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Full Length Research Paper

Identifying elite rhizobia for soybean (*Glycine max*) in Kenya

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Bio-prospecting was conducted in Kenya to identify elite isolates of rhizobia capable of effectively nodulating promising soybean varieties. One hundred isolates were recovered from nodules of wild and cultivated legume hosts. These isolates were authenticated and tested for effectiveness on soybean (*Glycine max*) var. SB 19 in sterile vermiculite, and the twenty-four most promising isolates screened in potted soil to assess their competitive abilities on two varieties ("promiscuously nodulating" SB 19 and specific SC Safari). The six best performing isolates were then evaluated under field conditions, comparing them to strain USDA110. Test isolates were classified into; non-infective, ineffective, partly effective, effective and highly effective based on their performance relative to controls and industry standards. In potted soil, native rhizobia isolates nodulated promiscuous soybean (SB19) but only 46% of them nodulated specific soybean (Safari). In the field experiment, isolate NAK 128 performed best on both promiscuous and specific soybean varieties, significantly ($p<0.05$) outperforming USDA110 by 29% and 24%, respectively. Partial economic return to inoculation with NAK 128 was about 21:1, justifying inoculation as a field practice and producing up to 2.5 million nodules (334 kg) ha^{-1}, significantly ($p<0.05$) more than USDA 110. The best isolates from this investigation have commercial potential.

Key words: African biodiversity, bio-prospecting, promiscuous soybean, rhizobia isolate selection, USDA 110.

INTRODUCTION

Rhizobia are soil-inhabiting bacteria that form root nodules where symbiotic biological nitrogen fixation occurs (Howieson and Brockwell, 2005; Weir, 2006). This process, where atmospheric N is captured for assimilation by plants is under-utilized by small-scale African farmers, in part because they do not understand its mechanism and management. Ninety-five per cent of farmers in East and Southern Africa are familiar with legume root nodules but only 26% considered them beneficial (Woomer *et al*., 1997). In Kenya, only one per cent of farmers use inoculants (Karanja *et al*., 2000).

Competitiveness of native rhizobia also poses a barrier to the benefits of inoculation (Shamseldin and Werner, 2004). Tropical soils are often rich in less-effective, native rhizobia and a key to overcoming their competitive advantage is through the composition and delivery of legume inoculants (Theis *et al*., 1991), especially for soybeans, a more specifically nodulating host legume (Sanginga *et al*., 2000). One pathway to improvement is to identify native rhizobia with superior symbiotic and competitive abilities and to use them in large doses within inoculants, building upon the biodiversity of indigenous rhizobial populations. The adaptability of indigenous rhizobia to their environment results in high levels of saprophytic competence. Therefore, continual identification of new, elite isolates offers the opportunity to improve BNF within fine-tuned geographical targets (Zengeni *et al*., 2006; Appunu and Dhar, 2006). In this way, a wide diversity of rhizobia isolates ensures a sustainable source of strains for commercial application into the future (Musiyiwa *et al*., 2005).

One empirical approach to rhizobia strain selection focuses upon the stepwise collection, isolation and authen-

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tication of native rhizobia, the screening of the isolates against reference strains for symbiotic effectiveness, the assessment of their competitive abilities and the evaluation of their performance under a range of field conditions (Howieson *et al*., 2000). Each step eliminates the worst performing isolates from further consideration. In this way, the identified elite rhizobial strains are likely to colonize the soil, tolerate environmental stresses, and compete with background populations (Slattery and Pearce, 2002).

Ideally, this empirical approach identifies the elite strains of rhizobia across a range of agro-ecologies, mass produces them as inoculant and makes them available to legume farmers so that they benefit from native microbial biodiversity. Kenya is an excellent location to test this approach. It has a wide range of ecosystems, legume communities (White, 1983) and soils (Sombroek *et al*., 1982), and a large population of farmers cultivating legumes, including soybean as an increasingly important cash crop. Moreover, these farmers are in the process of advancing from subsistence to market-based agriculture and seeking to improve their field practices and yields (Woomer *et al*., 1998). This study evaluates the effectiveness of Kenya's native rhizobia on farmer accepted varieties of soybean and is ultimately intended to result in improved legume inoculants by identifying elite indigenous rhizobia.

