Rhizobia Strain Isolation and Characterisation Protocol

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N2Africa

Putting nitrogen fixation to work for smallholder farmers in Africa

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Background

Rhizobia selection and inoculant delivery activities within the N2Africa project fall under Objective 3 as follows:

Objective 3: Select superior rhizobia strains for enhanced BNF and develop inoculum production capacity in sub-Saharan Africa through collaboration with private sector partners

Activities

3.1 Assess the need-to-inoculate for the target legumes and identify elite strains across the impact zones.

3.2 Establish and characterize a rhizobium germplasm bank in the impact zones.

3.3. Formulate improved inoculant products and develop cost-effective production and delivery methods, including standardized quality assurance procedures.

3.4 Expand and upgrade inoculant production capacity in sub-Saharan Africa.

3.5 Conduct and advocate policy review on inoculant quality and cross-border movement.

The activities include the recovery, evaluation and distribution of elite strains of nitrogen-fixing bacteria that perform well with those legumes and the inoculant delivery systems that make them more accessible to their respective host. Rhizobia used in this project will be isolated from African soils while others will be provided by cooperating laboratories from other parts of the world. The project will renovate existing laboratory facilities and facilitate the commercial production of legume inoculants.

This section provides information on nodule recovery and rhizobia isolation.

General approaches to nodule collection and storage

1. Nodules will be collected and isolated primarily for selection of superior rhizobial strains. For this purpose, nodules should be collected within uninoculated plots from healthy green plants that are suspected of high N2-fixation activity. Such plants usually have medium to large nodules with pink-red interiors which may indicate active fixation. In contrast, where the purpose for sampling is for BNF estimation, nodule weight and/or nodule number, plants are sampled randomly within specified plots.
2. Nodulation may be assessed several times during early growth of the crop when nodule number, size and distribution can be indications of rhizobial infectiveness and effectiveness. However, for the purpose of this project, a single observation made at mid- to full podding is recommended. Plants to be examined should be identified randomly from the sampling row, which should be either of the outermost rows of the net plot. Preferably an area of the plot (0.25 m by 3-4 rows) can be allocated for nodule observations as it is important that a number of plants are sampled (±10) and not only single plants.
3. Although nodule number and nodule dry mass are the nodulation parameters most often measured, these can be complemented with a nodule scoring system (e.g., Figure 1) which takes into account nodule number and distribution. In fact nodule dry mass is problematic as it is hard to clean nodules completely from adhering soil and a single sand grain is often heavier than one nodule! Nodule mass measurements rarely give significant differences between treatments except in extreme cases. Nodule effectiveness is determined by nodule colour and nodule size; ineffective nodules are not considered

(Effective nodules generally have pinkish red internal tissue, while ineffective nodules have green or white tissue).

