, cowpea seeds were sown at a rate of three seeds per hill and thinned to two seedlings per hill two weeks later. A maize variety, obaatampa, was used as the reference crop for nitrogen fixation estimation. Maize was planted at 75 cm x 40 cm at a rate of three seeds per hill and thinned to two seedlings after germination. Five grams of the inoculant was applied to 1 kg of seeds after gum arabic had been added to the seeds. The seeds were air dried under shade for 30–45 minutes with intermittent stirring to enhance the drying and sticking of the inoculant onto seeds before planting.

Experimental design and treatments

The experiments were laid out in a Randomized Complete Block Design (RCBD) with four replications for soybean and cowpea. Three treatments were applied to each crop. The treatments were 100 kg N ha⁻¹ as urea, *Bradyrhizobium japonicum* strain 532c and uninoculated control. All plots received basal application of 30 kg P and 30 kg K ha⁻¹. The P and K were from triple superphosphate (TSP) and muriate of potash (MOP). The nitrogen treatment was applied in two splits: 50 kg N ha⁻¹ two weeks after planting and 50 kg N ha⁻¹ at 50% flowering for both crops. The mode of fertilizer application was band placement at 5 cm away from the plant. The band placement maximizes fertilizer use efficiency.

Assessing the effectiveness of rhizobia isolates at the greenhouse

The greenhouse experiment was conducted in pots arranged in randomized complete block design with three replications. Acid-washed river sands were placed in the 5 kg pots to the brim [27]. Prior to filling the pots with the river sand, it was autoclaved at 121 °C, under 1.33 bar pressure for 1 hour. For each experiment, each pot received four seeds of either soybean or cowpea, which were thinned to two after germination.

Acquisition of nodules and culturing of isolates

Fifty and thirty root nodules of cowpea and soybean obtained from the MPN test were surface sterilized with 95% ethanol and 3% H₂O₂ for 10 seconds and 3 minutes respectively. The root nodules were rinsed thoroughly with sterilized distilled water.

Sterilized forceps were used to crush the root nodules in a Petri dish. Inoculating loop was used to streak the discharge of each root nodule onto Congo red yeast extract mannitol agar plates [27]. The Petri dish was sealed with parafilm to prevent contamination and then labelled accordingly. The Petri dishes were incubated at a temperature of 28 °C for up to 7 days. Petri dishes with colonies similar to

rhizobia were selected and colonies picked for repeated streaking and purification. A loopful of the isolates from each pure culture was transferred into sterilized 30-ml yeast extract mannitol broth and incubated at 28 °C in an orbital incubator at 125 revolutions per minute until the mid-exponential growth phase with an optical density of 600 nm (O.D.₆₀₀) at approximately 0.8, as determined with a spectrophotometer [21].

Inoculation of plants in sterilized river sand

One millilitre of liquid culture of each isolate was used to inoculate either cowpea or soybean seeds at planting. Soybean and cowpea seeds were sterilized with 95% ethanol for 1 min and rinsed thoroughly with sterile distilled water before planting. Nineteen isolates were obtained from the fifty cowpea nodules, while nine were obtained from the thirty soybean nodules. Thus, a total of twenty-eight isolates were evaluated (19 on cowpea and 9 on soybean) alongside a positive control (*Bradyrhizobium japonicum* strain 532c) and uninoculated treatments with or without nitrogen. The nitrogen treatments in each experiment received 0.5 g l⁻¹ KNO₃. Soybean and cowpea plants were irrigated with nitrogen-free nutrient solution. The experiment was set up for 6 and 8 weeks for cowpea and soybean respectively. Peak nodulation occurs within each period of growth stage for both crops.

