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TSBF-CIAT

Conservation and Sustainable Management of Below-Ground Biodiversity Project



Standard Methods for the Assessment of Soil Biodiversity in the Context of Land Use Practices

Inventory of below-ground biodiversity in eleven benchmark areas, within seven tropical countries









Standard Methods for the Assessment of Soil Biodiversity in the Context of Land Use Practices

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PREFACE

Agenda 21 calls for the conservation of biodiversity as well as actions to ensure sustainable development in the agricultural and other sectors. A major feature of global change in the tropics is that of land-use associated with agricultural intensification (Lavelle *et al.* 1997). In addition to plants, soil is the habitat of a diverse array of organisms: archaea, bacteria, fungi, protozoans, algae and invertebrate animals, the activities of which contribute to the maintenance and productivity of agroecosystems by their influence on soil fertility (Hole 1981, Lavelle 1996, Brussaard *et al.* 1997). This is mediated through four basic activities:

1. Decomposition of organic matter, which is largely carried out by bacteria and fungi, but greatly facilitated by soil animals such as mites, millipedes, earthworms and termites which shred the residues and disperse microbial propagules. Together, the microorganisms and the animals are called <u>decomposers</u>, but the term <u>litter transformers</u> has now come to be used to describe these animals, where they are not also ecosystem engineers (see below). As a result of decomposition, organic C can be released as CO_2 or CH_4 , but also incorporated into a number of pools within the soil (soil organic matter or SOM) which vary in their stability and longevity, but are generally in equilibrium with each other and the inflows and outflows of C from the system.

2. Nutrient cycling, this is closely associated with organic decomposition. Here again the microorganisms mediate most of the transformations, but the rate at which the process operates is determined by small grazers (micropredators) such as protozoa and nematodes. Larger animals may enhance some processes by providing niches for microbial growth within their guts or excrement. Specific soil microorganisms also enhance the amount and efficiency of nutrient acquisition by the vegetation through the formation of symbiotic associations such as those of mycorrhiza and N2-fixing root nodules. Nutrient cycling by the soil biota is essential for all forms of agriculture and forestry. Some groups of soil bacteria are involved in autotrophic elemental transformations, i.e. they do not depend on organic matter directly as a food source, but may nonetheless be affected indirectly by such factors as water content, soil stability, porosity and C content, which the other biota control.

3. Bioturbation. Plant roots, earthworms, termites, ants and some other soil macrofauna are physically active in the soll, forming channels, pores, aggregates and mounds, or by moving particles from one horizon to another, in such ways as affect and determine physical structure and the distribution of organic material. In doing so, they create or modify microhabitats for other, smaller, soil organisms and determine soil properties such as aeration, drainage, aggregate stability and water holding capacity.

Therefore they are also called **soil ecosystem engineers** (Stork and Eggleton, 1992; Jones *et al.* 1994, Lawton 1996, Lavelle *et al.* 1997). For animal soil ecosystem engineers it is usual to add to the definition that they also form faeces which are organo-mineral complexes, stable over periods of months or more (Lavelle *et al.* 1997).

Beyond the challenge of assessing the soil fertility benefits of retaining or enhancing the biodiversity of the soil community lies the question of giving this economic value at the level of the farm, the nation and the globe. We can distinguish between the short term benefits of soil fertility after slashand-burn conversion of forests in a long-fallow rotation and the value of the deforested land for permanent agriculture or plantation use. Apart from these values of the forest for local users and newcomers, there are additional "environmental service" functions, such as biodiversity conservation, watershed protection and miligation of greenhouse gas emissions, which are values for the outside world, though difficult to quantify. At the soil functional level, the short-term benefits of some types of soil biodiversity may be relatively easily evaluated, for example the gain in nitrogen from introducing N-fixing bacteria, but others are less easily specified, for example the improvements to structure resulting from the introduction of earthworms. While many farmers maintain above-ground biodiversity for a variety of reasons, the linkage between this and soil fertility may or may not be part of the perceived value. The enhancement of soil biodiversity by the retention of crop residues and other organic matter and by limitations in the use of pesticides will also have associated labour costs which are part of the assessment.