MATERIALS AND METHODS

Three sets of experiments were performed to identify elite rhizobia from our collection: authentication and effectiveness screening of rhizobial isolates in potted sterile media under greenhouse conditions, subsequent evaluation of the better strains in a representative potted soil also in the greenhouse, and finally on-farm testing of the best strains in an area where soybean enterprise is being rapidly adopted by small-scale farmers. Greenhouse studies were conducted at University of Nairobi field station, Kabete Campus, situated about 15 km to the west of Nairobi at $1^0$15'S and 36^0 44' E, in the Central Kenyan Highlands (Sombroek *et al*., 1982.). The field experiment was conducted at Nyabeda in a smallholder farming community in west Kenya, located at 00^0 08'N and 034^0 24' E, 1331 m above sea level.

A Complete Randomized Design with three replicates consisting of 104 treatments was established in a greenhouse, 100 indigenous test isolates, two reference strains (SEMIA5019 and USDA110), and non-inoculated plants with and without mineral N. Promiscuous soybean (SB19) was used as the test crop. Clean three liter plastic pots containing heat-treated gravel for drainage were filled with 750 g rhizobia-free vermiculite and covered with a clean plastic plate with two access holes to accommodate the test crop sprouts and a watering tube.

Soybean seeds were surface sterilized (Somasegaran and Hoben, 1994), pre-germinated in vermiculite and three uniform sprouts transplanted per pot, later thinned to two. Test isolates were cultured in Yeast Extract Mannitol broth (YMB) after Vincent (1970), incubated at 28 ^{0}C until turbid and 1 ml of broth was applied to the roots of each plant. For the mineral nitrogen control, KNO_3 (0.05%) was applied following (Broughton and Dillworth, 1971). After eight weeks, nodulation was observed by careful recovery of roots and shoots were harvested, oven dried and weighed. An Effectiveness Index was calculated by dividing shoot biomass of test isolates by that of USDA 110. With this index and isolate performance relative to experimental controls, isolates were categorized as non-infective, ineffective (less than -N control), partly effective (<75% of USDA 110), effective (75% or equal to USDA 110) or highly effective (>USDA 110) and ranked in ascending order.

A red clayey loam collected from a farm in Butula, west Kenya was used as media for competitive screening in the greenhouse. The soil was characterized for its chemical characteristics as follows. Nitrogen was determined using a steam distillation method (Bremner and Keeney, 1965) and organic carbon (C) by wet oxidation using a modified Walkley-Black procedure as described by Nelson and Sommers (1982). Phosphorus (P) and potassium (K) were extracted by Mehlich-3 procedure (Mehlich, 1984) and then measured by automated colorimetry using an Inductively Coupled Plasma Atomic Emission Spectrophotometer (Kalra and Maynard, 1991).

A second experiment was established in the greenhouse at the Kabete Campus using this soil with the 24 best performing isolates from the first screening. This experiment utilized a similar approach, three liter plant containers, sprout transplants, YMB isolate preparation and non-inoculated and industry standard controls, but included two soybean varieties, a promiscuous (SB19) and specific soybean (SB97) variety. Indigenous rhizobia populations in the test soil were determined using the plant infection technique (Somasegaran and Hoben, 1994). Experimental units were arranged as a Split-Plot by varieties with four replicates. The soil was fertilized with Sympal, a commercially-available blend for legumes (0-23-15 + Ca, Mg and S) at a rate of 500 kg ha^{-1} mixed with two kg of soil pot^{-1}. Pots were regularly irrigated with rhizobia-free water. After eight weeks, plants were carefully uprooted, nodules observed, shoots, roots and nodules recovered, oven dried at 70°C for 48 hours, plant biomass recorded, summary statistics calculated and two-way ANOVA (variety x N source) performed. The best performing isolates were selected for field testing.

In addition to USDA110, six indigenous rhizobia isolates (6%) were selected from the potted soil experiment to test their effectiveness in field under farmer conditions. A field experiment was established in west Kenya at Nyabeda during 2012-2013 short rains (Septem-