Data assessment

Nodule number and shoot biomass were recorded at 50% flowering stage for cowpea and soybean. Five cowpea and soybean plants were randomly harvested. Shoots were separated from the roots and prepared for drying by placing them in an envelope. Roots were washed with water to remove adhering soil. The detached nodules and fresh shoots were placed in an oven at 60 °C for 72 h for dry weight estimation. At physiological maturity, soybean and cowpea pods were harvested. The pods were threshed and winnowed to obtain the grains. Both cowpea and soybean seeds were dried to constant weight at 60 °C. The grain yields were estimated on per hectare basis. Dried shoot samples of cowpea and soybean were taken to the Faculty of Agriculture Chemistry Laboratory, KNUST, milled and % N in shoot was determined using standard protocols. Nitrogen fixation in both crops was estimated using the modified equations of [30] as follows:

$$\text{Total N in plants} = \frac{\text{Shoot dry weight} \times \% \text{ N in shoots}}{100} \quad (2)$$

$$\text{Amount of N fixed} = \text{Total N in legume} - \text{Total N in reference crop} \quad (3)$$

For the greenhouse study, growth and nodulation parameters were assessed, as well as N_2 fixed. Fresh shoots were placed in an oven at 60 °C for 72 h to estimate the dry weights. The shoot biomass of the isolates was compared with that of the nitrogen-treated plants [4]. Roots were carefully removed from the growth medium and washed. The nodules were detached, counted, and dried at 60 °C for 72 hours. The relative effectiveness of the isolates was estimated based on the formula proposed by [10]:

$$E = \frac{X - Y}{Z - Y} \quad (4)$$

where X = shoot biomass of isolates, Y = shoot biomass of uninoculated control, Z = shoot biomass of nitrogen-treated plants.

The calculated relative effectiveness (E) for the isolates was grouped according to the level of effectiveness [4].

Statistical analysis

The data from the experiments were subjected to analysis of variance (ANOVA) using the general treatment structure in GenStat statistical software, 12th edition. Significant differences between treatments were assessed at 5% ($p = 0.05$) level of probability. The least significance difference (LSD) method was used to separate treatment means. All count data for the field experiment were logarithm transformed [14].

3. Results

Field evaluation of rhizobia inoculant

Soil physical and chemical properties and most probable number count

The soil's textural class at the study site was sandy loam. The soil was strongly acidic and had low levels of available P, very low organic carbon, very low levels of total N, and high levels of exchangeable K (*Table 1*). The rhizobia numbers estimated for soybean and cowpea were 57 and 75 cells g^{-1} soil respectively.

Table 1. The soil's physical and chemical analysis and the most probable number of indigenous rhizobia at the experimental site

Soil parameters	Soybean field	Cowpea field
pH (1:2.5 H ₂ O)	5.04	4.87
Total nitrogen (%)	0.013	0.012
Available phosphorus (mg kg ⁻¹)	10.64	12.12
Exchangeable potassium (cmol ₍₊₎ kg ⁻¹)	1.39	0.95
Textural class	Loamy sand	Loamy sand
MPN (Rhizobia cell g ⁻¹ soil)	57	75

Nodulation of soybean and cowpea

The nodule numbers produced by soybean and cowpea plants amended with *Bradyrhizobium japonicum* strain 532c were 1.98 and 2.03 respectively. These numbers were high compared with the numbers produced by the nitrogen-fertilized soybean (1.72) and cowpea (1.95) – though not significantly different. The uninoculated soybean and cowpea controls also produced nodule numbers that were statistically on a par with the treated plots (Table 2). In soybean, the nitrogen-fertilized plots produced the lowest number of nodules (1.72 plant⁻¹), whereas in cowpea, the lowest number of nodules was recorded for uninoculated plants (Table 2). *Bradyrhizobium japonicum* strain 532c increased cowpea and soybean nodule numbers by 5% and 15%, respectively, over the nitrogen-fertilized plots. Similarly, the application of *Bradyrhizobium japonicum* strain 532c increased cowpea and soybean nodule numbers by 5% and 9%, respectively, over the uninoculated controls; however, the differences were not significant ($P > 0.05$) (Table 2).

