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Comparative evaluation of *Bradyrhizobium japonicum* USDA110 transferors for greener and economic soybean (*Glycine max* L.) agronomy in Southwestern Nigeria

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Abstract. Nigeria is the largest producer of soybean in sub–Saharan Africa plus soybean is one of the cheapest sources of protein in the developing world. Hence, substantial raise in soybean cultivation in Nigeria is pertinent. This study aims at evaluating *in–vitro* inoculation technology for soybean cultivation using local charcoal ($0.02 per hectare) against popularly imported peat ($3.00 per hectare) as carriers. *Bradyrhizobium japonicum* USDA110 was assayed for caseinase, lysine decarboxylase, citrate, lipase, and starch hydrolysis then scaled–up in yeast mannitol broth. Charcoal and peat carriers were prepared, inoculated, cured for 15 days at 28 °C and analysed on congo red agar. Soybean seeds were treated with inoculated charcoal and peat using gum Arabic, and cultivated on sterilized loamy soil samples. Untreated seeds served as negative control. *Bradyrhizobium japonicum* USDA110 was positive to bromothymol blue, catalase and oxidase, but negative to caesinase, lysine decarboxylase and starch hydrolysis. The height of the cultivated soybean plants was 37.10 ± 2.94 cm, 35.00 ± 1.27 cm and 17.70 ± 1.33 cm for the peat carrier, charcoal carrier and untreated seeds respectively, and the number of root nodules formed were 24.00 ± 1.00, 23.00 ± 1.00 and 5.00 ± 1.00 and for peat carrier, charcoal carrier and untreated seeds accordingly. There was no significant difference between the number of root nodules formed in charcoal and peat carrier plants at $p < 0.05$ value. This study reveals potentials for greener and economic soybean production in Nigeria.
Keyword: *Bradyrhizobium japonicum* USDA110 inoculation, charcoal carrier, peat carrier, soybean agronomy.

Introduction

Rhizobia are gram negative bacteria which inhabit the root nodules of most leguminous crops. They are soil bacteria that fix nitrogen (diazotroph) after becoming established inside root nodules of legumes [ROYCHOWDHURY *et al.*, 2015].

There are several different genera of *Rhizobia*, all of them belong to the Rhizobiales, a probably–monophyletic group of proteobacteria and they are soil bacteria characterized by their unique ability to infect root hairs of legumes and induce effective nitrogen–fixing nodules to form on the roots [MATIRU AND DAKORA, 2004].

Species of *Rhizobium* such as *Mesorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium* and *Sinorhizobium* form intimate symbiotic relationships with legumes by responding chemotactically to flavonoid molecules released as signals by the legume host.

These plants compounds induce the expression of nodulation genes in *Rhizobia*, which in turn produce lipochitooligosaccharide signals that trigger mitotic cell division in roots, leading to nodule formation [LHUISSIER *et al.*, 2001; DAKORA, 2004].

The legume–*Rhizobium* symbiosis is a classic example of mutualism whereby *Rhizobia* supply ammonia or amino acids to the plant and in return receive organic acids as a carbon and energy source [ROYCHOWDHURY *et al.*, 2015].

Rhizobia are grouped in two main genera; the fast–growing *Rhizobium* species which are usually isolated from pea, bean, clover, alfalfa, chickpea, and leucaena, and the slow–growing *Bradyrhizobium* species which are usually isolated from the soybean and cowpea [PAUL *et al.*, 2011; GIONGO *et al.*, 2008].

In the era of sustainable crop production, the plant–microbe interactions in the rhizosphere plays a pivotal role in

transformation, mobilization and solubilization of nutrients from a limited nutrient pool, and subsequently uptake of essential nutrients by plants to realize their full genetic potential [ESTRADA–DELOS *et al.*, 2001]. Presently, the use of biological approaches is getting prevalent as a complement or substitute to chemical fertilizers for increasing crop yield in an integrated plant nutrient management system [STURZ *et al.*, 2000].

Therefore, In this regard, the application of plant growth promoting rhizobacteria has gotten a vital role in promoting maintainable systems in agronomy [SHOEBITZ *et al.*, 2009].

Biofertilizers are known as microbial inoculants that can improve soil fertility and crop productivity [DATTA et al., 2015].

Biofertilizers achieve improved agronomy through colonization of rhizosphere or plant interior which leads to accessibility of essential nutrients to the host plant when it is applied to seed/plant faces or soil [VESSEY, 2003].