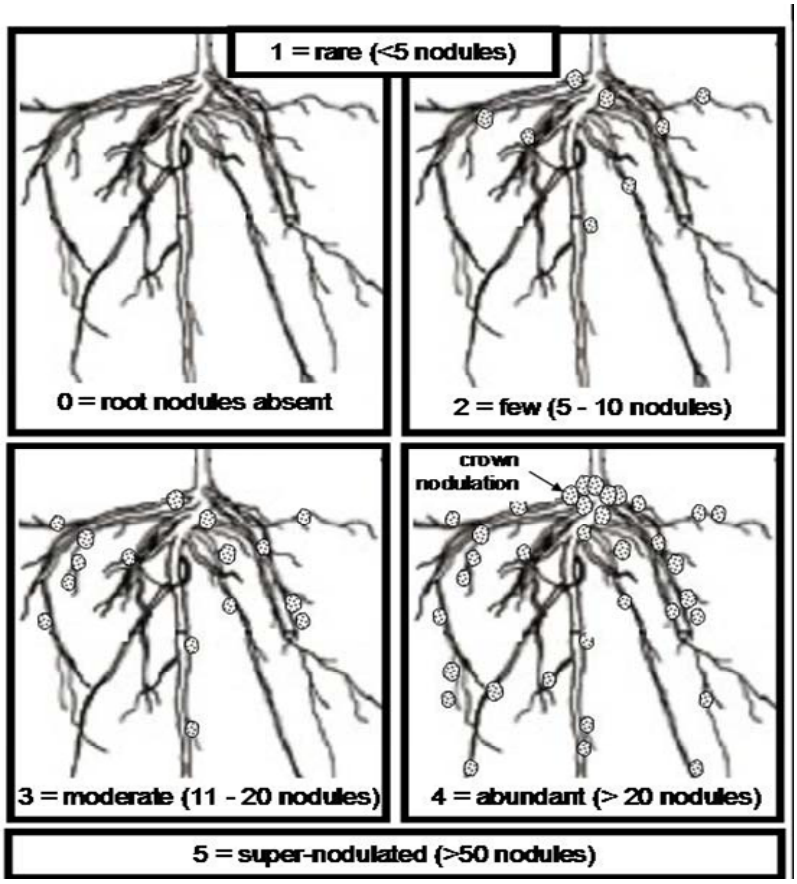

Figure 1. Nodulation scoring system used within the N2Africa Project based on 0 to 5 ranking. The system is suitable for most annual species but may need to be modified for different locations and legumes.

4. To retrieve root nodules, plants should not be uprooted or pulled by hand as this could lead to some or most of the roots and nodules being detached and left behind in the soil. Therefore, whole plants should be excavated, preferably using a sturdily built spade. Care needs to be taken on excavating plants to avoid detachment of the nodules from the roots which is particularly problematic in heavier, clayey soils and for some legume species (e.g. pigeonpea). Often only the top 10 cm of the root system is exposed whereas most grain legumes root to 50 cm or more and many of the nodules can be found at depth, particularly in non-inoculated plots. In particular cowpea is known to be drought-tolerant due to its capacity to root deeply. Of course woody legumes, and many forage legumes have strong tap roots that extend to several metres depth. Careful excavation is key to getting an accurate impression of the nodulation status of a plant.
5. Between 6 and 10 plants should be sampled from each treatment and replication and about 10-15 nodules sampled per plant. Note that these numbers are arbitrary but are guided by the need to get a representative sample that can also be easily handled. Dark or pinkish red medium sized nodules are recommended for sampling as these are more easily sterilised during isolations than the bigger nodules.
6. All nodules from host plants of the same species and variety sampled from the same plot represent one unit of collected material and can be stored in the same vial.
7. For storage and isolation purposes, nodules should not be plucked from the roots; rather, the nodules should be severed from the root by cutting the root about 0.5 cm on each side of the nodule. This keeps the nodule intact and improves the chances of getting a

clean viable culture of rhizobia. When handling the nodules, use forceps on the root appendages to reduce the risk of damaging the nodule.

8. For field trips of more than 2 days, the root nodules collected must be prevented from decomposing and prevented from invasion by soil microorganisms which interfere with subsequent isolation procedures. Therefore, the root nodules collected are preserved in a vial with desiccated silica gels as shown in Figure 2. If the gel colour turns pink, the desiccant must be changed for blue one as soon as possible.