Table 2. Effect of the *Bradyrhizobium japonicum* strain 532c on the nodule count of soybean and cowpea

Treatments	Nodule number plant ⁻¹	
	Soybean	Cowpea
<i>Bradyrhizobium japonicum</i> strain 532c	1.98 ^a	2.03 ^a
100 kg N ha ⁻¹	1.72 ^a	1.95 ^a
Control	1.82 ^a	1.93 ^a
CV %	12.4	7.4

Notes: Values are log-transformed. Values within columns with the same letters are not significantly different from each other at 5% probability.

Nitrogen fixation of soybean and cowpea

Bradyrhizobium japonicum strain 532c did not elicit significant differences in nitrogen fixation in soybean and cowpea. The inoculation with *Bradyrhizobium japonicum* strain 532c increased N_2 fixation in cowpea by 11% over control (Figure 1). For soybean, uninoculated plants recorded a higher amount of N_2 fixed (10 kg N ha^{-1}) than the *Bradyrhizobium japonicum* strain 532c (Figure 2).

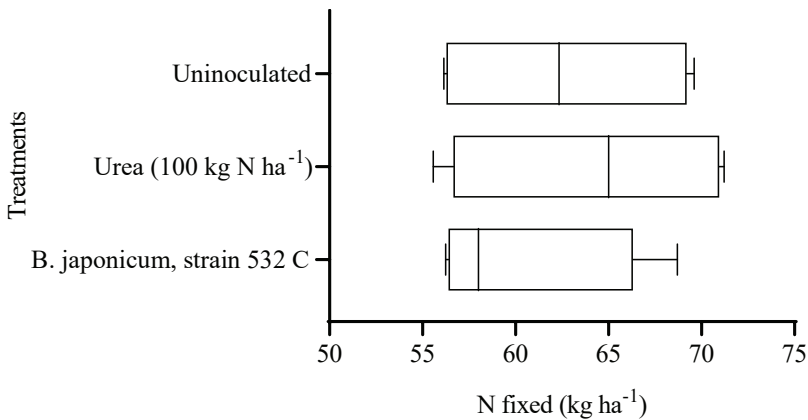


Figure 1. Nitrogen fixation as affected by *Bradyrhizobium japonicum* strain 532c on cowpea

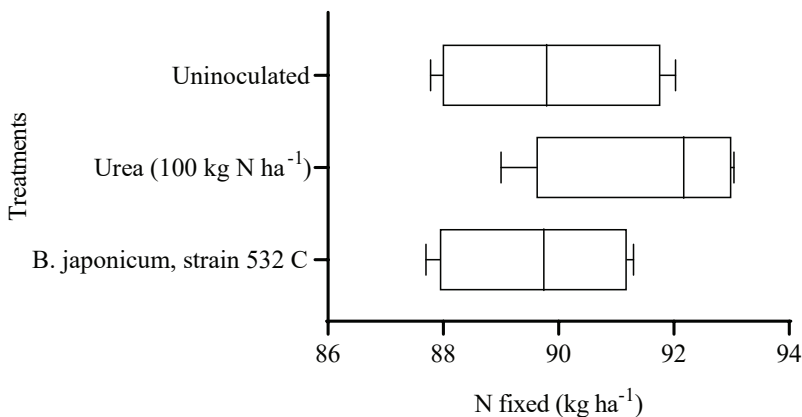


Figure 2. Impact of *Bradyrhizobium japonicum* strain 532c inoculation on the nitrogen fixation of soybean

Grain yield

Nitrogen-fertilized soybean plants significantly increased grain yields relative to *Bradyrhizobium japonicum* strain 532c inoculated plants (Figure 3). The grain yield of soybean plants that received *Bradyrhizobium japonicum* strain 532c was not different from the uninoculated control. Unlike soybean, cowpea inoculated with *Bradyrhizobium japonicum* strain 532c produced the highest grain yield of 797 kg ha⁻¹; however, it was not significantly different from the grain yield of nitrogen-fertilized plants (Figure 4).

