SYMBIOTIC EFFECTIVENESS OF BRADYRHIZOBIUM JAPONICUM USDA 110 AND SINORHIZOBIUM FREDII USDA 191 ON TWO DIFFERENT SOYBEAN CULTIVARS

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Abstract

Green house experiments were conducted to investigate the symbiotic effectiveness of two bacteria that fix nitrogen to soybeans: Slow growing bacteria (*Bradyrhizobium japonicum* USDA 110) and fast growing bacteria (*Sinorhizobium fredii* USDA 191). Two varieties of soybeans were used: Gazelle (non promiscuous variety) and TGx 1740 (promiscuous variety). The experimental design was a randomized complete design arrangement replicated four times. Nodulation, acetylene reduction activity and dry matter accumulation by nodulated plants growing in a nitrogen-free culture system were used to compare the symbiotic effectiveness of the fast-growing *Sinorhizobium fredii* USDA 191 with that of the slow-growing *Bradyrhizobium japonicum* USDA 110 in symbiosis with two soybean (Glycine max (L.) Merr.) Cultivars. Measurement of the amount of nitrogen accumulated 30 day period of vegetative growth showed that *Sinorhizobium fredii was* more effective in nitrogen fixation in TGX variety than in Gazelle variety while *Bradyrhizobium japonicum* was more effective in Gazelle variety than in TGx. The superior N_2 fixation capability of *Sinorhizobium fredii* with TGx variety as host resulted primarily from higher Nitrogenase activity per unit nodule mass (specific acetylene reduction activity) and higher nodule number per plant. The higher N_2-fixation capabilities of

Bradyrhizobium japonicum with unimproved Gazelle variety as host resulted primarily from higher nodule mass per plant which was associated with was associated with higher nodule numbers.

Keywords: Symbiotic effectiveness, rhizobium, soybean

Introduction

Soybean has become an important crop in Kenya due to its supply of proteins and oil. In spite of its importance as a source of protein and oil, researchers have noted that its production is way below potential (Borget, 1992). In the past, researchers have been emphasizing on inorganic fertilizer to foster the production of crops including soybean. However, declining soil fertility and high fertilizer costs are major limitations to crop production in smallholder farms in Kenya (Maobe *et al.,* 2004). Requirements for nitrogen in soybeans exceed any other major nutrients. Consequently, soils in the tropics rarely have enough of this nutrient to produce high sustainable yields (Wringley, 1982). There is need to seek for ways of supplying adequate amount of nitrogen in most soils with a goal of increasing soybean yield per unit area. It has been documented that through inoculation the production of soybean increases but no investigation has been done on the effectiveness between the slow growing *Bradyrhizobium japonicum* and fast growing *Rhizobium fredii*. To clarify the question of the symbiotic effectiveness of *S. fredii* compared with that of the *B. japonicum* strains, the symbiotic effectiveness of USDA 191 was compared with that of *B. japonicum* USDA 110 on two soybean cultivars (TGx 1740 and Gazelle). Nodulation, Nitrogenase activity, plant growth, nitrogen accumulation and concentration, and xylem sap nitrogen composition were considered in the comparison of symbiotic effectiveness.

Materials And Methods

Seed and Rhizobia Procurement

S. fredii USDA191 and *B. japonicum* USDA 110 were from Kabete campus Department of Agriculture, University of Nairobi, Kenya. Strain USDA 191 was grown in yeast extract-mannitol broth and strain USDA 110 was grown in yeast extract-gluconate broth. Seeds of soybean cultivars TGx 1740 and Gazelle were obtained from Kenya Agricultural Research Institute (KARI) Njoro, Kenya.

Leonard Jar Assemblies

The Leonard jar assemblies used were a modification of that described by Vincent (1970). The assembly was composed of a plastic cup, 8 cm mouth (brim) diameter which tapered to a bottom diameter of 4 cm. The

cup containing the rooting medium (vermiculite) was inserted into a larger plastic vessel containing the nutrient solution. 800 mls of plant nutrient solution was added into the lower container of each Leonard jar assembly. A sponge connecting the upper and the lower units of the jar irrigated vermiculite with the nutrient solution. The whole set up was insulated with a khaki paper bag.