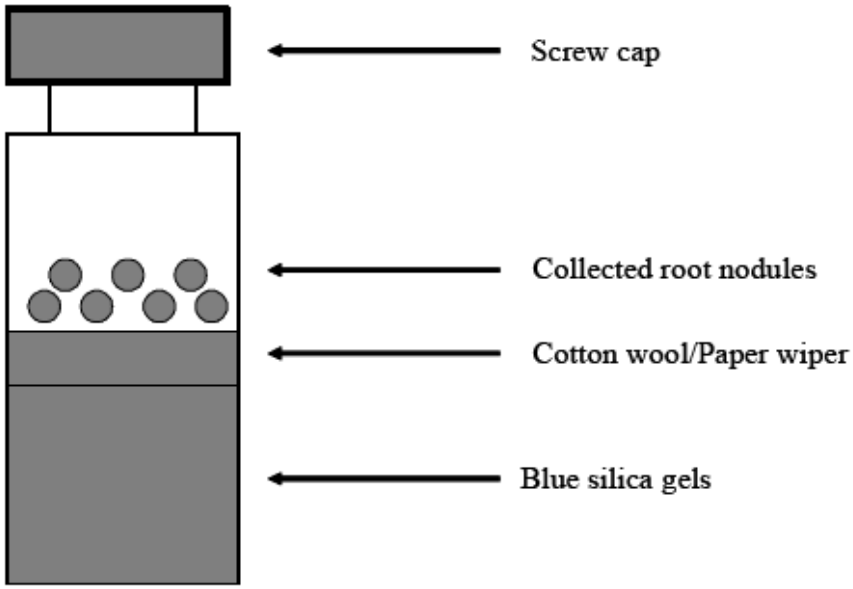

Figure 2. A preservation vial for root nodules

9. Do **not** oven-dry nodules that are sampled for rhizobia isolation as oven-drying will kill the rhizobial cells.

10. Fresh nodule sample may be stored in a refrigerator (4 oC) for up to 48 hrs. To store for longer periods, however, nodules should be placed in vials containing dry silica gel before being kept in a refrigerator. Nodules preserved in this way can be kept for up to 6 months, although recovery of the rhizobium during isolation after rehydration may be difficult after long-term storage. Nodules should not be frozen as the formation of ice crystals within the nodule may kill the bacteroids.

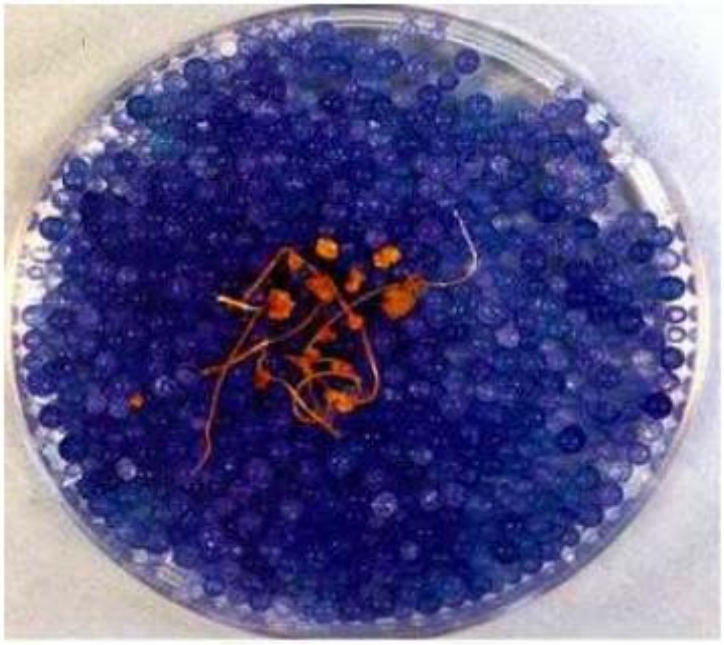

Figure 3. Dried root nodule in blue silica gels

Nodule recovery and rhizobium isolation procedure

1. With a spade, describe a circle of radius of approximately 20 cm around a legume plant and cut out this section at a depth of at least 20 cm.
2. Use the spade to lift the soil/root clump.
3. Carefully and gently remove soil from root materials with your hands. In case of heavy soils, wash the roots, by carefully immersing in a bucket of water, to remove the soil.
4. Explore the presence of nodules on main and lateral roots.
5. Carefully sample (sever the nodule from the root by cutting the root at 0.5 cm on each side of the nodule from main or lateral roots) and place roots in a vial containing silica gel previously dried at 110ºC.
6. Record date of collection, plant host and variety, location, soil type, , cropping system, soil fertility conditions and keep this as a permanent record for the nodule isolate.
7. For immediate isolation of rhizobia, preserve nodules in the refrigerator overnight (do not freeze nodules because ice crystals may rapture and kill the bacteriods).
8. Note the shape and size of nodules recovered from designated legume plant.
9. Examine nodule for effectiveness by cutting through (subsample of) nodule with a razor blade and recording interior pigmentation (red, pink, brown, green, grayish-green, black) in relation to location of nodule on the root system (upper 5cm portions of tap root, primary laterals).
10. Transport vials of nodules to the laboratory and store properly until isolation is done.
11. Rehydrate desiccated nodules by immersing in clean beaker of cool water and leaving the container in the refrigerator to imbibe overnight (NB: a one hour soaking at room temperature is sufficient for nodules that have been desiccated for only a short time).
12. Wash nodules thoroughly to remove soil.