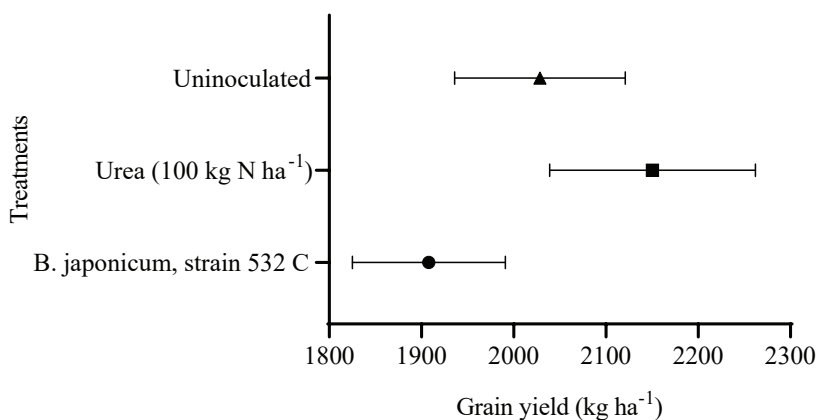


Figure 3. Grain yield of soybean as affected by *Bradyrhizobium japonicum* strain 532c inoculation and nitrogen

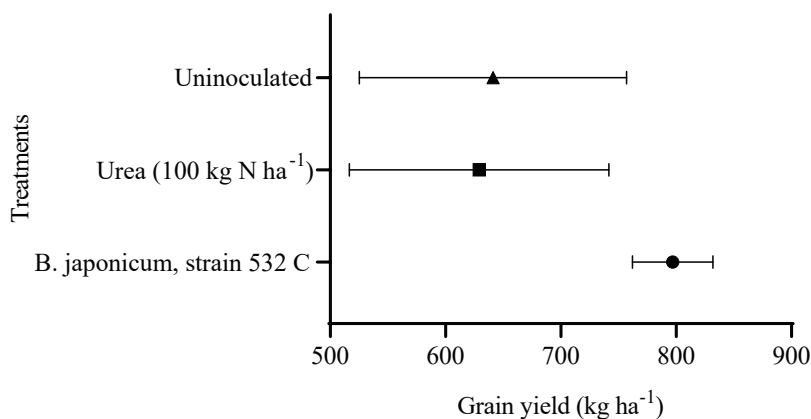


Figure 4. Grain yield of cowpea as affected by *Bradyrhizobium japonicum* strain 532c inoculation and nitrogen

Assessing the effectiveness of rhizobia isolates at the greenhouse

Nodulation of isolates on cowpea

Out of the nineteen isolates evaluated on cowpea, thirty-two percent produced more than fifty nodules per pot (Table 3). Isolate 9 significantly produced the highest number of nodules. About 21% of the isolates significantly increased nodule numbers relative to the *Bradyrhizobium japonicum* strain 532c. However, the nodule dry weight of 37% of the isolates was significantly lower than that of *Bradyrhizobium japonicum* strain 532c. The remaining 63% of the isolates had similar nodule dry weight to that of *Bradyrhizobium japonicum* strain 532c. The nitrogen-fertilized plants and the uninoculated control did not produce any nodule.

Table 3. Cowpea nodulation in response to rhizobia

Rhizobia isolates	Nodule number pot ⁻¹	Nodule dry weight (mg pot ⁻¹)
1	63.3	110.0
2	35.3	53.3
3	17.0	120.0
4	2.3	26.7
5	29.7	53.3
6	48.0	80.0
7	37.7	20.0
8	33.0	23.3
9	83.0	146.7
11	6.0	40.0
12	24.3	196.7
14	36.7	160.0
15	45.7	186.7
16	60.3	143.3
17	30.3	143.3
18	45.3	163.3
19	53.0	133.3
20	67.0	186.7
21	51.3	136.7
<i>Bradyrhizobium japonicum</i> strain 532c	34.7	170.0
LSD (0.05)	20.58	64.83
CV %	34.3	37.7

