Rhizobium collection, testing and the identification of candidate elite strains

Milestones 3.1.3, 3.1.4, 3.1.5 and 3.2.2

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N2Africa

Putting nitrogen fixation to work for smallholder farmers in Africa

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1 Summary

This report combines several sequential milestones related to the recovery, characterization and effectiveness testing of rhizobial isolates by program partners. Originally cooperators were to bio-prospect a proscribed number of nodules and sites for isolation (MS 3.2.2) but this proved too rigid and was adjusted to "targets of opportunity", where large individual healthy plants bearing large nodules, native legumes and soil trapping were employed. A N2Africa data base was developed to formalize our collection (MS 3.1.3) and 1360 isolates entered, with another 84 to be entered, resulting in 72% of the original program target. The empirical, stepwise approach to strain selection was adopted that first evaluated large number of test isolates under rhizobium-free greenhouse conditions and systematically reduced them to a few, highly effective and competitive "elite" strains using potted soils and comparative field trials (MS 3.1.4). Several candidate elite strains (12) have emerged from several countries but their characterization remains incomplete, and a more systematic and detailed approach is recommended. To achieve this goal, the best strains should be consolidated and tested side-by-side. These best isolates are now available to interested parties outside of the program (MS 3.1.5).

2 Background

This report describes three sequential milestone achievements within Objective 3: Rhizobiology. At Milestone (MS) 3.2.2, each of eight country teams was expected to collect 250 nodules and prepare isolates from them, leading to 2000 test isolates. At MS 3.1.3, these isolates were to be tested for their effectiveness on target legumes under greenhouse conditions, selecting the best performing 5% "candidate elite strains" for further testing under field conditions. At MS 3.1.4, these strains were to be further reduced to 2% by testing under field conditions, leading to the identification of 40 isolates with documented potential to be used in inoculants, focused initially upon bean and soyabean as hosts most likely to respond to inoculation. It was hoped that MS 3.1.5 would identify the best five strains that outperform widely-distributed industry standards currently used in inoculants (e.g. CIAT 899 for bean and USDA 110). These planned activities were built upon three other "early" milestone achievements, upgrading rhizobiology facilities (MS 3.4.2, Report 031), training technical staff in essential microbiological skills (MS 5.1.1, Reports 011 and 029) and the development of practical field protocols for nodule sampling, rhizobium isolation, laboratory characterization, greenhouse evaluation for effectiveness in biological nitrogen fixation (MS 3.1.1, Reports 011 and 026). Clearly this suite of milestone achievements were central to the success of the overall program in terms of advancing biological nitrogen fixation (BNF) over the longer term and a test of its abilities to coordinate sequentially related milestone tasks.

3 Milestone 3.2.2 and bio-prospecting strategies

An important goal of the N2Africa Program, and indeed rhizobiologists in general, is to discover new and better strains of rhizobia for use in legume inoculants. This pursuit entails the collection of isolates, strain characterization, assessment of symbiotic capacity and comparison to strains currently included within inoculants. During the process of bio-prospecting, care should be taken not to simply collect strains that were obtained through past inoculation. As illustrated through the following approach, the process of rhizobium exploration and characterization is somewhat arduous, and efforts must remain focused upon relatively few legumes of interest in an unbiased manner so that elite strains emerging from this work must be recognizably superior. Several considerations were included in rhizobial bio-prospecting.

The initial approach toward isolate recovery was to collect root nodules from uninoculated plots of the Objective 2: Legume Agronomy trials, with 10 isolates prepared from each of 200 sites, leading to 2000 test isolates. While expedient in theory, this approach had several limitations. Nodule collection could only occur during the cropping season, and in some cases the Year 1 planting season was missed or covered an incomplete range of available agro-ecologies. The need to more continuously collect rhizobia was felt. Also, collecting rhizobia from non-inoculated plots risks the recovery of strains originating from adjacent inoculated plots, so sampling from these plots alone appeared to be a weaker strategy than also including farmers' fields and natural communities.

As a result, isolates were obtained directly from root nodules collected in a wider range of conditions (Figure 3.1). In some cases, rhizobia were trapped by collecting soils and growing legume hosts of interest in the greenhouse. In general, larger, effective nodules were sampled rather than smaller, less effective ones. Usually, a large, green host legume was identified, carefully uprooted, a large nodule dissected and inspected for red pigmentation (leghaemoglobin) associated with effective BNF, and then other similar nodules collected. Sometimes, it was unnecessary to test the interiors of the best nodules, because pigmentation could be seen through the nodule cortex.

Figure 3.1: Bio-prospecting in action (clockwise):

Coastal Kenya (top left), bio-prospecting team (DR Congo), farmer-assisted *Erythrina* sampling strategies (Rwanda), sampling mixed farming systems, sampling field experiments, carefully sampling drainage ditch during dry season, helpful woman farmer in DR Congo (left).

Trapping native rhizobia from soil was conducted as a dry season activity, or where nodules are difficult to recover, or even where no legumes are present. Trapping was sometimes combined with soil dilutions and MPN in order to gain insight into the relative abundance and competitiveness of isolates (Woomer 1994). Target grain legumes are the best trapping hosts, in this case common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), groundnut (*Arachis hypogaea*) and specific and promiscuously nodulated soyabean (*Glycine max*) (Abaidoo et al. 2000), but in some cases siratro (*Macroptilium atropurpureum*), noted for its promiscuous and rapid nodulation (Stowers and Elkin 1980), was used.

Cultivated legume fields that are recognized by local farmers as consistently producing superior crops may be sampled but care was taken not to sample fields with a history of inoculation One advantage to this approach is its participatory nature, allowing farmers to become involved in sampling strategy. Prospectors sought permission from farmers before uprooting their crops and in some cases provided modest compensation for crop loss. Often local farmers knew best where to recover root nodules from both cultivated and native legumes, and any history of inoculation. This approach also allowed for the recovery of rhizobia from other grain legumes, such as bambara groundnut (*Vigna subterranea*), green gram (*Vigna radiata*), and runner bean (*Phaseolus coccineus*), that cross-nodulate with target grain legumes.

Legume communities containing hosts belonging to the same cross-inoculation group or taxonomic tribe (Stowers and Elkin 1980) as the target legumes were also sampled. This approach was most applicable to legumes nodulated by more promiscuous legumes, such as cowpea and groundnut, which are in turn less likely to respond to inoculants in soils containing large numbers of native rhizobia. Common bean also nodulates in Africa with several species of rhizobia including strains indigenous to Africa (Giller et al., 1994; Anyango et al., 1995; Shamselin and Werner 2004). Similarly, soyabean is associated with diverse rhizobia with promiscuous varieties associated with bradyrhizobia common to many soils (Abaidoo et al. 2000; Maingi et al, 2006; Musiyiwa et al. 2005).