13. Randomly select and immerse intact, undamaged nodules in sterile glass or plastic beakers containing 95% ethanol (or isopropanol) for 5-10 s to break surface tension and to remove air bubbles from nodule tissues.
14. Transfer nodules to a 3-5% (v/v) solution of sodium hypochlorite for 5 min.
15. Rinse in 5-6 changes of sterile water using sterile forceps for transferring, ensuring that forceps are sterilized in between transfers by dipping in alcohol and flaming.
16. Crush each surface sterilized nodule with a pair of blunt-tipped forceps in a large drop of sterile water in a Petri dish. A well sterilized nodule would most often produce pure cultures. This number is lowered if desiccated nodules are being used.
17. Streak one loopful of the nodule suspension on a yeast-mannitol agar (YMA) plate containing bromothymol blue (BTB) or Congo Red (CR). **Note** that the use of indicator media could camouflage real morphologies and distort growth rate of the 'non-conventional' rhizobia, such as Bukholderia and pigmented strains. Hence, some laboratories isolate rhizobia on YMA plates without indicator media. In that case, the main thing is to watch the plates closely (daily) and note whether colony growth is uniform across the plate (indicating relatively pure cultures) or whether colonies of different appearance and growth rate emerge (indicating contaminants). If the latter, either throw it away and start again, or select across the range of colony types and authenticate after purifying.
18. Deposit culture on agar with inoculation loop, then streak out on the agar surface along one direction. Resterilize the loop, cool by touching the agar surface near the side of the Petri dish, then streak from end of (and in direction perpendicular to) the first streaked area to a second area, and then repeat for a third and a fourth area.
19. Incubate at 28 ºC for 7-10 days and make daily observations for the appearance of typical colonies of rhizobia. Distinct colonies of fast-growing rhizobia begin to appear within 3-5 days, while those of slow-growers require 7-10 days to appear. Typical rhizobia colonies should show little or no CR absorption. Colonies of slow-growing rhizobia are characterized by a blue colouration, which indicates alkaline reaction on BTB. A yellow colour, indicating acid reaction, is produced by fast-growing rhizobia.
20. Select isolated typical colonies and restreak on YMA-BTB and YMA-CR; observe for characteristics described in step 19 and then perform a Gram stain to check for purity of culture and authenticate the isolates as rhizobia by testing the capacity of typical isolates to form nodules on roots of legumes from which the isolates were obtained.
21. Store authenticated isolates on YMA slant in screw-cap tubes for short-term storage and day-to-day use of the culture.

The needle method of isolating rhizobia

The second method by which rhizobia can be isolated from nodules is the needle method. This method of isolation is especially useful with freshly harvested nodules 2 mm or larger in diameter. The nodule is initially washed in water, then in alcohol, and then held with forceps and briefly passed through a flame. This surface-sterilized nodule is placed on a small piece of sterile filter paper (2 x 2 cm) in a sterile Petri dish. A new piece of filter paper should be used for each nodule. The same Petri dish can be used for several nodules. The blunt-tipped forceps are dipped into 95 % alcohol and flamed momentarily. While holding the nodule with the forceps and resting the nodule on sterile filter paper, a small section is quickly sliced off with a flamed, hot scalpel. While still holding the nodule with the forceps on the filter paper, the tip of a sterile inoculation needle (with a 1-mm loop) is inserted into the cut surface. The loop is then loaded with inoculum, and then streaked directly onto a YMA plate containing CR and a YMA plate containing BTB. When using the needle method, the nodule can also be held in the fingers of one hand while inserting the needle with the other hand. The heels of the hands are braced together to steady them.