Biofertilizers are prepared as carrier–based inoculants which enable easy handling, long–term storage and effectiveness [SOMASEGARAN and HOBEN, 1994].

Among the various biofertilizers, bacterial inoculant is a major group which includes N_2 fixing Rhizobacteria that fix atmospheric nitrogen symbiotically in leguminous plants [BROWN and WALKER, 1970].

Peat materials are mostly used as a carrier for *Rhizobia* but it is expensive due to its limited availability in some part of [CHAO and ALEXANDER, 1984] including Nigeria.

Alternative materials such as sawdust [ARORA *et al.*, 2008], rice husk [KALJEET *et al.*, 2011], fly ash [KUMAR *et al.*, 2010] and biochar [SARANYA *et al.*, 2011] have been tested for bio–inoculants preparation.

Charcoal is indigenous in Nigeria, its application as carriers of *Bradyrhizobium japonicum* will be environmentally friendly and economically responsive.

Therefore, in this study, we focused on the use of native charcoal as an alternative to peat as carriers/transferors of *Bradyrhizobium japonicum* USDA110 which is approved and commercially used in International Institute of Tropical Agriculture (IITA) Nigeria, during the cultivation of soybean.

Material and methods

Sample collection. A pure strain of *Bradyrhizobium japonicum* USDA110 and peat were collected aseptically from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The bacterial culture was transferred to the Microbiology Department of the Federal University of Technology, Akure and kept in the fridge at 4 ºC for further studies.

Colonial and morphological identification of *B. japonicum* USDA110

The *B. japonicum* USDA110 count was carried out on selective media of yeast extract Congo red agar. For the preparation of yeast extract Congo red agar, 10 g of mannitol, 0.5 g of yeast extract powder, 0.5 g of potassium phosphate, 0.2 g of magnesium sulphate, 0.1 g of Sodium chloride and 15 g of agar powder were weighed and dissolved in 1000 mL of distilled water.

Ten millilitre of Congo red dye was added to the solution, while the pH was adjusted to 6.8. The end solution was sterilized at 121 ºC for 20 minutes [PAUL *et al.*, 2011]. The sterilized medium was allowed to cool to 47 °C degrees before pouring in sterile petri dishes.

The spread plate technique was used with little modification [PAUL *et al.*, 2011]. With the aid of sterile inoculating loop, a loopful from the *B. japonicum* culture was added into already prepared 9 mL of sterile normal saline and vortex.

Using a Pasteur pipette, 0.1 mL of 10^{-1}, 10^{-2}, 10^{-4}, 10^{-8} serial dilutions of the suspension was aseptically poured, spread by a spreader on the prepared yeast extract Congo red agar plates and incubated at 37 °C for 5 – 7 days.

Biochemical characteristics of *B. japonicum* USDA110.

Lysine Decarboxylase Test. *Bradyrhizobium japonicum* was streaked on Bromocresol Purple Falkow medium (peptone 5 g, yeast extract 3 g, glucose 1 g, Bromocresol purple 0.02 g, 1000 mL distilled water).

The bacterial strain was inoculated on the media and was left for incubation at 34 °C for 1 day.

Catalase Test. This test was performed to study the presence of enzyme Catalase which hydrolyzes Hydrogen peroxide (H_2O_2) into H_2O and O_2 in bacterial strains. *Rhizobial* colonies (2–3 days old) were taken on glass slide and one drop of H_2O_2 (30 %) was added.

Lipase Test. Lipase presence around bacterial colonies was detected by supplementing Yeast Mannitol Agar with 1 % (w/v) Tween 80.

Citrate Test. Citrate utilization as a carbon source was examined by replacing mannitol from yeast mannitol agar with equal amount of sodium citrate and Bromothymol blue (25 mg/l). Plates with modified media were inoculated and incubated for 24 – 48 hours.

Urease Test. Urease Test was performed by inoculating the bacterial strain on Urease medium (peptone 1 g, dextrose 1 g, NaCl 5 g, KH_2PO_4 2 g, Phenol red 0.012 g). The plates with modified media were inoculated with *Rhizobial* strain and incubated for 5 days at 30 °C.

Caesinase Test. The bacterial strain was inoculated on skimmed milk agar medium (caesin 5 g, yeast extract 2.5 g, glucose 1 g and agar 15 g). Components were mixed with distilled water in a conical flask and were boiled and sterilized.