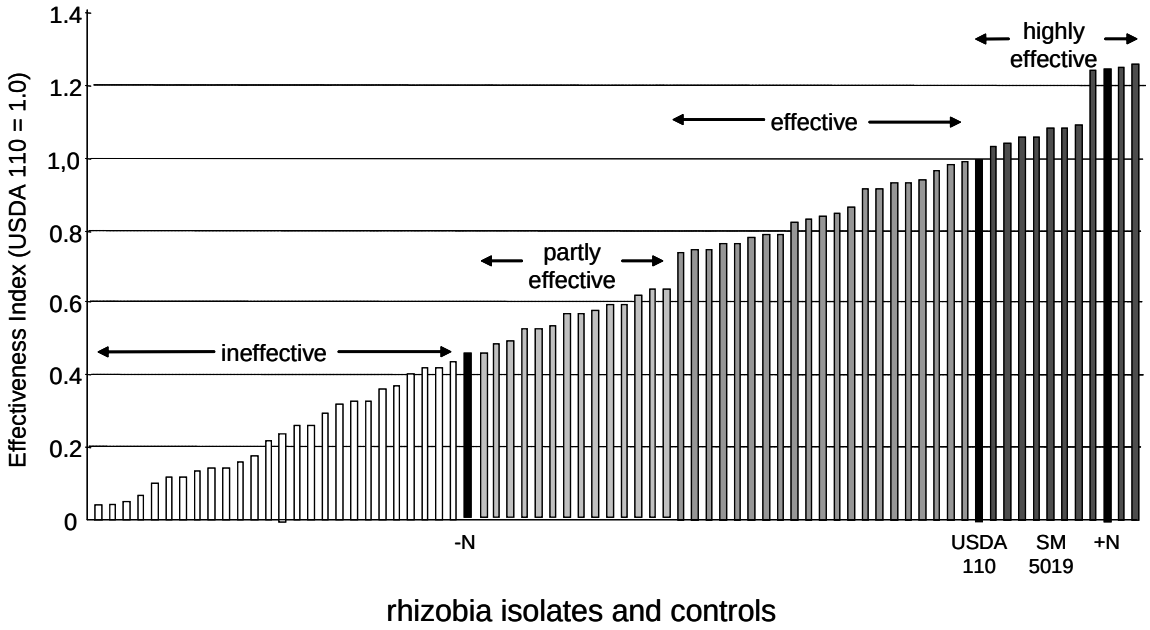

Figure 1. Effectiveness Index of 80 Kenya isolates on soybean variety SB 19 grown in rhizobia-free vermiculite for 56 days.

ber to January). The red clayey loam at Nyabeda has the following characteristics: pH=5.7, organic C=2.32%, total N=0.21%, extractable P=5.0 ppm and exchangeable K=398 ppm. Six indigenous isolates and USDA110 were compared on promiscuous (SB19) and specific (SB97) soybeans. Non-fertilized maize was grown the previous season to reduce soil N. Indigenous rhizobia populations were determined using the plant infection technique (Woomer *et al*., 1994). Sympal fertilizer was applied at the rate of 200 kg ha^{-1}. Sugarcane bagasse was also applied at a rate of 2 t ha^{-1} to immobilize soil N. Calcium ammonium nitrate (CAN) fertilizer was applied at the rate of 78 kg N ha^{-1} to one treatment (+N). Plots were arranged as a Randomized Complete Block with each consisting of 6 rows 45 cm apart and seed planted at 5 cm intervals. Each plot was separated by three non-inoculated rows to reduce cross-contamination. The test isolates were NAK 84, 89, 115, 117, 128 and 135 with USDA110 included as a reference strain. Legume inoculants were prepared from these isolates using sterilized sugar mill filter mud as a carrier, cured for 14 days and applied at 10 g kg $seed^{-1}$ with 16% gum arabic adhesive using the two-step inoculation method of Woomer (2010). Soybeans were managed according to usual farmer practice, weeding twice by hoe prior to canopy closure. Plants were sampled for nodulation eight weeks after emergence coinciding with 50% flowering in 0.225 m^2. Plants were carefully uprooted, root systems recovered, washed, nodules recovered, counted, oven-dried for 24 hours at 70^0C and dry weight recorded. At grain maturity, soybean grain was harvested from 5.4 m^2, dried and weighed. Data was compiled on a spreadsheet, inspected and then summary statistics calculated and ANOVA performed.

RESULTS

One hundred isolates obtained from diverse agro-ecologies were tested for effectiveness on SB 19 in the greenhouse using rhizobia-free sterile vermiculite as a rooting media. Negative (-N) and positive (+N) controls were not nodulated. Of the test isolates, 20% did not form nodules and were eliminated from further consideration. The remaining isolates were classified as ineffective (26%), partly effective (26%), effective (17%) or highly effective (11%) based upon their performance compared to the non-inoculated control and USDA 110, the local industry standard (Figure 1). Total plant biomass ranged from 0.5 to 13.5 g per pot and nodule number from zero to 151 per pot (data not presented), suggesting that the growth system allowed for large differences between treatments.