Origins of this manual

The manual describes sampling and laboratory assessment methods for the biodiversity of a number of key functional groups of soil biota. The methods were initially assembled and the protocols drafted by a number of scientists affiliated with the Tropical Soil Biology and Fertility Institute of CIAT (TSBF), the EU-funded Macrofauna Network, the NERC (UK)-funded Terrestrial Initiative in Global Environmental Research (TIGER), and in particular, the UNDP-GEF funded Alternatives to Slash and Burn Project (ASB). Under the subsequent CSM-BGBD project (Conservation and Sustainable Management of Below-Ground Biodiversity), methods were progressively refined in workshops held in Wageningen (2002), Sumberjaya (2003), Embu (2004) and finally Manaus (2005). The last of these meetings was also able to draw on the experiences of field sampling in 7 countries participating in the project, as well as the recommendations of taxon-specific workshops held in Cali (on microsymbionts, Cali 2003; on soil fungi, Bangalore 2003; on earthworms, Nairobi 2004 and on termites and ants, Nairobi 2005), and on several in-country workshops held since the inception of the project in 2001.

The objectives and working hypotheses are as follows; the methods were assembled to provide a standardized basis for achieving the first objective.

Objectives and working hypotheses

1) To characterize soil biodiversity occurring in natural forest, current landuses resulting from slash and burn agriculture and the "best-bet" alternative sustainable regimes of land management.

2) To establish the relationship between the above-ground and the belowground biodiversity across current and alternative land-use systems.

3) To identify "entry points" for improved land management through introduction and/or management of soil biota. The "entry points" might include better understanding of indigenous knowledge and more effective utilisation of available technologies.

INTRODUCTION

CHARACTERIZATION OF THE SOIL BIOTA AND METHODOLOGICAL APPROACHES

Key functional groups

The taxonomic diversity of the soil biota is so high that inevitably some selection must be made. The taxonomic groups described below were selected on the basis of their diverse functional significance to soil fertility (hence the term "target taxa"); and their relative ease of sampling (Fig. 1).

1) **Earthworms,** which influence both soil porosity and nutrient relations through channeling, and ingestion of mineral and/or organic matter, and which act as regulators of soil biotic populations at smaller special scales, for example mesofauna, microfauna and microsymbionts.

2) **Termites and ants,** which influence a) soil porosity and texture through tunnelling, soil ingestion and transport, and gallery construction; b) nutrient cycles through transport, shredding and digestion or organic matter.

3) **Other macrofauna** such as woodlice, millipedes and some types of insect larvae which act as litter transformers, with an important shredding action on dead plant tissue, and their predators (centipedes, larger arachnids, some other types of insect).

4) **Mesofuana,** such as collembolans and mites, which act as litter transformers and micropredators (grazers of fungi and bacteria, and predators of other soil animals), thus contributing to smaller-scale organic comminution processes and exerting a strong regulatory role within the soil biota.

5) **Microfauna**, principally nematodes, which a) influence turnover in their roles as root grazers, fungivores, bacterivores, omnivores and predators b) occupy existing small pore spaces in which they are dependent on water films c) usually have very high generic and species richness and d) have a strong role in the regulation of microbial abundance and activity.

6) **Arbuscular mycorrhizas,** which associate with plant roots, improving nutrient availability and reducing attacks by plant pathogens.

7) Legume-nodulating bacteria and, when relevant, other N-fixing microsymbionts which transform atmospheric N_2 into forms available for plant growth.

8) **Phytopathological**, saprophytic and antagonistic fungi, which determine crop viability and the turnover of organic carbon during decomposition.

Sampling design: overall strategy

Under the ASB protocols, based on long-standing TSBF-CIAT approaches, macrofauna, microbiota and soil (for physical and chemical analyses) are sampled in transects, for which the optimum size was suggested as 40 x 4 m.

However, for the quantitative sampling of termites and for a number of aboveground studies (particularly plant functional attributes and C sequestration) in ASB work, quadrats of 40 x 5 m were deployed, and it seemed advisable to standardize both above-ground and below-ground work at 40 x 5 m (Fig. 2). In further amendments to the procedures, pitfall trapping of surface-active invertebrates and a 100 m qualitative transect for termites were added to the sampling. These can take place along one flank of the transect (pitfalls) or in parallel at about 5-10 m distance (termite transect). These modifications were intended, in part, to contribute elements of true biodiversity to the dataset by achieving resolution at the species level, but also to mitigate the variability of data from short transects on groups with typically patchy distributions. Replication of transects in each site was also considered desirable, as it facilitates statistical analysis of the data obtained, though this may not always be practical where time and funding are limited.