Alternatively, skimmed milk powder was prepared by dissolving 1 g of skimmed milk in 100 mL distilled water and was autoclaved. Sterilized skimmed milk was mixed into the first flask and was poured over the plates. Inoculated plates were incubated at 34 °C for 3 days and color change was examined.

Starch Hydrolysis. This test was performed to determine the capability of the *Rhizobium* to use starch as a carbon source. Starch Agar Medium was inoculated with *Rhizobium* and analyzed for starch utilization Iodine Test was used to determine the capability of microbes to use starch. A drop of iodine (0.1 N) was spread on 24 hours old culture and clear zone of inhibition were formed.

Bromothymol Blue Test. The YMA was enriched with 1 % (w/v) Bromothymol blue to determine if the *Rhizobium* is fast or slow growing and incubated for 48 hours at 28 °C.

Preparation of Peat as *Bradyrhizobium japonicum* Carrier. Fifty grams (50 g) of unsterile peat was weighed into polypropylene bag and then sealed. The peat was transferred to Ghana to be sterilized by Gamma radiation; the essence of Gamma radiation was to ionize the peat (powdered peat) in order to kill the microorganisms or any form of life that might be present in it [PAUL *et al.*, 2011].

After sterilization by Gamma radiation, the bags of peat were transferred back to Nigeria for further processing. The 50 g of sterilised peat was diluted with 50 mL of sterile water and kept.

Preparation of Charcoal as *Bradyrhizobium japonicum* Carrier. During the preparation of charcoal as rhizobacteria carrier and to attain the consistency of the diluted peat**,** 50 g, 60 g, 65 g and 70 g of charcoal were weighed into polypropylene bags.

Each of the weighed charcoal was diluted with sterile water to measure up to 100 mL. The 60 g of charcoal and 40 mL of water attained the consistency of the diluted peat, all other mixtures formed lumps or too viscous. The diluted charcoal was sterilized by autoclaving at 121 °C for 30 minutes [ARGAL *et al.*, 2015].

Inoculation of *Bradyrhizobium japonicum* into Yeast Mannitol Broth. One loop–full from the confirmed pure strain of *Bradyrhizobium japonicum* USDA 110 was taken and transferred aseptically to a 250 mL flask containing 100 mL of Yeast Mannitol Broth. This was covered with a flamed cork and placed on a rotary shaker for 7 days until the liquid is quite cloudy. This procedure was repeated for several other bottles [PAUL *et al.*, 2011].

Injection of Yeast Mannitol Broth into Sterilized Peat and Charcoal Bags. Yeast Mannitol Broth was aseptically injected into the sterile peats in ratio 1:1 i.e. 50 mL of the broth was dispensed into 50 g bag of sterile peat; for charcoal, 40

mL of the broth was dispensed into 60 g bag of sterile charcoal.

After the injection of broth into the sterile peat and charcoal, the bags were gently and thoroughly massaged for homogeneity before being arranged in trays. The inoculants were then transferred into the incubator for curing [PAUL *et al.*, 2011].

Curing of Inoculated *Bradyrhizobium japonicum* Carriers after Injection. The inoculants were incubated at 28 °C for 15 days. This temperature is optimum for the growth of *Bradyrhizobium japonicum*. Curing also helps to prevent the growth of the hyphae of fungi mycelium [PAUL *et al.*, 2011].

Quality Analysis Inoculated *Bradyrhizobium japonicum* Carriers and Broth. The essence of quality analysis is to estimate the number of *Bradyrhizobium japonicum* viable cells to nodulate soybean at planting, as well as the enumeration of contaminants present in the inoculant.

One millitre of the cured inoculant (carrier and rhizobacterium) was aseptically pipetted into 9 mL of sterile physiological water to achieve 10^{-1} dilution. The serial dilution was repeated until 10^{-8} dilution was attained.

With a Pasteur pipette, 0.1 mL from each of the solutions (10^{-1}–10^{-8}) was aseptically dropped on the prepared sterilized Congo red agar plates and incubated at 37 °C for 5 – 7 days for *Bradyrhizobium japonicum* cells count.

The spread plate method was repeated on the nutrient agar plates to test for quality test against contaminants; microbial growth on the nutrient agar plates will indicate the presence of contaminants in the inoculants.

The inoculated nutrient agar plates were incubated at 37 °C for 24 hours.