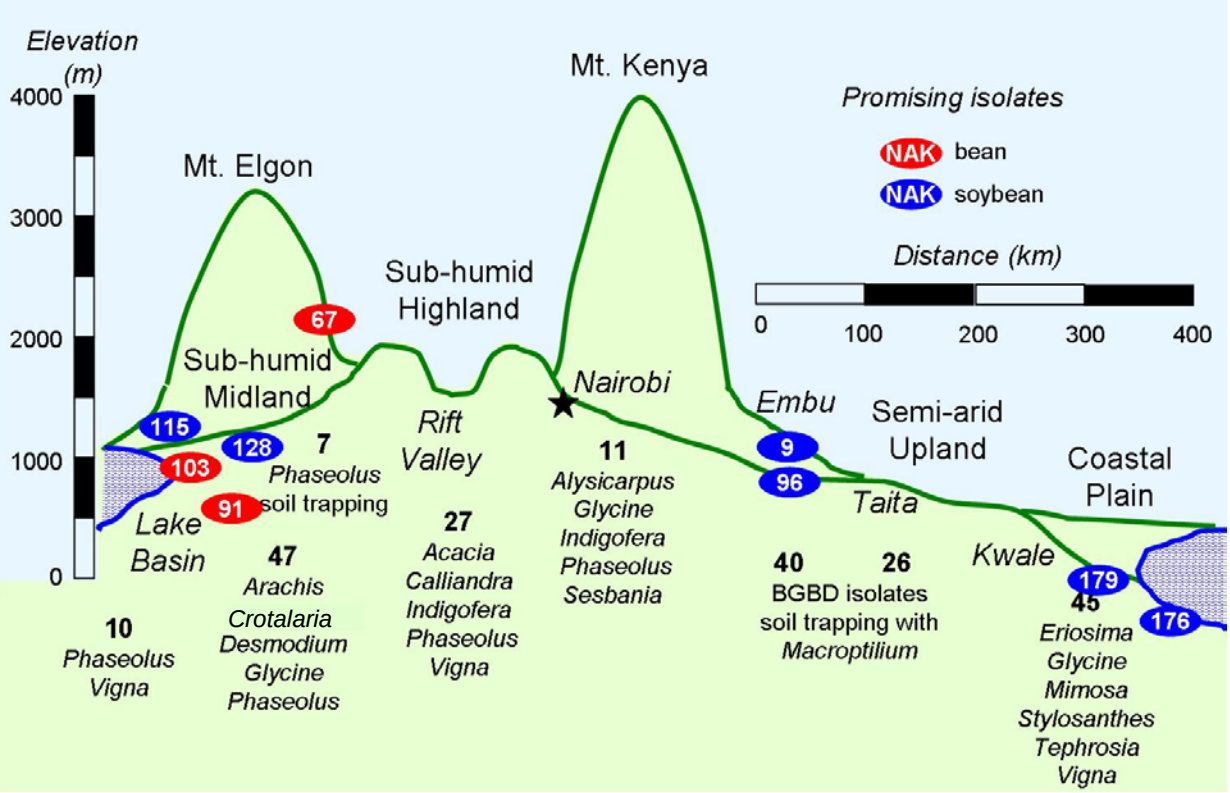

Figure 3.2: Bio-prospecting in Kenya sampled from the shores of the Indian Ocean to the Lake Victoria Basin and all major agro-ecologies along a 1050 km transect. Numbers in shaded ovals indicate the source of some candidate elite isolates for bean and soyabean

Different sampling locations may be stratified based upon agro-ecological zones, extreme climatic and soil conditions or along transects incorporating these gradients. An example is sampling along an elevation transect crossing a series of land uses and plant communities. Soil samples comprised of several composite sub-samples are better than those obtained from fewer ones. For example in Kenya a transect was sampled through a series of field campaigns that included all major agro-ecological zones in the country (Figure 3.2).

Finally, a great place to collect nodules year round was identified, within semi-cultivated, wetland margins where moisture conditions are such that nodules occur year-round and close to the soil surface. Another easy to sample location was drainage ditches, but extra care must be taken as dangerous animals often hide there. Often it was difficult to recover nodules from tree legumes and re-sprouting perennials, so bio-prospectors learned to watch for understorey seedlings and hosts growing on steep slopes or beside road cuts.

4 Isolate characterization and curation

Isolates were prepared from nodules as described in MS 3.1.1 Report (Bala 2011). Briefly, nodules were placed into a vial containing silica gel, refrigerated when possible, removed and rehydrated, surface sterilized, crushed, a loop streaked across YMA and resulting colonies purified in a second culture. These clean cultures were re-streaked on YMA slants and saved as working cultures. The full protocol called for additional characterization on diagnostic media (Congo Red, Bromothio Blue) but these steps were not followed by all cooperators.

A MS Excel data base was developed to compile the results from bio-prospecting, characterization and effectiveness screening of rhizobia among the collaborators. It has 16 descriptors (Table 4.1) that cover isolate origin including taxonomic position of host legume, performance on diagnostic media and in effectiveness tests, and its eligibility as a candidate elite strain. To date, seven countries have entered information on 1360 isolates into the database with another 11 to be entered (1371 total). This is 69% of the program target (MS 3.1.3). Legume host taxonomy range from 1 to 11 Tribes, 2 to 20 genera and 2 to 27 species per country (Table 4.2). This taxonomic spread reflect large differences in sampling strategy, such as Rwanda and Zimbabwe that only sampled cultivated fields of one or two target grain legume (bean and soyabean, data not presented) and DR Congo and Kenya that sampled all cultivated and natural legume communities encountered across a wide range of agro-ecologies. Nigeria not only sampled its three target legumes, but also Bambara groundnut (*Vigna subterranea*), an important indigenous food legume. Malawi included *Desmodium* spp. in its sampling strategy. Ghana sampled some legumes belonging to *Mimosoideae* and *Caesalpinioideae* families as well.

Table 4.1: Parameters included on the N2Africa Rhizobium Database

Source country: NAC = DRC, NAG = Ghana, NAK = Kenya, NAM = Malawi, NAQ = Mozambique, NAN = Nigeria, NAR = Rwanda, NAZ = Zimbabwe
Entry: strain number in chronological order
Contributor: Organization holding isolate
Alternate Code: strain designation of contributing organization
Longitude and Latitude
Host Sub-family: M = Mimosoideae, P = Papilionoideae
Host Tribe: taxonomic group of host legume at Tribe level
Host Genus: Original host legume genus
Host Species: Original host legume species
YMA Growth rate: S = slow, I = intermediate, F = fast
CR YMA: colony characteristics on Congo Red
BTB YMA: Reaction on bromothiol blue
Test Host: legume host used in effectiveness testing
Reference: reference rhizobium strain in effectiveness

Table 4.2: Entries and host taxonomic of isolates entered into the N2Africa rhizobium database (as of April 2013)

Country[1]	Entries[2]	Tribes	Genera	Species
DR Congo	104	11	16	25
Ghana	168	5	7	8
Kenya	387	9	20	27
Malawi	170	2	4	6
Nigeria	250	2	3	4
Rwanda	252	1	2	2
Zimbabwe	29	1	2	2

[1] No database entries from Mozambique. [2] Additional isolates reported for DR Congo (7), and Rwanda (4)

Observations of colony growth rates and performance on diagnostic media have not been particularly revealing (Table 4.3). A majority of isolates are fast growing, do not absorb Congo Red and produce a range of pH effects when grown on BTB YMA. It is difficult to explain why so few observations on Congo Red absorption were conducted considering that our protocols call for routine isolation on CR YMA. If nothing else, the range of legume hosts and colony characteristics indicates that many different types of isolates appear in the N2Africa culture collection.