Note that in small field plots, highly dissected cropping systems or on difficult terrain, it is not necessary for the transect to be both linear and contiguous. For example, where the greatest linear dimension of a particular land-use is <40 m, two parallel transects of

20 m sample with the same theoretical efficiency as one of 40 m. Similarly, a transect can be bent through angles up to 90o to sample plots of irregular shape or to avoid significant natural features such as streams, steep slopes or rock outcrops. Tree falls should, however, be included in the transect if this is appropriate to its existing line and length, and not bypassed.

In the ASB regime, there was a heavy emphasis on the digging of monoliths (8 recommended, with 5 as a minimum), arranged along the transect. Monoliths are used to address the macrofauna (ants, termites and earthworms), and when used in relatively large numbers (5 or more per location), can generate quantitative data on biomass, as well as diversity. Sampling of microfauna, microsymbionts and other microorganisms was arranged in various appropriate schemes along or around the transect, and pitfall traps were added to widen the range of macrofauna sampled.



country and each is sampled through a number of windows defined via satellite imagery. Within each window a large number of prospective sampling sites (individual locations) are identified by a grid-intersection method. Sites actually sampled are chosen to be representative of the major land-uses found within the benchmark locality, and to provide roughly the same number of samples per land-use. Benchmarks, windows and individual sites provide a scheme of replication for the sampling of each land-use.



Fig. 3. Minimum point sampling scheme for all soil biota. Sampling can be extended by using one or two additional transects for termites, ants and beetles, by casual sampling for termites (1 hour) and by additional monoliths to capture more earthworms.

MACROFAUNA

Procedures follow Anderson and Ingram (1993), making use of pitfall traps together with the digging of soil monoliths of dimensions $25 \times 25 \times 30$ (depth) cm. An additional 20×2 m sampling transect is used for termites. This transect can also be used for ants and beetles, although it is better if the litter quadrats are arranged in a separate parallel line, as shown in Fig. 3.

Sampling procedure

a. Isolate the monolith by cutting down with a spade a few centimetres outside the quadrat and then digging a 20 cm wide and 30 cm deep trench around it. NB, in a variant of the method, all invertebrates longer than 10 cm excavated from the trench are collected; these will be mainly large collectors) and the following microniches are investigated in detail: **surface soil and litter** down to a depth of about

5 cm; deep accumulations of litter and soil between large buttress roots; dead wood in all stages of decay; termite carton or soil runways on tree trunks and other vegetation; subterranean, epigeal and arboreal termite nests and mounds to a height of 2 m above ground level (including purse nests suspended on vegetation). Soil, litter and woody items can be rapidly dissected in trays; this is often helpful in revealing termites in cryptic niches, or when light is poor. Sticks should be broken into pieces and tapped onto the trays with moderate force to displace any termites they contain. Larger items of dead wood should be chopped up, bearing in mind that they may be infested in one part and not another. Rotting wood partly incorporated into the topsoil or covered in soil sheeting will frequently contain termites. Mounds and nests may be inhabited by species other than, or in addition to those building them; it is therefore advisable to check the periphery and the base of the structure, as well as its central chambers.

iv) Representatives of the termites discovered should be preserved in alcohol, wherever possible including soldiers as well as the worker caste. One specimen tube should be used for each population (or apparent colony) encountered. A label should be added into the alcohol on which is noted (NB, in pencil or waterproof ink, not ball-point!):

The information is important in establishing the nature of the termite community (especially functional group diversity) and for constructing a species-accumulation curve. To avoid confusion in termite-rich sites, the labels should be written as soon as the termites have been placed in the specimen tubes. Elsewhere, labels can be written when the searching of each section of the transect is completed. However, the 30 minutes allocated should include labeling time.

v) A short training or orientation period is usually necessary before inexperienced collectors can sample with the same efficiency as experts. Ideally, a training transect, 50 or 100 m should be sampled first, with the guidance of an experienced collector. Collectors should work steadily (rather than frantically) through each 30 minute sampling period and aim to maintain the same level of sampling efficiency in all sections of the transect. For this reason, and to minimize the necessity of having to work in poor light, it is recommended that no more than 12 sections be completed in any one day. It is also helpful to rest for a few minutes between sections. Two experienced collectors can therefore complete a transect comfortably in two days. In most sites there is no need to collect every termite found, and very common species might be passed over after being initially sampled in order to search for rarer or more cryptic forms, or to find soldiers in species that have relatively low soldier/worker ratios (but bearing in mind that some species are soldierless). It is impossible to sample efficiently in heavy rain, so it is permissible to interrupt collection until better conditions return. Work can be divided between the collectors in any mutually agreed way, for example for each to work in the 1 m belt on opposite sides of the line, to divide each section into two subsections each of 2.5 x 2 m, for one to sample wood and mounds, while the other examines soil and tree roots, etc. depending on the nature of the site and the section-to section topography. It is recommended that soil should be dug up in at least a dozen well separated places per section of transect. In transects where few termites are encountered, it is important to observe the sampling protocol exactly, in spite of the small returns, and not to curtail the work.

termites are usually more conspicuous than other feeding types, by reason of the numerous galleries or soil sheets constructed over wood, litter and the surface of the ground, foraging holes opening to the surface from subterranean passages or the formation of uncovered columns of individuals (usually mixtures of foraging workers and soldiers).