Cultivation of Treated and Untreated Soybean seeds. Loamy soil samples were gotten from FUTA environs and were subjected to sterilization by autoclaving at 121 °C for 30 minutes.

This was done to kill all forms of live, including native *Rhizobia* so as to be able to estimate the efficacy of the product.

The sterilized soil was shared into eight planting pots. A portion of the soybean seeds were treated with the inoculants containing charcoal as carrier coated with gum Arabic, while other portion of the soybean seeds was treated with the inoculants containing peat as carrier using gum Arabic.

The soybean seeds were planted in duplicate according the treatments. The control pots contained seeds without treatment.

Results and discussion

Morphological and Biochemical Characteristics of *Bradyrhizobium japonicum* USDA110

The colonial, morphological and biochemical characteristics of *Bradyrhizobium japonicum* USDA110 are shown in Table 1.

Table 1.
Morphological study and Biochemical Characteristics of *Bradyrhizobium japonicum* USDA110

Colonial and morphological characteristics	
Size on plate	3.1 mm
Colour	Whitish pink
Opacity	Translucent
Bacterium shape	Rod shape
Gram nature	Gram negative
Motility	Motile
Size	Circular
Biochemical characteristics	
Bromothymol blue	–
Caesinase	–
Citrate	–
Catalase	+
Lipase	+
Urease	+
Starch hydrolysis	+
Lysine decarboxylase	+

Colonial and morphological observation showed that the bacterial isolate has a rod shape, translucent opacity, whitish pink colour and it is motile. The bacterial isolate produced gas bubbles and effervescence indicating a positive catalase test.

The *Rhizobium* also showed a positive result for urease, lipase, lysine decarboxylase and starch hydrolysis test while blue colour due to alkali production showed a negative test to bromothymol blue. The result for caesinase and citrate tests on the bacterial isolate was negative.

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Growth of *Bradyrhizobium japonicum* USDA110 Treated and Untreated Soybean Seeds

Tables 2, 3 and 4 show the growth of *Bradyrhizobium japonicum* treated and untreated soybean seeds after 28 and 42 days respectively, and the total counts of soybean root nodules after 42 days cultivation.

Table 2.

Growth of *Bradyrhizobium japonicum* USD110 treated and untreated soybean seeds after 28 days

Treatment	Height (cm)	Width (cm)	Size (cm)
Charcoal with *Bradyrhizobium japonicum*	28.25±1.01	1.70±0.58	49.16±1.36
Peat with *Bradyrhizobium japonicum*	30.75±1.25	1.70±1.00	61.64±2.84
Control	13.20±1.17	1.18±0.03	14.79±0.75

Values are Mean±Standard error

Seeds treated with *Bradyrhizobium japonicum* with peat as carrier performed best in terms of height (37.10±2.94), width (1.85±0.03) and total count of root nodules (24.00±1.00) after 42 days.

However, seeds treated with *Bradyrhizobium japonicum* with charcoal as carrier had a very close performance with respect to height (35.00±1.27), width (2.00±0.06) and total count of root nodules (23.00±1.00).

Table 3.

Growth of *Bradyrhizobium japonicum* USD110 treated and untreated soybean seeds after 42 days

Treatment	Height (cm)	Width (cm)	Size (cm)
Charcoal with *Bradyrhizobium japonicum*	35.00±1.27	2.00±0.06	58.05±1.37
Peat with *Bradyrhizobium japonicum*	37.10±2.94	1.85±0.03	71.39±1.97
Control	17.70±1.33	1.47±0.03	18.23±1.10

Values are Mean±Standard error

The untreated seeds performed so poorly compared to the treated seeds [ROYCHOWDHURY *et al.*, 2015, SAMFIRA, *et al.*, 2015].

Environmental stresses are becoming a major problem and crop productivity is declining in an unprecedented rate. Biofertilzers can solve the problem of feeding an increasing global population as it is eco–friendly and less expensive thus, marginal farmers can benefit from its adoption in agricultural practices.

This work revealed the significant improvement in soybean biomass production as well in crop yield after inoculation with *Bradyrhizobium japonicum* USDA110.

Table 4.

Total count of treated and untreated soybean root nodules after 42 days cultivation

Treatment	Count (cfu/mL)
Charcoal with *Bradyrhizobium japonicum*	23.00±1.00
Peat with *Bradyrhizobium japonicum*	24.00±1.00
Control	5.00±1.00

Values are Mean±Standard error

All inoculated plants were found to have properly formed root nodules; they showed the significant increase in plant matter and crop yield.