Relative effectiveness index of isolates on cowpea

Out of the 19 isolates evaluated on cowpea, 16% were classified as effective, 42% as moderately effective, and 42% as ineffective based on their performance (shoot dry weight produced) relative to the uninoculated control with (+N) (*Figure 5*). Isolates 19, 1, and 7 were grouped as being effective. Isolates 9, 16, 6, 20, 21, 18, 7, and 2 were classified as moderately effective. The remaining isolates were classified as ineffective (*Figure 5*).

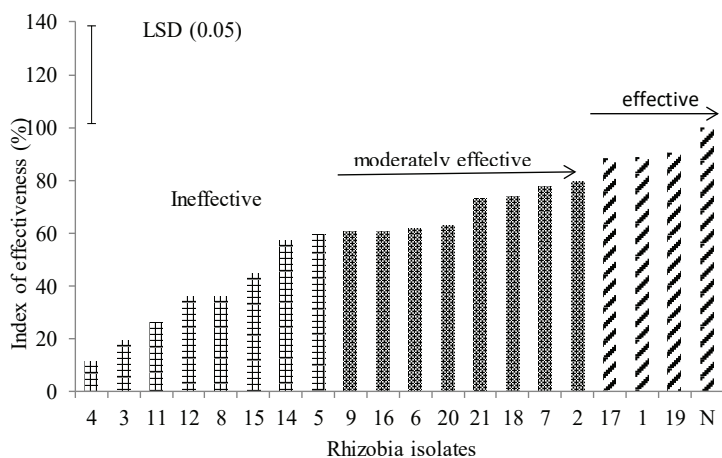


Figure 5. Index of effectiveness of rhizobia isolates on cowpea

Nodulation of rhizobia isolates on soybean

About 78% of the rhizobia isolates had significantly lower nodule number than *Bradyrhizobium japonicum* strain 532c (*Table 4*). The nodule dry weight of 11% of the rhizobia isolates was significantly higher than the nodule dry weight of *Bradyrhizobium japonicum* strain 532c (*Table 4*).

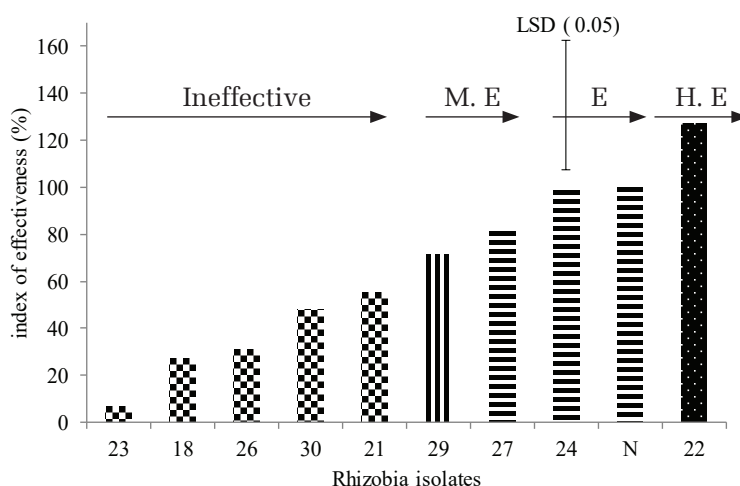
Table 4. Soybean nodulation response to rhizobia

Rhizobia isolates	Nodule number pot ⁻¹	Nodule dry weight (mg pot ⁻¹)
18	11.3	93.0
21	20.3	167.0
22	49.0	283.0
23	23.3	90.0
24	22.3	123.0
26	13.3	90.0
27	15.3	153.0

Rhizobia isolates	Nodule number pot ⁻¹	Nodule dry weight (mg pot ⁻¹)
29	16.7	163.0
30	39.3	153.0
<i>Bradyrhizobium japonicum</i> strain 532c	62.0	157.0
LSD (0.05)	20.38	99.9
CV (%)	43.5	39.5

Index of effectiveness of isolates on soybean

Rhizobia isolate performances on soybean were classified as highly effective (11%), effective (22%), moderately effective (11%), and ineffective (55%) (Figure 6).