Rhizobium exploration and testing

A goal of rhizobium scientists is to discover new and better strains 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 collection, care should be taken not to simply collect strains that were obtained through past inoculation, and the elite strains identified through exploration and characterization should be distinguished as original through strain identification procedures. Symbiotic performance is key but the ability of rhizobia to survive stress conditions or to utilize less expensive growth media are also important considerations. As illustrated through the following procedure, 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 by others. There are several considerations in designing rhizobium exploration strategies:

1. Isolates may be obtained directly from collected root nodules or may be trapped by applying soils to legume hosts of interest and then isolating rhizobia from the nodules formed. Nodule collection in the field is more direct and is best conducted during growing (rainy) seasons in drier climates. Alternatively, trapping native rhizobia from soil may be conducted as a dry season activity. Trapping may be combined with soil dilutions and MPN assays in order to gain insight into the population size of the rhizobial population and broaden the range of rhizobial types isolated.
2. Legume communities containing hosts belonging to the same cross-inoculation group as the target legumes may be sampled. This approach is most applicable to legumes nodulated by more promiscuous rhizobia which are in turn less likely to respond to inoculants in soils containing large numbers of native rhizobia. However, no two legumes nodulate and fix nitrogen with exactly the same range of rhizobia. Even within closely-related legumes (e.g. different species of Trifolium) there are large differences in numbers of effectiveness between rhizobial strains that nodulate them.
3. Sample within centers of biological diversity and, when possible, in the target legume center of origin. Keep in mind, however, that numerous elite rhizobia were discovered in areas far from these centers of diversity.
4. Cultivated legume fields that are recognized by local farmers as consistently producing superior crops may be sampled. Care must be taken not to sample fields with a history of inoculation unless the intent is to establish the saprophytic competence of elite strains that had earlier been applied to the field (in which case the strains are not truly discovered). One disadvantage of this approach is confounding BNF and crop performance with overall soil health, particularly the absence of pathogens, and hosts should be examined closely during collection. An advantage is its participatory nature, allowing farmers to become involved in sampling strategy.
5. 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.
6. While the opportunity to collect root nodules from uninoculated plots of rhizobium strain and legume variety screening exists, this approach risks collecting inoculant strains originating from adjacent plots. Sampling from these plots alone is a weak strategy. Collection is better performed from trapping rhizobia from soils taken before the plots are inoculated during preliminary studies such as MPNs.
7. Integrating several of these considerations is best, collecting nodules and trapping from soils, collecting from natural habitats and cultivated fields, sampling extreme environments and croplands. It is important that collection procedures remain flexible and open to opportunities as they emerge during field expeditions.

Table 1. Rhizobium exploration report form

1. Sample identification
2. Collector
3. Date
4. Location
5. Coordinates longitude _____ E W latitude _____ N S
6. Ecological zone
7. Land use
8. Vegetation type
9. Legume community
10. Material ☐ nodules ☐ rhizosphere sample ☐ soil sample
11. If nodules are collected, legume host
12. If rhizosphere is collected, root source
13. Describe texture and color of soil
14. Related sample numbers
15. Prepare a sketch map of the sample area on reverse side

Recovery

Identify an area of interest and technique for rhizobium exploration based on the above considerations. Assemble collection materials including collection vials, plastic bags, digging tools, markers, notebooks, maps and Geographic Positioning System (GPS). Develop a sampling schedule and route and assign two-person teams to each route.

Complete a simple report form for each sample (Table 1). Quickly identify which legumes are present and whether nodule, rhizosphere or soil samples are to be collected. Indicate if this sample is related to other nearby ones.

If nodules are to be recovered, then excavate roots carefully and remove attached soil by hand and gentle shaking. Recover three to eight nodules and place into vials containing silica gel as described in Activity 1. Enter legume host description into the report form. If rhizosphere or soils are collected, recover 0.25 to 0.50 kg to a depth of 15 to 25 cm, place in plastic bag and describe soil and rhizosphere as requested in Table 1.

Rhizobia are isolated from nodule samples by streaking on YMA as described in Activity 1.

Rhizosphere and soil samples are used for rhizobium trapping. This may be performed using hosts grown in Leonard jars, pouches or pots containing sterilized sand or rhizobium-free soil.

The “sloppy” MPN option. Calculate or estimate moisture content and prepare a 1:5 soil:diluent dilution. Further dilute the sample in two 1:100 steps, resulting in a three-step five-fold, 500-fold and 50,000-fold dilution series. Inoculate two sets of host legumes with 5 ml of each dilution as described in Activity 4. Different legumes belonging to different cross-inoculation groups may be planted in the same pot. This results in a 1, 100 and 10,000-fold dilution series. After nodules form, observe nodulation, recover a sample of healthy nodules for isolation and generate population estimate using MPNES software. Nodules from the lowest dilution step represent the more competitive and infective strains while those from the lower dilutions are more abundant and, perhaps, more saprophytically competent.