Table 4.3: Cultural characteristics of isolates entered into the N2Africa Rhizobium Database[1]

Test	Total	Categories (%)		
Growth Rate	731	slow	intermediate	fast
		27%	16%	57%
Congo Red absorption[1]	221	none	partial	full
		37%	60%	3%
BTB reaction	583	basic	none	acidic
		28%	34%	38%

[1] This summary does not include late arriving entries from Ghana and Malawi.

5 Milestone 3.1.3: Effectiveness testing and candidate elite strains

Effectiveness testing of the collected strains involves greenhouse trials where host legumes are raised in rhizobium-free media and then test cultures applied, and growth response measured. These trials require non-inoculated controls and the results are best compared to current industry standards. Different growth systems were employed by various cooperators ranging from standard Leonard jars, the Huye jar offspring made from bottled water (Figure 5.1), and three liter pots containing horticultural-grade vermiculite. Growth units are over-planted, and then selected for uniform hosts and then test isolates are applied to host legumes as broth. In general, smaller units suffer less contamination but restrict plant growth while larger units must be carefully protected from contaminants and permit much larger test plants that allow for greater differentiation of test isolates. Effectiveness testing was performed on both soyabean and bean using as many as 100 test isolates at a time, depending upon clean greenhouse space and the availability of materials. The goal is to identify which isolates outperform industry standards.

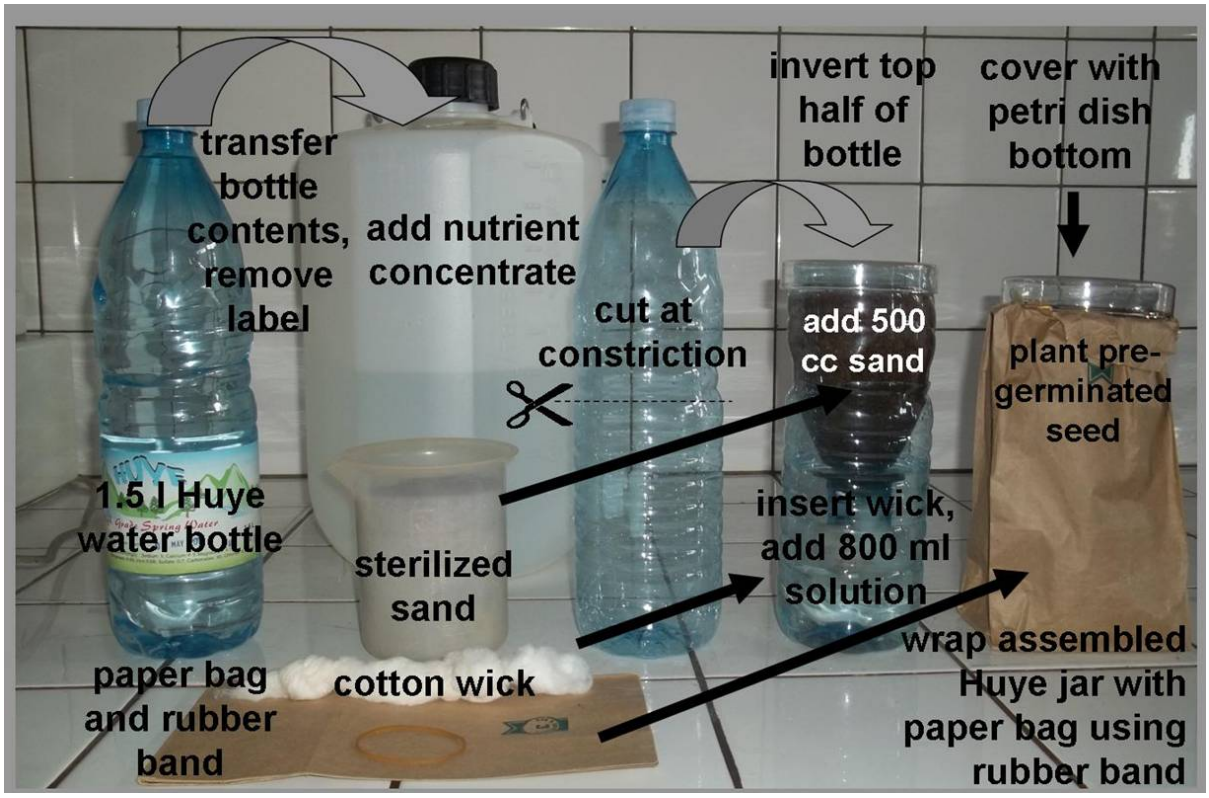

Figure 5.1: One innovation was the design of the Huye jar that allowed for authentication and preliminary strain effectiveness testing without autoclaving the growth unit

6 Tests of competitive ability

The next step is to evaluate the competitive ability of the better isolates under representative soil conditions (Stowers and Elkin 1980; Howieson et al. 2000). Host legumes are planted into potted test soil in the greenhouse, and then test isolates applied as broth. In this case, the native rhizobia serves as the baseline control so it is important to select test soil based upon MPN results (e.g. between 50 to 1000 indigenous rhizobia per g soil) as "non-hardened" test isolates cannot be expected to overcome massive native populations (e.g. > 5000 rhizobia per g). Test units that outperform the control soil are considered to be competitive and those that do not are less competitive. The best isolates, those that are both highly effective and competitive are considered to be candidate elite strains used in later field testing.

The strength of this approach is illustrated through results from DR Congo. Bintu Ndula, an MSc student, first identified 15 highly effective isolates for soyabean (out of 107 test isolates) under rhizobium-free culture (Huye jars, see Figure 5.1) and then evaluated them in potted soil (Figure 6.1). This approach permitted the isolates to be separated into two categories , highly effective/less competitive and highly effective/competitive, with five isolates performing very well in both assays. Note that all of these test isolates outperformed USDA 110 in effectiveness as the effectiveness performance index is based upon the ratio of test isolate to industry standard. Results from Kenya suggest that isolates performing well in potted soils also do well in the field (Table 6.1).