The best performing 24 isolates were then compared to USDA110 using the same greenhouse growth system, but substituting a red clayey loam soil from western Kenya for of vermiculite. MPN using SB 19 as host estimated that the soil contained 2.7 x 10^3 native rhizobia g^{-1} soil. In this way the non-inoculated treatment serves as a useful control allowing the most competitive and effective isolates to distinguish themselves (Figure 2). Plant biomass and nodulation were greater for SB 19 than cv. Safari ($p < 0.001$ and 0.001, respectively). Only 25% of the isolates outperformed the native population in terms of plant biomass on SB 19 and 16% did so on cv. Safari. In contrast, many isolates formed more nodules than the native population, 67% and 38% on SB 19 and cv. Safari, respectively (data not presented). The native population and many of the test isolates failed to nodulate Safari, while all formed nodules on SB 19, reaffirming the

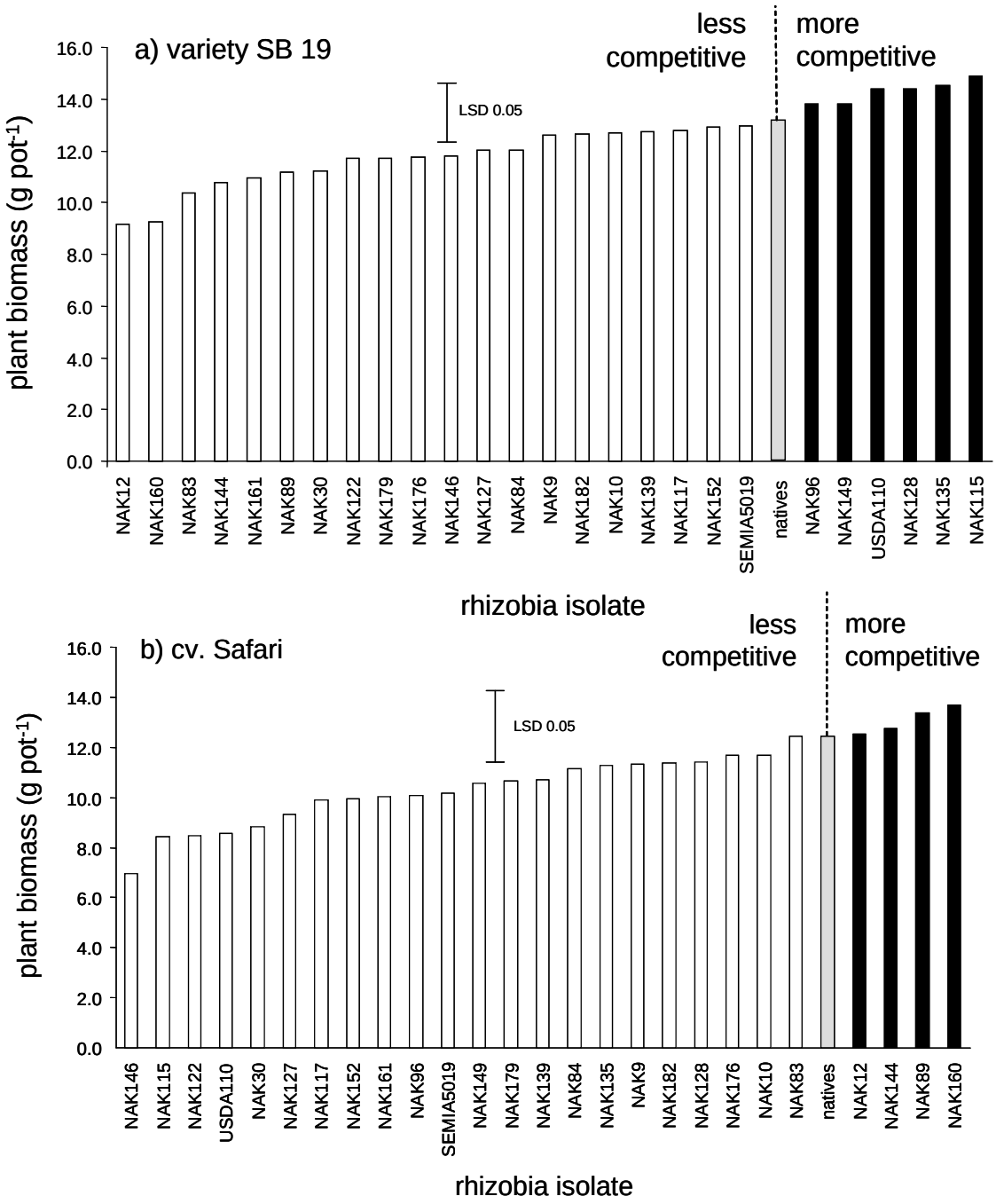

Figure 2. Plant biomass of two soybean varieties, SB 19 (a) and SC Safari (b) in potted soil receiving different N sources including 24 test isolates of Kenyan indigenous rhizobia.