The soybean seeds inoculated with *Bradyrhizobium japonicum* USDA110 with peat as carrier had a slightly higher plant height (37.10±2.94 cm) compared with the seeds as with charcoal as carrier (35.00±1.27 cm).

Charcoal being indigenous, accessible and relatively cheap can be used as an alternative to peat– a non–indigenous, non–easily accessible and expensive raw material.

This study showed that soybean nodulation by this efficient *B. japonicum* strain was low–cost in terms of energy for the plant, with the best benefits in terms of nitrogen fixation yield.

There are important criteria for isolate selection since use of mineral fertilizers can be significantly reduced which would contribute to economical and sustainable agricultural practises [HUNGRIA *et al*., 2003].

Gibson (1980) suggested that plant weight, grain yield and nitrogen content are the more reliable measures of symbiotic effectiveness for rhizobial isolates of undetermined capacity; the variations in biomass and crop yield suggest the variable effectiveness of the native *Bradyrhizobia*.

Arroyo amd collab., also discovered and discussed the variability in N_2–fixation efficiency among native *Bradyrhizobia* [ARROYO *et al,* 1998].

Pant and Prasad found the variable effect of native *Bradyrhizobia* for N_2–fixation in soybean. Minamisawa (1999) observed the field site variation in indigenous population of soybean *Bradyrhizobia* in Japan [PANT and PRASAD, 2004]. The results of the study revealed that inoculation of effective B. *japonicum* strain significantly increased the plant biomass and crop yield in soybean.

Plants inoculated with peat and charcoal based *Rhizobia* showed significant increase in the number of nodules over uninoculated control. Highest nodule number (24.00±1.00) was recorded in case of peat, with charcoal having 23.00±1.00 root nodules.

The results are in agreement with Khattak and collab., Shahzad and collab., and Rokhzadi and Toashih, charcoal can be comfortably used in place of peat in countries where peat is expensive to access such as Nigeria [KHATTAK *et al.* 2006, SHAHZAD *et al.* 2008, ROKHZADI and TOASHIH 2011].

Effective nodulation is dependent not only on the presence of a large number of viable *Rhizobia* on the seed but also on the conditions which favour growth and development of *Rhizobia* on the planted seed in soil, and in the case of biofertilizer production, the type of carrier used is a determinant of outcome [GUPTA, 2005; JADHAV *et al*., 1988, CAUNII, *et al.,* 2015].

Plant yield is dependent on nutrients, such as nitrogen (N), and farmers usually need to apply at least 100 kg of N per hectare [DEAKER *et al.* 2004].

However, N fertilizers are expensive, and chemical fertilization may promote soil pollution.

In contrast, biofertilizers are gaining importance in sustainable agriculture.

The term biofertilizer specifies that the fertilizer meets the nutritional requirements of a crop through microbiological means. In several countries, biofertilizers are synonymous with bacterial inoculants [BRAHMAPRAKASH and SAHU, 2012, IANCULOV, *et al.,* 2004].

During this study, an alternative carrier, charcoal has been tested to be a satisfactory substitute.

The formulation step is a crucial aspect for producing microbial inoculants and determines the success of a biological agent.

Formulation typically consists of establishing viable bacteria in a suitable carrier together with additives that aid in the stabilization and protection of microbial cells during storage and transport and at the target [BRAHMAPRAKASH and SAHU, 2012, SAMFIRA, *et al.,* 2014].

The formulation should also be easy to handle and apply so that it is delivered to the target in the most appropriate manner and form and should also protect bacteria from harmful environmental factors and maintain or enhance the activity of the organisms in the field [XAVIER *et al*., 2004, , RASHED and BUTNARIU, 2014].

Conclusions

This study revealed the potential of charcoal as competitive alternative to renowned peat as carriers of rhizobacteria in soybean agronomy. Also, charcoal is indigenous, biological and cheap ($0.02 per hectare) against popularly imported peat ($3.00 per hectare).

The benefits involved include accessibility to local and poor farmers and continuous accessibility. This will in turn generate high yield of soybean production; mostly needed in developing countries. Further studies are recommended on the large–scale application of *B. japonicum* USDA110 in soybean agronomy. The government and

other stakeholders are ensued to sensitize and train farmers on these options.

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