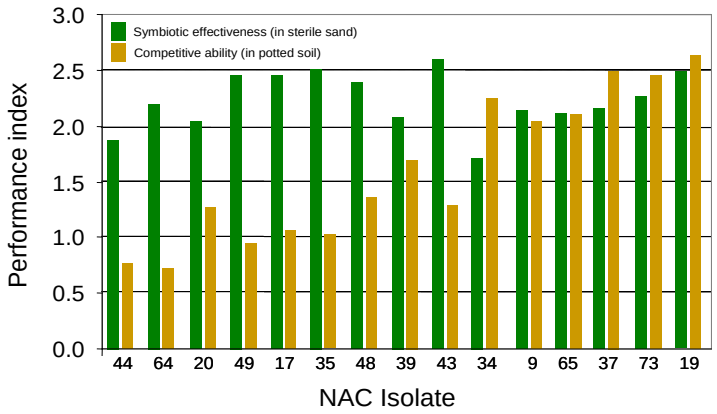

Figure 6.1: Symbiotic and competitive abilities of selected NAC isolates on Soyabean 24 under greenhouse conditions

Table 6.1: Grain yield by two soyabean 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	4.4
NAK 115	1153	5.2	1140	4.5
NAK 117	1210	8.2	1086	1.6
NAK 135	1212	8.3	1230	9.3
N-fertilizer applied	1299	0.7	1304	0.7
NAK 89	1317	13.9	1230	9.3
NAK 84	1339	15.1	1182	6.7
NAK 128	1462	21.7	1416	19.3
$LSD_{0.05}$	293		162	

[a] Partial return calculated as increased soyabean value/cost of N source with soyabean valued at \$0.613 kg^{-1}, inoculant at \$11.40 ha^{-1} and CAN-N at \$2.38 kg^{-1}.

7 Milestone 3.1.4: Field testing of the elite strains

The so-called 2% candidate elite strains are to be tested in the field under farmer conditions. The only country to reach this point was Kenya, in large part because it's team rapidly embarked upon bio-prospecting and isolation from nodules, its greenhouse is large and specifically designed for rhizobium studies, and its two growing seasons per year allowed for rapid movement of the very best isolates from the greenhouse to the field. Several useful experimental approaches were employed. The field trials include industry standard strains local isolates, non-inoculated controls and managements receiving 78 kg N ha^{-1}. Field work was conducted in N-deficient soils and an additional high C:N material (sugar cane bagasse) was applied to reduce N mineralization. Different strains were well separated by non-inoculated boundary rows, resulting in a “rolling effect” of healthy green and smaller chlorotic rows (Figure 7.1). Results from one field trial in west Kenya clearly illustrate the strong performance of isolates forwarded as N2Africa elite strains (Table 7.1)

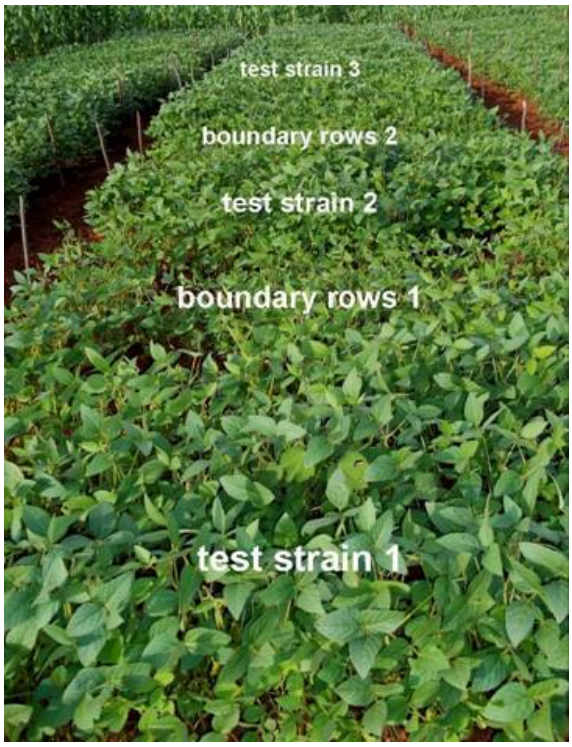

Figure 7.1: The rolling effect achieved by separating elite isolates with non-inoculated boundary rows under N-deficient soil conditions

Table 7.1: Greenhouse, pot and field testing of isolates has led to the identification of Kenyan elite soyabean strains

NAK isolate	Stage 1: sterile media	Stage 2: Potted soil		Stage 3: field trials
Test variety	SB24	SB19	Safari	SB19
NAK 96	1.24	0.96	1.18	0.93
NAK 115	1.09	1.03	0.97	1.16
NAK 128	1.06	1.01	1.34	1.20
NAK 9	1.06	0.88	1.32	1.07
USDA 110	1.00	1.00	1.00	1.00

8 Country-by-country activities

8.1 DR Congo

Progress in bio-prospecting and strain selection was slow to start because delays in construction of the laboratory within the Kalambo Agricultural Center near Bukavu, but it made rapid advances after laboratory establishment. In DR Congo, 213 nodule samples were collected leading to 107 tested isolates originating from 41 cultivated fields and 66 natural legume communities. These cultures are currently held on agar slants under refrigeration. To date, 104 isolates are entered into the program database. One hundred and seven (107) isolates were screened for effectiveness on soyabean against USDA 110 in potted, sterilized sand under greenhouse and the best 15 then compared in potted field soil to assess competitive abilities with native rhizobia (Figure 6.1). The most effective strains were NAC 35 and 43, but when competitive abilities were considered the four most promising strains are NAC 19, 37, 65 and 73.

8.2 Ghana

The laboratory at SARI was quick to upgrade its facilities and then examined tests for inoculation (MS 3.1.2) rather than initial bio-prospecting and strain testing. More recently it collected 194 isolates, assigned them NAG labels and entered 168 isolates into the program's Rhizobium Database. It has reported screening the symbiotic effectiveness of 53 indigenous rhizobia against USDA 110 using soyabean under greenhouse conditions (Figure 8.1). None of these isolates exceeded the performance of the industry standard strains so that none moved forward as candidate elite strains.