latter's "promiscuous" pedigree. The best six isolates were then evaluated under field conditions at Nyabeda farm for comparison to USDA 110 and non-inoculated and N-fertilized managements, again with promiscuously nodulating (SB 19) and specific (SB 97) varieties of soybean. With both varieties, NAK 128 emerged as the most promising isolate, producing an average 1439 kg grain ha^{-1} and outperforming both the non-inoculated control and USDA 110 by 22% and 27%, respectively (Table 1). Nodule number and biomass from the field experiment are presented in Table 2.

DISCUSSION

The empirical, stepwise approach to strain selection employed was largely successful in that it started with a large number of test isolates and systematically reduced

Table 1. Grain yield by two soybean varieties under different nitrogen management at the Nyabeda field experiment in west Kenya.

N source	------ SB 19 ------		------ SB 97 ------	
	grain yield kg ha^{-1}	partial return[a] $ per $	grain yield kg ha^{-1}	partial return[a] $ per $
Non-inoculated	1057	n.a.	1301	n.a.
USDA 110	1129	3.9	1139	-
NAK 115	1153	5.2	1140	-
NAK 117	1210	8.2	1086	-
NAK 135	1212	8.3	1230	-
N-fertilizer applied	1299	0.2	1304	0.0
NAK 89	1317	13.9	1230	-
NAK 84	1339	15.1	1182	-
NAK 128	1462	21.7	1416	6.2
$LSD_{0.05}$	364		294	

[a] Partial return calculated as increased soybean value/cost of N source with soybean valued at \$0.613 kg^{-1}, inoculant at \$11.40 ha^{-1} and CAN-N at \$2.38 kg^{-1}. Not calculated when non-inoculated management outperformed inoculants (-).

Table 2. Nodule number (x10^2) and biomass per ha by soybean after 58 days at the Nyabeda field experiment in west Kenya.

N source	Nodule number		Nodule biomass	
	---- no ha^{-1} (x10^2) ----		---kg ha^{-1} (10^{-1})-----	
	SB 19[a]	SB 97[a]	SB 19	SB 97
N applied	37.0	0.0	7.0	0.0
Native rhizobia	181.2	82.9	21.0	60.0
NAK 135	344.1	509.5	20.0	79.0
USDA 110	600.4	372.8	67.0	29.0
NAK 117	924.1	793.8	99.0	80.0
NAK 115	1222.2	1124.1	126.0	78.0
NAK 128	2469.9	1446.0	334.0	207.0
NAK 84	3999.7	6908.0	291.0	538.0
NAK 89	4227.0	3323.1	326.0	306.0
$LSD_{0.05}$	--------- 2779.7 --------		---------- 200 ---------	

[a] SB 19 is promiscuously nodulating and SB 97 is more specific.

them to a few, highly effective and competitive strains. Part of this success is due to reliance upon large pots used in our greenhouse experiments, and the greenhouse design and sanitation that permits these units not to become contaminated. Another component is the comprehensive strategy to bio-prospecting throughout Kenya's diverse wild and cultivated legume communities, but this aspect will be covered in a later paper. Part of this success is perhaps due to luck, because bio-prospecting for rhizobia intended for a moderately specific legume host away from its Center of Origin is risky, but Africa is an ancient land mass and Kenya embraces considerable biodiversity. One indicator of our success is the performance of the best isolates compared to long-time industry standard USDA 110.

There was considerable variation in nodulation and plant biomass among the test isolates in sterile media, indicative of varying ability as symbiotic partners of soybean. Other investigations (Terpolilli *et al*., 2008; Karaca and Uyanöz, 2012) noted that efficiency of nitrogen

fixing symbioses can vary from those that fix little or no nitrogen to those that fix at levels equivalent to even greater than plants provided mineral N (Figure 1). It is essential that both negative (-N) and positive (+N) controls were not nodulated as the former indicates that contamination was minimized and the latter suggests that sufficient mineral N was applied to meet soybean demand for nitrogen.