Rooting Medium

The rooting medium, which was used in this study, was vermiculite. This material was washed thoroughly for three successive days by changing the water three times per day and stirring frequently. The final rinse was with distilled water and the pH of the medium was adjusted to about pH 6.8. After attaining the correct pH, water was drained off and the vermiculite packed into the small plastic cups of the Leonard jar assemblies. To reduce contamination and entry of water, the top cups were covered with lids and the assemblies were then steamed for one hour, twice in an autoclave to get rid of microorganisms.

Plant Growth Medium

Plant nutrient solution was prepared as described by Beck *et al.*, (1993). For each litre of full-strength plant growth solution, 0.5ml was added from each of the five stock solutions. The pH of the solution was adjusted to 6.8 using NaOH (1.0 M) or HCL (1.0 M). All solutions were sterilized by autoclaving at 121◦C for 15 minutes.

Sterilization and Pregermination of Seeds

Soybean seeds of good viability (more than 80 %), undamaged and of uniform colour and size were selected as described by Maingi *et al.* (1999). The seeds were surface sterilized by immersing them into a 3 % solution of sodium hypochlorite for 5-10 minutes (3 % sodium hypochlorite solution was prepared by adding 10 parts of commercial bleach [5.25 % sodium hypochlorite] to 7.5 parts of water). The seeds were rinsed with 8 changes of sterile distilled water after surface sterilization. They were then soaked in clean sterile distilled water and allowed to imbibe it for one hour. They were transferred aseptically to 2 % water agar plates with a spoon-shaped spatula. Twenty seeds were placed in each plate. The plates with the seeds were incubated upside down at 28^0 C to enable the radicles to grow away from the water agar. The incubation period was four days. Seedlings whose radicles attained a length of 1-2 cm after the incubation period were considered ready for transferring to Leonard jar assemblies.

Planting in Leonard Jar Assemblies

A pair of flame sterilized forceps was used to prepare one hole in the rooting medium in each Leonard jar. Seeds with radicle length of 1-2 cm were picked up with the sterile pair of forceps and placed two per hole, with the radicle facing downwards. The holes were deep enough to accommodate pre-germinated seeds 0.5 cm below the surface. The seedlings were maintained for eight days in the Leonard jar assemblies before inoculation. The Leonard jar assemblies with the seedlings were set in quadruplicates and inoculated with 1 ml of 5 day old respective rhizobia (approximately 10^8cells ml^{-1}) broth culture onto the radicle base following the procedure described by Somasegaran and Hoben (1994). The control was left uninoculated but fed with plain yeast extract mannitol broth. All Leonard jar assemblies were arranged in a randomized complete block design in the greenhouse of the department of plant and Microbial sciences at Kenyatta University, Kenya. Each treatment had 10 Leonard jar assemblies and they were replenished with sterile N-free nutrient solution daily as required (Odee *et al.,* 1995). At 14 days after transplantation, the seedlings were thinned to one per Leonard jar assembly. Composition of the nitrogen-free nutrient solution was as described by McClure and Israel 1979, except that 1.0 mM KH2PO4 was the sole source of phosphorus and the initial solution pH was 6.2. Experiments were conducted during the month of August of 2012 in a greenhouse. Natural light intensity was supplemented for 18 h each day with metal halide lamps that provided 150 microeinsteins * m-2 * S-1 of photo synthetically active radiation at pot level. The 18-h photoperiod was sufficient to prevent the flowering of all cultivars.

Acetylene reduction assay

Acetylene reduction assay was performed at Kenya Tea Research Foundation, Kericho, Kenya. The plant roots were packaged in a khaki paper bag to prevent dessication of the nodules. Acetylene reduction was performed on the excised root systems of 30-day-old plants by the methods of Sloger 1969; the roots were cut then washed to remove the sand. The nodulated portion of roots were removed and placed in 60 ml plastic syringes and incubated with a gas mixture prepared according to the method of Hardy *et al.* (1968). The syringe was then connected to the Gas Chromatography (G.C) which was equipped with a hydrogen flame detector. The sampling was taken after every 15 minutes for 1 hour, where 5 ml were injected into the G.C and amount of ethylene released was recorded. After assay procedure, the syringe was opened and carefully all the nodules were removed from the roots with a razor blade, counted and their number recorded, they were then oven dried at 70^oC for 48 hours to obtain their dry weight.