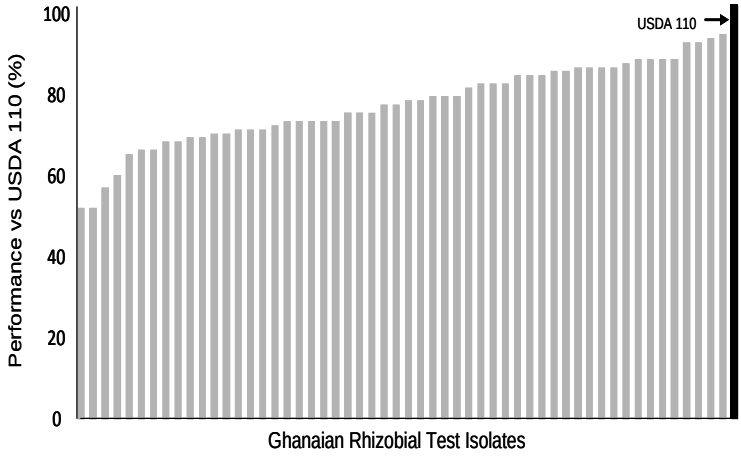

Figure 8.1: Effectiveness testing in Ghana where no indigenous isolate exceeded the performance of the industry standard 532c

8.3 Kenya

Expansion of the Rhizobium data base continues. The MIRCEN laboratory collection currently stands at 387 NAK isolates, all entered into the program database, with the last 179 entries being tested for effectiveness under greenhouse conditions but unlikely that the best will reach field testing. Of these isolates, 76% originated from cultivated fields and 24% from wild plant communities within 13 different ecological zones. The first three-stage round of strain testing for soyabean is completed. Starting with 186 authenticated isolates, 100 were selected for testing in sterile media for symbiotic effectiveness, the best 24 then retested in potted soil for competitive ability and the top six tested under field conditions (Table 8.1). The best two

Table 8.1: Greenhouse, pot and field testing of isolates has led to the identification of Kenyan elite soyabean strains

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Test variety	SB24	SB19	Safari	SB19
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NAK 128	1.06	1.01	1.34	1.20
NAK 9	1.06	0.88	1.32	1.07
USDA 110	1.00	1.00	1.00	1.00

are NAK 115 and NAK 128 both outperforming USDA 110, the current industry standard). NAK 84 and 89 are also emerging as promising isolates for soyabean (Waswa et al. submitted). The elite strains are currently undergoing molecular characterization in collaboration with COMPRO 2.

A similar evaluation process for bean rhizobia is nearing completion. Fast growing isolates were tested in the greenhouse for their effectiveness with bush (cv. Rose Coco) and climbing bean (cv. Tamu) and the best strains taken to the field. These isolates were compared to USDA 2667 and CIAT 899 as industry standards. Host-strain interactions were observed with NAK 45 and NAK 104 performing best with bush bean and NAK 67 performing best on climbing bean. Molecular characterization of the eight most promising bean rhizobia is also on-going at the COMPRO laboratory in Nairobi.

8.4 **Malawi**

The laboratory and greenhouse at Chitedze required considerable improvement that was completed in June 2013. The laboratory has since obtained 217 isolates from six legume hosts and entered 170 of them into the program data base. Besides routine characterization (e.g. Congo Red and BTB response), these isolates were screened for pH and salinity tolerance. For reasons not explained, only the 26 most "resilient" isolates were authenticated and tested for symbiotic effectiveness. The laboratory has focused more on conducting inoculation response tests and pilot production of inoculants, than on strain evaluations and has no candidate elite strains to offer at this time. Its cultures are stored under refrigeration in agar slants. Effectiveness and competitive assays using these isolates will be conducted during October 2013.

8.5 **Mozambique.**

Little progress was made in Mozambique in terms of bio-prospecting, isolate characterization or strain effectiveness testing. Laboratory upgrading is considerably delayed. The group has collected over 2,161 nodules and sent them to either the Microbiology Laboratory at IITA or to EMBRAPA in Brazil for isolation, authentication and characterization without further engagement, leading to only 10 isolates held at IITA, Nigeria. It has not reported any strain evaluation activities and hence has not identified any candidate elite strains.

8.6 **Nigeria.**

Bio-prospecting and isolate testing is led by the Institute of Agricultural Research in Zaria and was conducted across two agro-ecological zone (Sudanese and Northern Guinea Savannas) at ten sites. It boasts 256 isolates obtained from cowpea, soyabean, groundnut and bambara groundnut and 250 of these entered into the Rhizobium Data Base. To date, 149 isolates were authenticated and evaluated in three sets of pot experiments in a screenhouse (see Table 4.2). Preliminary effectiveness testing was performed on soyabean using 90 isolates with results available for the first 50 isolates being compared to USDA 110 and other reference strains. Few isolates failed to perform better

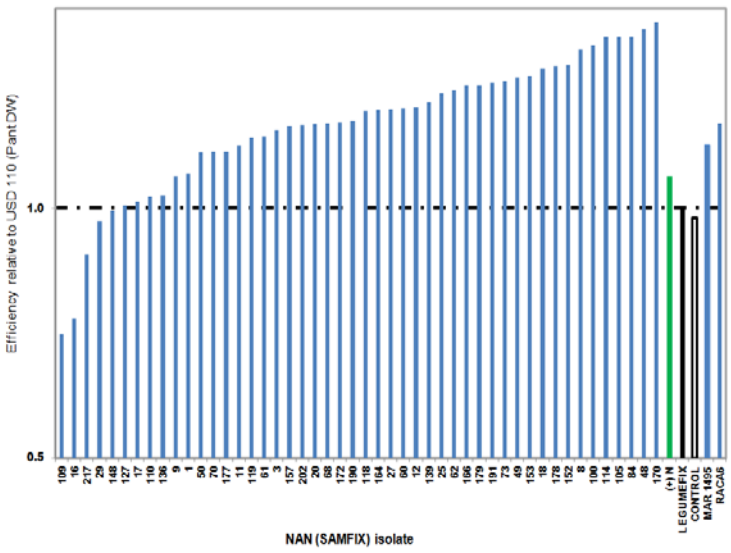

Figure 8.2: Effectiveness testing of rhizobium strains on soybean in Nigeria where almost all indigenous isolates outperformed the industrial standard USDA 110 and other reference strains

than the standard strains, which makes all potential elite strains (Figure 8.2).

8.7 **Rwanda**

The laboratory at RAB-Rubona was quick to upgrade its facilities and apply the technical training provided by the program. It has isolated and characterized a total of 259 rhizobia (94 from soyabean and 165 from bean). All the isolates were entered into the Rhizobium Data Base. Greenhouse screening of the isolates identified 15 soyabean isolates that outperformed the commercial standard USDA 110 and 13 bean isolates that outperformed the commercial standards CIAT 889 and UMR1957. These 28 candidate elite strains are scheduled for further testing in potted soils and at two field locations. Forty isolates from Rwanda collected by MIRCEN early in the program from a variety of cultivated and wild hosts are also held in Kenya, bringing the total isolates from Rwanda to 292. All of these isolates are stored on Yeast Mannitol Agar slants at 4°C.