Specialized- and incidental-feeders This category follows the listing of termite foods given by Wood (1978), and includes species feeding on fungi, algae, bryophytes and lichens on tree bark (for example *Hospitalitermes hospitalis* in SE Asia; species of *Constrictotermes* and *Ruptitermes* in S. America), dung-feeders and scavengers of vertebrates corpses (probably consumed opportunistically, although dung is arguably a form of decayed litter), and also certain secondary inhabitants of termite mounds which feed on the organic-rich linings of internal chambers as obligate inquilines (for example *Ahamitermes* and *Incolitermes* in Australia: Gay and Calaby, 1970; *Ophiotermes* and *Tuberculitermes* in West/Central Africa: Eggleton and Bignell, 1997).

The categories are not mutually exclusive and many species will take food from at least two sources, especially under unfavourable conditions. Identification of the functional group can be made by reference to abdominal colour in live specimens (soil-feeders and soil/wood interface-feeders are darker), site of discovery (in wood, in soil, etc) and other aspects of biology such as nesting site (arboreal nesters are usually non soil-feeders), absence of soldiers (generally indicates soil-feeders) and taxonomic affiliation (e.g. all Macrotermitinae are non soil-feeders; most Apicotermitinae are soil-feeders or soil/wood interface-feeders). If allocation to functional group is difficult, a useful approximation is to divide the species into "soil-feeders" (soil-feeders and soil/wood interface-feeders as defined above) and "non-soil-feeders" (all the rest). The distribution of species between these categories indicates termite community structure; forest communities are often dominated by soil-feeders, but disturbance or drying-out generally increases the proportions of other functional groups.

Similarly, it is possible to compare termite assemblages on the basis of nesting types by allocating species to the following categories:

Wood nesting Termites whose colony centres are normally within dead logs or standing trees. Sometimes the dead wood is gradually replaced with carton material or fungus comb.

Hypogeal nesting Termites whose colony centres are below the ground. The centres are often poorly defined and amorphous (especially in the soldierless Apicotermitinae), with little obvious internal structure, although some have complex underground nests (e.g. many Macrotermitinae). This group includes many species which are facultative secondary inhabitants of epigeal mounds.

Epigeal mounds Termites whose colony centres are above ground (but excluding arboreal mounds), free-standing or associated with tree buttresses. These mounds are usually well defined and highly complex structures, built to species-typical specifications but with a tendency to become more irregular as they age through erosion, additions and occupation by secondary inhabitants.

separates smaller macrofauna and some mesofauna from the organic material, and the animals can then be colleted from the sheet using a handheld aspirator (pooter). In this case a search limit of 30 mins should be observed per 1m2 of litter sampled. Dried litter from the Winkler bags should be weighed at the conclusion of the extraction.

d. 3 or more pitfall traps are installed at roughly 4m intervals along one flank of the ant transect line, approximately 14 m from the monolith. The traps are put in during the afternoon or early evening and emptied 24 hours later. Each trap contains a little water, with a few drops of detergent added to immobilize specimens by drowning. Glass jars of about 10-15 cm mouth diameter make suitable traps, or use plastic sups as shown in Fig. 5 and Fig. 6. Depth of the traps is not critical, but the mouth must be exactly flush with the surface of the ground. A sloped cover (for example an inverted petri dish, or a piece of plywood or plastic), supported on twigs over the jar, is useful to keep rain out.

Pitfalls will collect a variety of soil animals, including some collembolans. Collembola filters (53 μ m and 38 μ m mesh) can be used to separate these from other fauna.

e. Record the number and fresh weight of all animals and identify to at least the taxonomic and functional levels indicated in Table 2 (but preferably further). The presence and weight of termite fungus combs (if any) should also be noted. If a balance is not available in the field, fresh weight can be approximated for preserved specimens by weighing them after light blotting.

f. Make a list of species, if possible grouped into subfamilies or families. Within each of these, use generic names to generate alphabetical orders. Combine the results from pitfall traps and monoliths to compile this list.