Nodule sampling for colouration

At 45-day after inoculation, the plants were harvested and the nodules collected from ten plants in the greenhouse. The plant shoot was removed and the roots washed. The nodules were removed from the roots, sectioned and their inner colouration noted whether dark pink or white and their number recorded for each case.

Authentication of the isolates

At both the 30- and 45-day harvests, two nodules from each plant were surface sterilized, crushed in sterile distilled water, and streaked onto yeast extract-mannitol broth plates containing bromthymol blue (pH 6.7) to characterize inocula as either acidic or basic in reaction. The nodules from plants inoculated with USDA 191 exhibited an acidic reaction that changed the medium from green to yellow, and nodules from plants inoculated with USDA 110 exhibited a basic reaction that changed the medium from green to blue. These tests indicated that all plants were nodulated by the appropriate bacterial strain.

Analysis of data

The data were statistically analyzed using analysis of variance and continued with least significant difference (LSD) for mean comparison.

Results

Nodulation and Nitrogenase activity

There was a significant effect ($p \leq 0.05$) on TGx 1740 variety in terms of the number of nodules produced and Acetylene reduction assay. The TGx 1740 inoculated with USDA 191 strains had the highest mean number of nodules (34) while the one inoculated with USDA 110 strains bacteria had the lowest (11), on TGx 1740 variety inoculated with USDA 191 strain had high ethylene production (18 µmol) while the one inoculated with USDA 110 had high ethylene production (6.57 µmol) as indicated in Table 1. However, no significant effect ($p \geq 0.05$) was found in the dry weights of the nodules, shoots and roots of the plants. There was a significant effect ($p \leq 0.05$) in the number of nodules in Gazelle variety when inoculated with USDA 191 and USDA 110. There were low mean formed when Gazelle variety was inoculated with USDA 191 strain (17) while the one inoculated with slow growing rhizobia had high number of nodules (38.75) as indicated in Table 1. Acetylene reduction assay also had a significant effect ($p \leq 0.05$) on Gazelle variety inoculated with USDA 191 strain had low ethylene production (3.55 µmol) while the one inoculated with USDA 110 strain had the high ethylene production (13.78 µmol) as indicated in Table 1.

Table 1: Symbiotic effectiveness of USDA 191 and USDA 110

Strain	Cultivar	No of nodules	Dry weight of nodules (mg)	Acetylene reduction (µmol of C_2H_4. $Plant^{-1.}$ h^{-1})
USDA 191	TGx 1740	34± 2.45a	0.48± 0.11a	18.22±1.12a
USDA 110	TGx 1740	11±3.10b	0.25±0.06a	6.57±1.54b
USDA 191	Gazelle	17±1.29b	0.45±0.12a	3.55± 0.27b
USDA 110	Gazelle	38.75±9.65a	0.57±0.26a	13.78±2.18a

The means with similar letters are not significantly different at p≥0.05: Means separated using Tukey's LSD

Table 2: Dry weight of shoots and roots

Strain	Cultivar	Dry weight of shoots (g)	Dry weight of roots (g)
USDA 191	TGx 1740	1.85±0.28a	0.78±0.18a
USDA 110	TGx 1740	1.03±0.45a	0.58±0.26a
USDA 191	Gazelle	0.88±0.14a	0.58±0.14a
USDA 110	Gazelle	1.3±0.31da	1.02±0.22a

Mean values denoted by similar letter (s) are not significantly different at p≥0.05; Means separated using Tukey's LSD

Number of effective nodules

There was a significant difference (p≤0.05) between USDA 191 and USDA 110 rhizobia strains in terms of nitrogen fixation into two soybean varieties. The TGx variety inoculated with USDA 191 (fast growing Sinorhizobium) had higher mean number of pink nodules (11.25) while Gazelle variety had a mean of 3.7(Table 3). The USDA 110 (slow growing Bradyrhizobium*)* had a significant effect (p <0 .05) on the two soybean variety. The Gazelle soybean cultivar had higher number of pink nodules 10.20 ± 0.40 while TGx soybean cultivar inoculated with USDA 110 had a mean of 4.75 ± 0.27 as indicated in Table 3.