8.8 **Zimbabwe**

Rhizobiology efforts are led by the Soil Productivity Research Laboratory (SPRL) under the Department of Research and Specialist Services in the Ministry of Agriculture. SPRL has many years experience in rhizobiology and inoculant production, distributing about 80,000 80-gram packets last year. All indigenous rhizobia isolations were collected from non-inoculated common bean and soyabean nodules collected from seven administrative districts. Ten isolates were prepared from agronomic and dissemination trials and 19 isolates originate from farmers' cultivated fields. Only healthy plants with nodules bearing a reddish interior were selected for plating. Authentication was performed using sterile growth pouches in the greenhouse yielding 20 isolates from soy bean and 9 from common bean). Isolates are stored on YMA slants under refrigeration and checked every 3 months. Most of these isolates (27) are entered into the program database. To date the group has conducted two rounds of greenhouse testing for symbiotic effectiveness with top ranked isolates being NAZ 15 and NAZ 21 for soyabean and NAZ 18 for bean. NAK 69 and NAK 91 from Kenya also performed well on bean in Zimbabwe under greenhouse conditions and were next tested in the field.

Zimbabwe field tested candidate elite strains for both soyabean and common bean during the 2012-2013 growing season. For soyabean, five isolates were tested at six sites and compared to non-inoculated, N-fertilized and

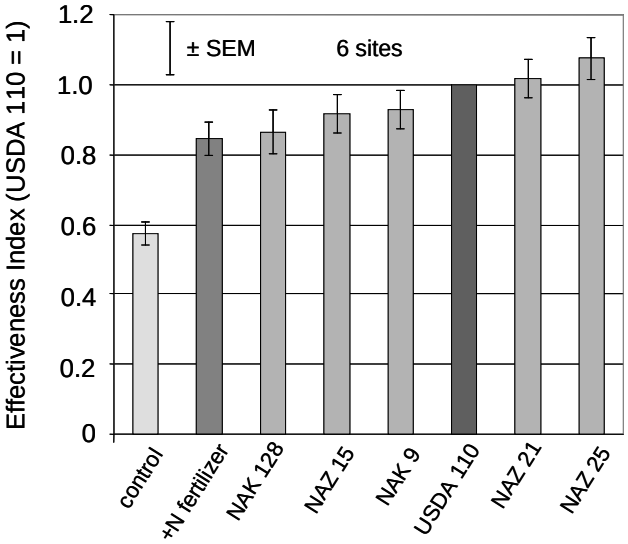

Figure 8.3: Field testing of candidate elite strains on soyabeans across six sites in Zimbabwe

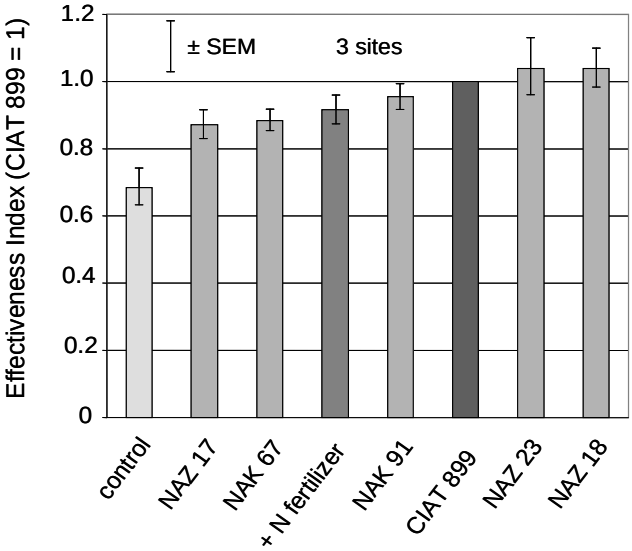

Figure 8.4: Field testing of candidate elite strains on common bean across three sites in Zimbabwe

industry standard (USDA 110 = MAR 1491) controls (Figure 8.3). Two of these isolates originated from Kenya (NAK 9 and NAK 128). Average yields for the industry standard averaged between 0.63 and 2.35 t ha^{-1}. Two candidate elite strains emerged (NAZ 21 and NAZ 25). For common bean, five isolates were tested at three sites and compared to non-inoculated, N-fertilized and industry standard (CIAT 899) controls (Figure 8.4). Two of these isolates originated from Kenya (NAK 67 and NAK 91). Average yields for the industry standard averaged between 1.07 and 1.44 t ha^{-1}. Two candidate elite strains emerged (NAZ 18 and NAZ 23). That these Zimbabwean candidate elite strains that compare so favourably to both industry standards and elite strains from Kenya from such a narrow germplasm base (29 isolates) suggests that bio-prospecting efforts there should be expanded.

9 Further characterization of elite strains

Isolates may be further characterized over a series of stages (Figure 9.1). Several soil factors influence the symbiotic relationship including extreme pH, salinity, and high temperature and these may be considered in more advanced selection activities as they limit the establishment of introduced *Rhizobium* (Slattery and Pearce 2002).

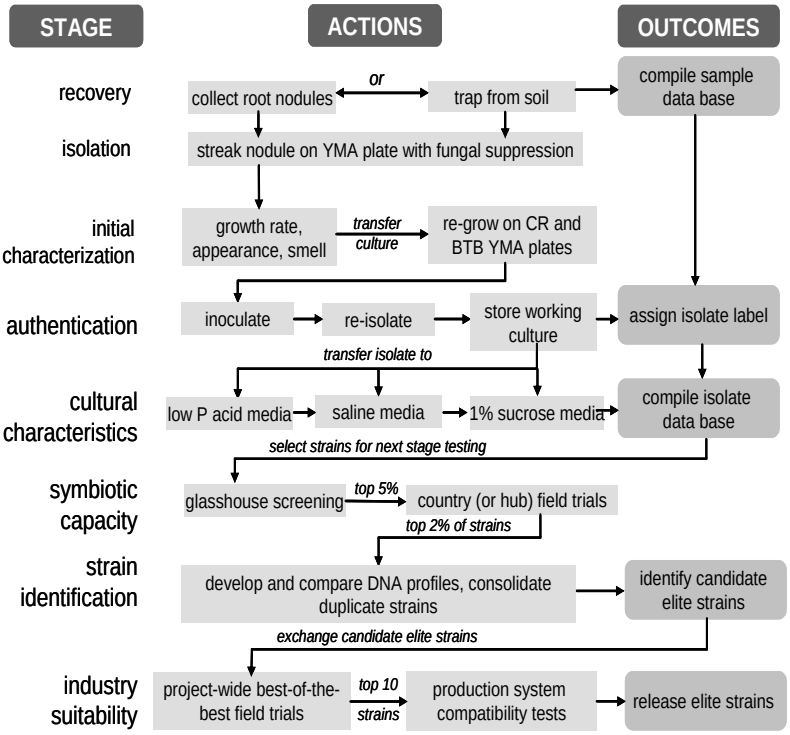

Figure 9.1: The stage-wise projection from rhizobium exploration, characterization and establishment as an elite strain for widespread use in legume inoculants

Initial characterization involved observation of colonies growing in YMA containing Congo Red and Bromothiol Blue indicators Somasegaran and Hoben 1994). Isolates were authenticated through inoculation (and sometimes re-isolation) from nodules of a test legume. More advanced colony characteristics may be obtained from culturing isolates in different "stress" broths containing saline, low pH or lower cost C sources (e.g. glycerol and glucose). This laboratory assessment leads to the compilation of a data base that describes the isolates and assists in the selection of which strains warrant testing for BNF capacity. BNF capacity was to be assessed in a three step manner; first under