Fig. 5 Demonstration of the pitfall trap.

means for untransformed data, together with the (back-transformed) geometric mean and confidence limits for log (x+1) transformed data. The transformed data can be used for histograms and site-to-site comparisons (Eggleton *et al.*, 1996).

Prepare a summary table, for example:

Table 1: Termite numerical density in 7 sites across a forest disturbance gradient in Jambi province, central Sumatra: (specimen data).

Site	Arithmetical mean, nos m ⁻² (n=5)	Geometric mean, nos m ⁻² (n=5) [*]	95% confidence limits [*]
BS1, Primary forest	2892	971	347-12827
BS3, Logged over	163	65	22-977
BS6,	512	47	5-16445
Paraserianthes			
BS8, Rubber	128	11	2-1046
BS10, Jungle	211	25	2-9772
rubber			
BS12, Alang-alang	3	2	0-20
BS14, Cassava	26	10	2-534
Stratum level (all	Arithmetical	Geometric mean,	95% confidence
5.055 2.157 2.9027	(n=5)	(n=5)*	
	· · ·	· ·	
Litter	46	15	3-64
0-10 cm	106	80	43-148
10-20 cm	55	44	24-78
20-30 cm	49	4	1-50

* back-transformed

Estimate biomass as g m-2 in a similar way. Use fresh weight or the mass of blotted preserved specimen, if possible. Avoid the use of dry weight because of the different oven temperatures used by different scientists and the variable water content of different types of organism. Where insect specimens in a range of sizes are available, an alternative method is to calibrate live biomass against head width in representative specimens covering the whole size range. The weight of unknowns can then be estimated from the curve. For log transformations of data, it is most convenient to work in (mg + 1), then back-transform and express as g.

Keystone species (e.g. termites and arguably some litter transformers) providing physical niche opportunities for lower-level organisms and determining the community structure of those organisms.

The following functional group classification for ants was agreed at the Nairobi Training Workshop on ant and termite taxonomy and ecology:

1. Carnivores: (specialists or generalists) with a subterranean colony centre.

2. Carnivores: (specialists or generalists) with colony centres in the superficial litter layers.

3. Carnivores: (specialists or generalists) with an epigeic or arboreal colony centre.

4. Herbivores: with a subterranean colony centre.

5. Herbivores: with colony centres in the superficial litter layers.

6. Herbivores: with an epigeic or arboreal colony centre.

Minimum datasets are required as follows:

Per point sampling

From all sampling

Species/morphospecies lists for ants, termites earthworms and baited beetles.

Family list for other beetles.

List of other invertebrates, to highest taxonomic resolution possible. For each taxon, please indicate the sampling method employed as:

- C casual
- T transect
- W Winkler
- P pitfalls

M monolith

(from the monolith, don't observe 3 x 10 cm stratification, except for earthworms)

Total abundance as numbers m-², separately for All ants (+ as functional group (FG) 1, FG2, etc.)

All termites (+ as FG1, FG2, etc. or by default as wood-feeders and soilfeeders))

All earthworms (+ as FG1, FG2, etc.) All beetles Other invertebrates

All invertebrates

From the transect

Relative abundance of termites = no. of encounters for FG1, FG2, etc.

total no. of encounters

(make one calculation for each FG, using all transect sections available for the sampling point)

From the Winkler bags

MESOFAUNA

The protocols described here are addressed primarily to mites and collembolans present in the mineral horizons. Mesofauna in the litter can be sampled by other means (see above). The principle is to take small cores or blocks and combine them to make a single bulk sample which is then subsampled for extraction by the Berlese funnel method (Fig. 7).



Fig. 7 Construction of the Berlese funnel.

Primary soil samples of $3.5 \times 3.5 \times 3$ (depth) cm are recommended, and can be dug with a small spade or trowel, or with a corer of approx 4 cm diameter. Bulk the soil in a large (5 kg) plastic bag and mix. Transfer 10 subsamples of about 500 ml into small cloth bags for temporary storage, before transferring to the Berlese funnel.

The funnel system works without lights or heating (Fig. 8, cf. Tullgren funnels), and should be run over a period of several days until the soil is dry. Weigh the soil at the conclusion of the extraction.