In later experiments, differences between soybean varieties were observed. Saeki *et al*. (2005) also reported differences in nodulation of different soybean varieties grown in soil with some rhizobia better able to overcome native background populations. Dhami and Nandan (2009) reported better nodulation where inoculants were applied in higher concentrations because they overwhelmed native rhizobia. Theis *et al*. (1991) identified a critical threshold of native rhizobia beyond which inoculation proved no longer effective. These mechanisms are presumable operative within our experimental conditions as well. Nonetheless, several of our isolates from a range of legume hosts and ecologies outperformed industry standard strains (Table 3) even in soils with relatively large native populations of rhizobia.

Several native isolates failed to nodulate soybean SC Safari, a non-promiscuously nodulating variety while all formed nodules on SB19, a promiscuous variety arising from the decades-long breeding program at IITA, leading to the TGx series (Sanginga *et al*., 2000). Based on recovery of isolates by TGx lines from 65 African soils, Abaidoo *et al*. (2000) concluded that bradyrhizobia nodulating promiscuous soybean are diverse and genetically distinct from those nodulating North Americam soybeans. Several isolates performing well on the TGx lines were obtained from a range of hosts not usually considered to cross-infect soybean (e.g. *Eriosema sp.*) reaffirming that TGx is more promiscuously nodulating. This conclusion is reinforced by a report by Maingi *et al*. (2006) on most-probable number obtained from cowpea, and TGx and Clark soybean using two soils where TGx recovers a greater proportion of native bradyrhizobia nodulating cowpea. Similarly, Salvucci *et al*. (2012) reported different response to rhizobial inoculation across soybean genotypes in Argentina. Certainly, a similar phenomenon operated within our rhizobia selection process, suggesting that our best isolates warrant effectiveness testing across a wider range of soybean germplasm. For example, one test isolate, NAK 179, performed very well with SB 19 in potted soil and poorly with Safari, and was dropped from further testing, an exclusion that may have been premature. From a more practical standpoint, however, we have empirically established which rhizobia perform best on soybean varieties that have achieved farmer and market acceptance.

Even promiscuous SB 19 responds to inoculation under field conditions. This response to inoculation by TGx soybean was also observed in Nigeria (Muhammad, 2010) and Ghana (Kumaga and Etu-Bonde, 2000), although in the latter case USDA 110 (=TAL 102) proved the best inoculant rhizobia. Promiscuous Asian soybeans are often nodulated by ineffective rhizobia (Eaglesham, 1985) and the same may be true in Africa, possibly explaining this response. Indeed, response to inoculation was not considered in the breeding program that developed the TGx promiscuous lines (Dashiell *et al*., 1985). Non-promiscuity in soybean is a dominant trait (Gwata *et al*., 2004), perhaps explaining why the development of the promiscuous TGx lines required so many years and is incomplete.

Despite differences in symbiotic relations by promiscuous and specifically nodulating soybean, isolate NAK 128 emerged as the most productive effective isolate on both SB 19 and SB 97 (Table 1) but without producing the most nodules or nodule mass (Table 2). The latter observation further reinforces our conclusion that this is a highly effective strain, certainly more so than USDA 110 under our field conditions, offering greater economic partial return to inoculation as a field practice (Table 1). Presumably, NAK128 outperformed both native rhizobia and USDA 110 because it was more effective and competitive. Appunu and Dhar (2006) and Appunu *et al*. (2008) also concluded that native rhizobia can be symbiotically more effective than reference strains of foreign origin. In addition to NAK 128, five other isolates have favorable abilities to fix nitrogen compared to USDA 110, NAK 84, 89, 115, 117 and 135. While rhizobia isolated from many legume species in Kenya were evaluated, all six of these superior strains were isolated from non-inoculated soybean growing in farmers' fields, and not from natural habitats. While the strategy that recovered, tested and identified these candidate elite strains was otherwise expedient, it was not particularly comprehensive. Our laboratory facilities did not allow us to characterize isolates using molecular tools, and these isolates may comprise identical strains or different species. The genetic stability of nodulation and nitrogen fixation under laboratory culture is not yet established, nor is the ability of the isolates to utilize lower cost carbon sources. Characterization and testing of these isolates continues, and we invite other laboratories and commercial inoculant producers to join us in exploring the potential of these elite rhizobia from Kenya.

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