Box 9.1: A summary of bio-prospecting and strain evaluation performed in Kenya (after Waswa et al. 2013)

Bio-prospecting and isolate characterization was conducted in Kenya to identify elite strains of rhizobia capable of effectively nodulating promising soyabean varieties. One hundred isolates were recovered from nodules of wild and cultivated legume hosts growing in different agro-ecological zones across a 1000 km transect. These isolates were authenticated and tested for effectiveness on soyabean (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, one promiscuously nodulating" and another specific. The six best performing isolates were then evaluated under field conditions, comparing them to Bradyrhizobium japonicum strain USDA110. Test isolates were classified into five categories, non-infective (20%), ineffective (26%), partly effective (26%), effective (17%) and highly effective (11%) based on their performance relative to controls and industry standards. The indigenous rhizobia that outperformed USDA 110 were considered highly effective. In potted soil, all the 24 native rhizobia isolates nodulated promiscuous soyabean (SB19) but only 46% of them nodulated specific soyabean (Safari). In the field experiment, strain NAK 128 performed best on both promiscuous and specific soyabean varieties, significantly ($p<0.05$) outperforming USDA 110 by 29% and 24%, respectively. Partial economic returns to inoculation with NAK were about 21:1, justifying inoculation as a field practice and producing up to 2.5 million nodules (333 kg) ha^{-1}, significantly ($p<0.05$) more than USDA 110. The three best isolates from this investigation, NAK 84, 89 and 128 outperformed the management receiving 78 kg N ha^{-1}, require further characterization and field testing but clearly have commercial potential and are available to interested parties.

rhizobium-free growth conditions, then in glasshouse pots containing a representative field soil and finally in the field under farmer conditions, with each allowing the number of test isolates to be narrowed to the most promising candidates (Halliday 1984; Howieson et al. 2005; Stowers and Elkin 1980). Field testing is conducted using inoculants prepared from the best isolates and compared to currently-available commercial inoculants. In most cases, N2Africa failed to reach the third and most important step.

Ideally, the elite strains identified through exploration and characterization should be distinguished as original through strain identification procedures, either molecular (Howieson and Brockwell 2005) or serological (Olsen and Rice 1989). Symbiotic performance is key but the ability of rhizobia to survive stress conditions (Slattery and Pearce 2002) or to utilize less expensive growth media (Halliday 1984; Somesegaran and Hoben 1994) are also important considerations.

10 Milestone 3.1.5: Adoption of the elite strains by inoculant producers

Elite strains are determined by their ability to replace those already in use as commercial legume inoculants. First it is important that the strains be identified and not confused with one another and those already in use. Second, more comprehensive field testing is required to assure that the candidate elite strains perform across a wide range of field conditions, with direct comparison to commercially-available inoculants. Finally, the compatibility of the strains within different inoculant production systems must be determined. Strains that are unique, consistently outperform currently available ones in the field and are readily incorporated into established production systems are eligible for release as elite strains. To date, there are three requests for an elite soyabean strain from N2Africa, NAK 128, by BIO-NEXT (Wichita, USA), Novozyme (Milwaukee, USA) and MEA Ltd. (Nairobi, Kenya) that are being processed as of the preparation of this report. Similar requests by others and for additional isolates will likely be received over the next few months.

11 Conclusions and recommendations

The empirical, stepwise approach to strain selection described in this report is partially successful in that it started with a large number of test isolates and systematically reduced them to a few, highly effective and competitive strains (Halliday 1984; Howieson et al. 2000). The first of these highly effective and competitive strains to be identified by N2Africa research appear in Box 11.1, with more certain to follow. Part of this success is due to reliance upon larger growth units in our greenhouse experiments, and the greenhouse design and sanitation that permits these units not to become contaminated. Another component is comprehensive bio-prospecting throughout many countries, and the additional testing of strains for competitive ability in potted soil. Another element of this success is perhaps due to luck, because successful bio-prospecting for rhizobia intended for a moderately specific legume host away from its Center of Origin is more risky (Appunu et al. 2008), but Africa is an ancient land mass and embraces considerable biodiversity (Maingi et al. 2006). One indicator of our success is the performance of the best isolates compared to long-time industry standards USDA 110 and CIAT 899. In one case, none of 53 locally obtained test isolates from Ghana outperformed USDA 110 but in other countries (e.g. DR Congo, Kenya and Nigeria) several more effective strains were identified.

Box 11.1: Candidate elite strains emerging from N2Africa bio-prospecting followed by effectiveness, competition and field testing

Climbing bean: NAK 67
Common bean: NAK 45, NAK 104, NAZ 18, NAZ 23
Soybean: NAC 19, NAC 73, NAK 115, NAK 128, NAN 109, NAN 177, NAZ 21, NAZ 25

With regard to the scope and execution of strain effectiveness testing, a wide range in performance exists between collaborators, from those that simply collected nodules and sent them away for isolation by other without further engagement (e.g. Mozambique) to the MIRCEN in Kenya where