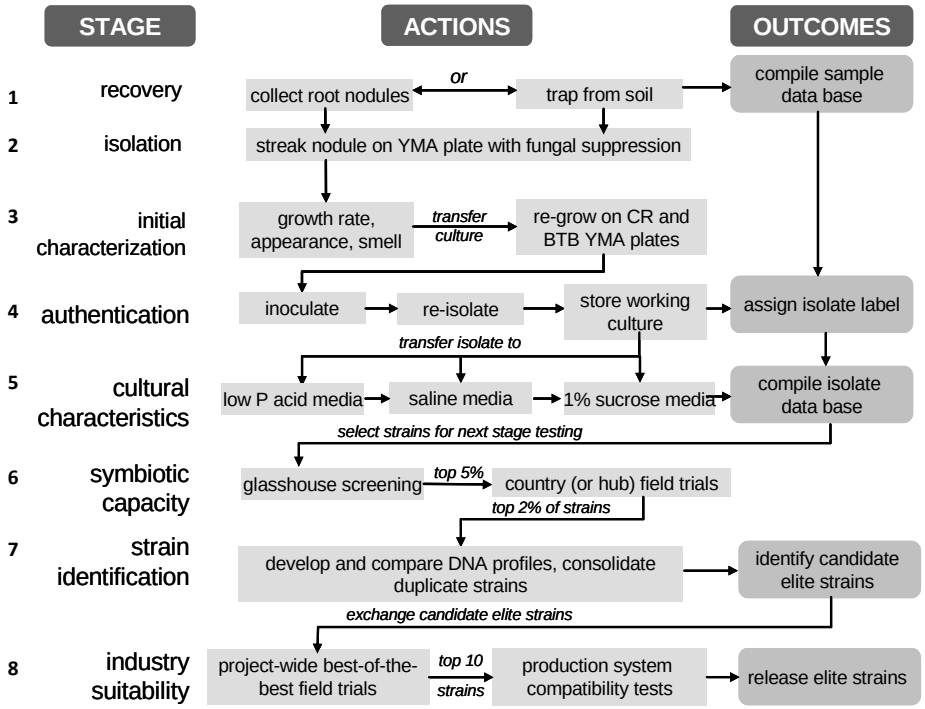

Figure 4. The stage-wise projection from rhizobium exploration, characterization and establishment as an elite strain for widespread use in legume inoculants.

Further characterization

Isolates may be further characterized over a series of stages (Figure 4). Initial characterization involves observation of colonies growing in YMA containing Congo Red and Bromothymol Blue indicators (Activity 3). Isolates must be authenticated through inoculation and re-isolation from nodules of a test legume (Activity 4). More advanced colony characteristics may be obtained from culturing isolates in different "stress" broths containing saline, low pH or sucrose as described in Activity 5. This laboratory assessment leads to the compilation of a database that describes the isolates and assists in the selection of which strains warrant testing for BNF capacity. BNF capacity is assessed in a three step manner as described in Activities 6, 7 & 8, under sterile plant growth conditions (e.g. Leonard jars or rhizobia-free soil), 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 2% as candidate elite strains.

Identifying and comparing elite strains

Elite strains are determined by their superior performance to currently used inoculant strains, such that it would be worth using them to replace those already in use as commercial legume inoculants. First it is important that the strains be identified and not confused with 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.

References

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Unkovich, M., Herridge, D., Peoples M., Cadisch G., Boddey, R., Giller, K.E., Alves, B. and Chalk P. 2008. Measuring plant-associated nitrogen fixation in agricultural systems. ACIAR Monograph No. 136, 258 pp.

List of project reports

1. N2Africa Steering Committee Terms of Reference
2. Policy on advanced training grants
3. Rhizobia Strain Isolation and Characterisation Protocol

Partners involved in the N2Africa project

Diobass

Université Catholique de Bukavu

University of Zimbabwe

- Programme d'appui au developpement durable **PAD** (DRC)
- Service d'Accompagnement et de Renforcement des capacities d'Auto promotion de la Femme en sigle – **SARCAF** (DRC)