Table 3: Mean number of pink nodules

Strain	**Cultivar**	**Mean number of pink nodules**
USDA 191	TGx 1740	$11.25±0.46^a$
USDA 110	TGx 1740	$4.75±0.27^b$
USDA 191	Gazelle	$3.7±0.21^c$
USDA110	Gazelle	$10.20±0.40^a$

Mean values denoted by similar letter (s) are not significantly different at p≥0.05; Means separated using Tukey's LSD

Discussion

There is a constant search for superior rhizobia strains, characterized by high rates of nitrogen fixation, high nodule efficiency and competitiveness, which would allow an increase in legume yields and nitrogen content (Dusica *et al.*, 2010). The two tested strains were capable of nodulating both soybean variety but there were different responses to the

strains inoculation. Differences in acetylene reduction activity per unit nodule mass imply differences in Nitrogenase activity which could result from differences in the ratio of bacteriodal to plant tissue in the nodules or from differences in Nitrogenase activity per unit bacteroid (Pankhust 1984). Nodulation and acetylene reduction activity showed that the symbiotic effectiveness of slow growing rhizobia isolate was superior to that of fast growing rhizobia isolate in Gazelle variety. This result is in agreement with another study which showed that the symbiotic effectiveness, as measured by acetylene reduction activities, of USDA 110 was superior to that of USDA 191 (Yelton *et al.,* 1983). Another study conducted by Israel *et al.* (1986) showed that the symbiotic *B. japonicum* USDA 110 was superior to that of *Sinorhizobium fredii* USDA 191 in terms of nitrogen fixation. The fast growing rhizobia showed high performance in TGx 1740 in terms of number of nodules and acetylene reduction activity as compared to slow growing rhizobia. The fast growing rhizobia (USDA 191), showed more pink coloured nodules after cross-section with TGx 1740 cultivar than when inoculated with unimproved gazelle cultivar, showing formation of more effective nodules with TGx cultivar than gazelle cultivar. But when gazelle cultivar was inoculated with slow growing (USDA 110) it produced more effective nodules than TGx 1740 when inoculated with USDA 110. The number of effective nodule formed by each strain correlate with the result obtained in Nitrogenase activity of reducing acetylene, number of nodules, nodule dry weight, shoot dry weight and root dry weight. These results confirms that fast growing *Sinorhizobium* is more active in nitrogen fixing with improved soybean cultivar as compared to unimproved cultivar, while slow growing is more active in nitrogen fixation with unimproved cultivar gazelle. The interior pinkish colour found in the effective nodules of both rhizobia strains had started changing into pink brown at 48 days of harvesting nodules. This showed that their stage of development lasted for six weeks, similar to results found by (Gwata *et al.,* 2003; Fujihara *et al.,* 2006 and Dusica *et al.,* 2010).

The colonies that attained full size in 3-5 days were fast growing rhizobia while the ones that attained full size in 4-7 days are slow growing rhizobia (Somesegaran and Hoben, 1994). All the isolates were Gram negative rods, which is a characteristic typical to rhizobia (Zakhia and DeLajudie, 2001). Rhizobia do not absorb Congo red when incubated in the dark (Maingi *et al.,* 1999). This is the reason why colonies appeared milky in media containing Congo red dye. The change in colour of YEMA containing bromothymol blue from dark green to blue indicated the production of alkaline substances, which diffused into the media hence colour change. This is a typical characteristic of slow growing rhizobia, which is *B. japonicum* and *B. elkanii* (Xu *et al.,* 1995). The change in colour of YEMA containing

bromothymol blue from dark green to yellow indicated the production of acidic substances which diffused into the medium (Somesegaran and Hoben, 1994). The production of acid substances is a characteristic of the fast-growing rhizobia (Beck *et al.,* 1993).

Conclusion

This study results shows that strain of *S. fredii* is more effective in nitrogen fixation with the improved cultivar of TGx 1740 as compared to strain of *B. japonicum* which is a slow growing bacteria. While for unimproved cultivar gazelle the strain for slow growing *B. japonicum* was more effective in nitrogen fixation than fast growing strain of *S. fredii*. These results indicate it will not be prudent to introduce *S. fredii* strains into Kenyan unimproved varieties like gazelle until more efficient nitrogen fixing symbiosis between Kenyan cultivars and these fast growing strains can be developed. *B. japonicum*, showing high potential of nitrogen fixation with local variety gazelle in greenhouse experiment, could be used in further investigation for their efficiency under field conditions for inoculation as microbiological nitrogen fertilizer.

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