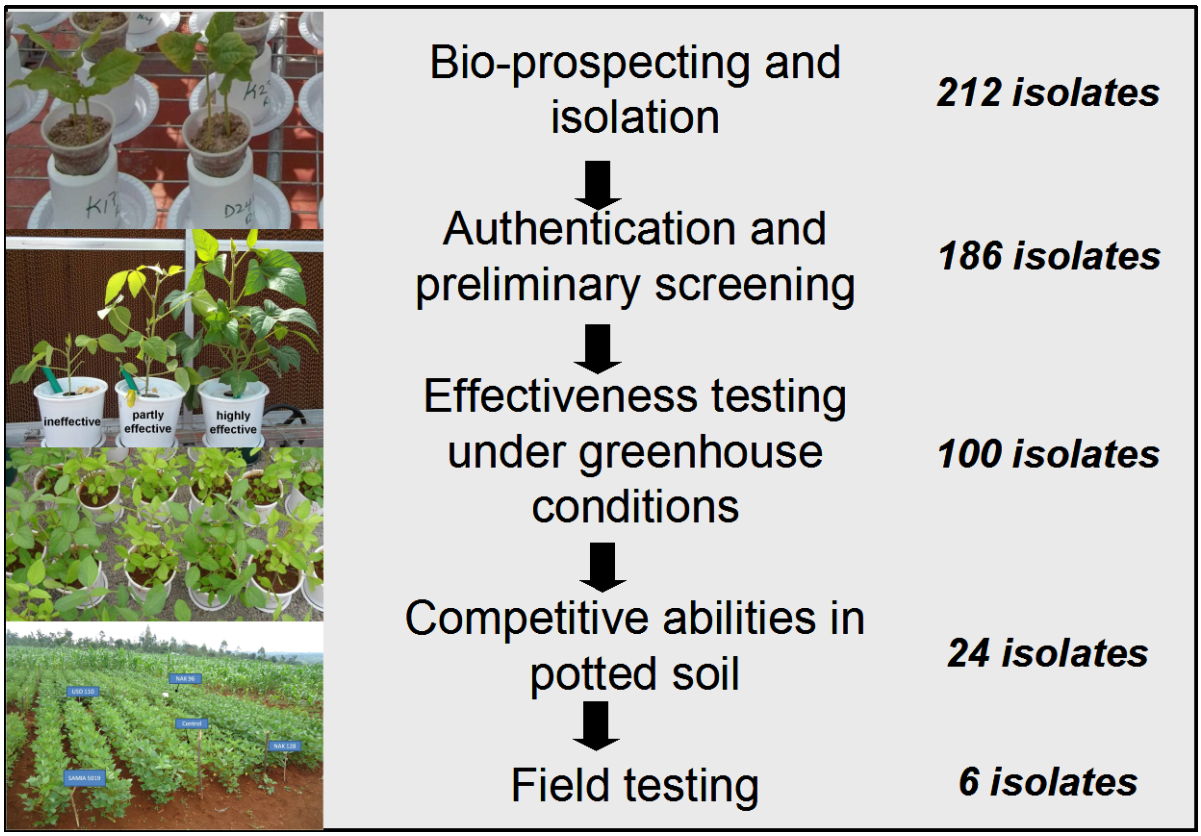

Figure 11.1: The stepwise approach to isolate evaluation that led to the identification of four elite strains for soyabean in Kenya (after Maureen Waswa)

greater than expected isolates were recovered and characterized, the program's rhizobium data base was developed, and the elite strains at both 5% and 2% were identified (Box 9.1, Figure 11.1).

Recommendations.

1. Bio-prospecting, isolate characterization and strain effectiveness testing should continue.
2. To date, effectiveness testing was conducted on soyabean, and to a lesser extent bean. Further work on bioprospecting and isolate testing should be targeted to cowpea and groundnut,
3. Cooperators must further advance the N2Africa virtual rhizobium collection by completing their entries into the program's N2Africa Rhizobium Data Base. The data base should then be posted on the program web site and distributed to other interested parties.
4. Strategies and protocols for long-term storage must be developed and the necessary equipment and skills obtained. Lyophilization is the most suitable approach. Decisions must be made on which isolates should be committed to long-term storage and where they should be held.
5. Characterization of candidate elite isolates should include molecular profiling to identify species and that these isolates are distinct strains. In addition, the genetic stability of candidate elite strains must also be considered as the loss of symbiotic effectiveness by cultures during storage may occur. Stability during repeated subculturing is a key characteristic for commercial use of rhizobium strains.
6. The elite strains identified by various N2Africa partners should be assembled within a single cooperator's laboratory for further comparison and release to other interested parties.

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List of project reports

1. N2Africa Steering Committee Terms of Reference
2. Policy on advanced training grants
3. Rhizobia Strain Isolation and Characterisation Protocol
4. Detailed country-by-country access plan for P and other agro-minerals
5. Workshop Report: Training of Master Trainers on Legume and Inoculant Technologies (Kisumu Hotel, Kisumu, Kenya-24-28 May 2010)
6. Plans for interaction with the Tropical Legumes II project (TLII) and for seed increase on a country-by-country basis
7. Implementation Plan for collaboration between N2Africa and the Soil Health and Market Access Programs of the Alliance for a Green Revolution in Africa (AGRA) plan
8. General approaches and country specific dissemination plans
9. Selected soyabeans, common beans, cowpeas and groundnuts varieties with proven high BNF potential and sufficient seed availability in target impact zones of N2Africa Project
10. Project launch and workshop report
11. Advancing technical skills in rhizobiology: training report
12. Characterisation of the impact zones and mandate areas in the N2Africa project
13. Production and use of Rhizobial inoculants in Africa
18. Adaptive research in N2Africa impact zones: Principles, guidelines and implemented research campaigns
19. Quality assurance (QA) protocols based on African capacities and international existing standards developed
20. Collection and maintenance of elite rhizobial strains
21. MSc and PhD status report
22. Production of seed for local distribution by farming communities engaged in the project
23. A report documenting the involvement of women in at least 50% of all farmer-related activities
24. Participatory development of indicators for monitoring and evaluating progress with project activities and their impact
25. Suitable multi-purpose forage and tree legumes for intensive smallholder meat and dairy industries in East and Central Africa N2Africa mandate areas
26. A revised manual for rhizobium methods and standard protocols available on the project website
27. Update on Inoculant production by cooperating laboratories
28. Legume Seed Acquired for Dissemination in the Project Impact Zones
29. Advanced technical skills in rhizobiology: East and Central African, West African and South African Hub
30. Memoranda of Understanding are formalized with key partners along the legume value chains in the impact zones
31. Existing rhizobiology laboratories upgraded
32. N2Africa Baseline report
33. N2Africa Annual country reports 2011

34. Facilitating large-scale dissemination of Biological Nitrogen Fixation
35. Dissemination tools produced
36. Linking legume farmers to markets
37. The role of AGRA and other partners in the project defined and co-funding/financing options for scale-up of inoculum (banks, AGRA, industry) identified
38. Progress Towards Achieving the Vision of Success of N2Africa
39. Quantifying the impact of the N2Africa project on Biological Nitrogen Fixation
40. Training agro-dealers in accessing, managing and distributing information on inoculant use
41. Opportunities for N2Africa in Ethiopia
42. N2Africa Project Progress Report Month 30
43. Review & Planning meeting Zimbabwe
44. Howard G. Buffett Foundation – N2Africa June 2012 Interim Report
45. Number of Extension Events Organized per Season per Country
46. N2Africa narrative reports Month 30
47. Background information on agronomy, farming systems and ongoing projects on grain legumes in Uganda
48. Opportunities for N2Africa in Tanzania
49. Background information on agronomy, farming systems and ongoing projects on grain legumes in Ethiopia
50. Special Events on the Role of Legumes in Household Nutrition and Value-Added Processing
51. Value chain analyses of grain legumes in N2Africa: Kenya, Rwanda, eastern DRC, Ghana, Nigeria, Mozambique, Malawi and Zimbabwe
52. Background information on agronomy, farming systems and ongoing projects on grain legumes in Tanzania
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54. N2Africa Project Progress Report Month 42
55. Market Analysis of Inoculant Production and Use
56. Identified soyabean, common bean, cowpea and groundnut varieties with high Biological Nitrogen Fixation potential identified in N2Africa impact zones
57. A N2Africa universal logo representing inoculant quality assurance
58. M&E Workstream report
59. Improving legume inoculants and developing strategic alliances for their advancement
60. Rhizobium collection, testing and the identification of candidate elite strains

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