Notes: M. E. = moderately effective, E. = effective, and H. E = highly effective.

Figure 6. Index of effectiveness of rhizobia isolates on soybean

4. Discussion

Field evaluation of rhizobia inoculant

Nodulation and biological nitrogen fixation of soybean and cowpea in the field

The success of rhizobia inoculation largely depends on the population of native rhizobia. There have been contradicting reports on the population of native rhizobia that can influence inoculation response. While [18] indicated that less

than 10 cells g⁻¹, [28] indicated that more than 100 cells g⁻¹ will negatively affect inoculation responses. The native rhizobia population from our experimental field falls within this range. However, the response to introduced rhizobia was not significant. This may indicate that, when conducting inoculation trials, not only the population of native rhizobia but their effectiveness should also be considered [15]. There have been contrasting reports on nodulation response to introduced strains. While [6, 9] reported no response at all, [2, 13] reported some nodulation response to introduced strain. The nitrogen fixations in this study are within the reported ranges [19, 34] even though the N-difference method of determining N fixation used in this study is less accurate compared to the other methods.

Grain yield of soybean and cowpea

Rhizobia inoculation in nitrogen-deficient soils is supposed to increase nitrogen fixation and enhance grain yield; however, this is not always the case, as factors such as pH, indigenous rhizobia population, and management may affect the introduced strains of rhizobia. The experimental field had low pH, which affects the survival and infectivity of introduced rhizobia strains, thereby hindering nitrogen fixation [5]. The native rhizobia at the study location were less than 100 cell g⁻¹, and no significant response to the introduced strains was observed. Our study indicated that the introduced strain *Bradyrhizobium japonicum* did not enhance the grain yield of cowpea and soybean. Although several studies [2, 13, 25, 28] have reported inoculation successes, some others [6, 28] did not observe any significant response to rhizobia inoculation. N-fertilized plants outperformed inoculated plants. [28] reported similar results. This indicates that nitrogen is a major yield-limiting factor in soybean. N-fertilized cowpea plants have similar yield to the introduced strains, indicating that N was not the only yield-limiting factor.

Assessing the effectiveness of rhizobia isolates at the greenhouse

Determining the need to inoculate often depends on the size of the native rhizobial population. High population densities of native rhizobia obviate significant response to introduced rhizobia strains [24, 26, 28]. Our study indicates that not only the size but also the effectiveness of the rhizobial population is an equally important consideration when deciding whether to inoculate. The effective isolates from the experimental sites outperformed the introduced strain *Bradyrhizobium japonicum* strain 532c. Native rhizobia are acquainted with local soil environment [9], which enhances their saprophytic competence. The negative and positive controls in the greenhouse experiment did not nodulate, confirming that nodulation can only occur in the presence of compatible rhizobia. Our results indicated that 16% of the isolates evaluated on cowpea plant was effective. This was higher than the 6% reported by [9].

5. Conclusions

Inoculation with *Bradyrhizobium japonicum* strain 532c at a rate of 5 g/kg⁻¹ of seed did not elicit any significant effect on the nodulation and grain yields of soybean and cowpea, and it did not affect nitrogen fixation in soybean and cowpea. The study location had low numbers of native rhizobia (57 and 75 rhizobia cells per gram of soil), as the populations did not exceed 100; however, it had some effective rhizobia as well. Sixteen percent of the isolates assessed on cowpea were effective, while 11% of the isolates assessed on soybean were highly effective. Results from the greenhouse evaluation of the native isolates enable a further characterization of the isolates to establish whether they are phylogenetically related to existing strains and to harness them for increased soybean and cowpea production in Ghana.

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