Collembolan specimens should be stored in 70% alcohol. However for identification, they should be cleared in Nesbitt's fluid (25ml distilled water, 40g chloral hydrate, 2.5ml of 1N HCl) and mounted in Berlese solution (20ml distilled water, 15g Gum Arabic, 50G chloral hydrate, 5ml glycerine, 5ml glacial acetic acid; harden at 70oC for 7 days). Mites can be cleared and preserved from alcohol in Hoyer's medium (50 ml distilled water, 30g Gum Arabic, 200g chloral hydrate, 16ml glycerine).

rpm for 5 minutes and the supernatant discarded. The residue is then resuspended in sucrose solution (45.6%) and centrifuged at 1000 rpm for 1 min. Nematodes are collected by passing the supernatant through a 37-mm screen and washed into additional Golden fixative, to make a final volume of 15 ml in which the formalin concentration is 3%.

iii) The nematode population is counted by randomly removing 1 ml of solution and determining the mean of 15 fields for each of three aliquots in a counting chamber. The remainder of the specimens are processed to pure glycerine by Seinhorst quick rehydration with ethanol (Hooper, 1970) and permanently mounted on glass slides for identification and photography. 100 nematodes from each transect replication are randomly selected and identified, wherever possible to genus level, and then allocated to trophic (= functional) group. Data should be expressed as the frequency and abundance of each trophic group per unit volume of soil and compared using standard soil nematode diversity indices.

The following functional groups are recognized:

- Bacterivores
- Fungivores
- Plant parasites
- Omnivores
- Predators

iv) Nematode populations can be described by the Maturity Index (MI), a measure based on the ecological characteristics of nematode taxa (Bongers, 1990; Freckman and Ettema, 1993). In the scheme, nematode taxa, except for plant-feeders, are classified on a scale of 1-5, with **colonizers** (short life cycles, high reproductive rates, tolerant to disturbance) = 1, and **persisters** (long life cycles, low colonization ability, few offspring, sensitive to disturbance) = 5. The Index is calculated as the weighted mean of the constituent nematode taxa values:

 $MI = \sum (v_i x f_i),$

Where v_i is the colony-persister (c-p) value assigned to taxon i, and f is the frequency (dominance) of taxon i in the sample. MI is a measure of disturbance, with lower values indicating a more disturbed environment and higher values characteristic of less disturbed sites.

MICROSYMBIONTS: LEGUME-NODULATING BACTERIA

Introduction

Economic and ecological importance of LNB symbiosis

Biological nitrogen fixation is one of the most important process for life maintenance on earth as it contributes with about 70 % of all Nitrogen

General Species	References
Rhizobium	(Frank, 1889)
R. leguminosarum	(Frank, 1879,1889)
Biovars phaseoli, trifolii, viceae	(Jordan, 1984)
R. galegae	(Lindström, 1989)
R. tropici	(Martinez-Romero et al., 1991)
R. etli	(Segovia et al., 1993)
<i>R. giardinii</i> biovars phaseoli, giardinii	(Amarger <i>et al.</i> , 1997)
R. gallicum	(Amarger et al., 1997)
R. hainanense	(Chen <i>et al.</i> , 1997)
R. mongolense	(van Berkun <i>et al.</i> , 1998)
R. huautlense	(Wang <i>et al.</i> , 1998)
R. etli	(Wang <i>et al.</i> , 1999)
R. yanglingense	(Tan <i>et al.</i> , 2001)
R. sullae	(Squartini et al., 2002)
R. indigoferae	(Wei <i>et al.</i> , 2002)
R. loessense	(Wei <i>et al.</i> , 2003)
 Bradyrhizobium	(Jordan, 1984)
B. japonicum	(Jordan, 1984)
B. elkanii	(Kuykendall <i>et al.</i> , 1992)
B. liaoningense	(Xu <i>et al.</i> ,1995)
B. yuanmingense	(Yao <i>et al.</i> , 2002)
B.canariense	(Vinuesa <i>et al.</i> , 2005)
B. spp.	
Azorhizobium	(Dreyfus et al.,1988)
A. caulinodans	(Dreyfus <i>et al.</i> ,1988)
A. johannae	(Moreira et al., 2005)
 Sinorhizobium	(Chen et al., 1988; De Lajudie et al., 1994)
Ensifer (?)	(Casida, 1982, Young, 2003)
S. meliloti	(Dangeard, 1926; Jordan et al., 1984; de Lajudie <i>et al.</i> , 1994)
S. fredii	(Scholla and Elkan, 1984; Chen <i>et al.</i> , 1988; de Lajudie <i>et al.</i> , 1994)
S. xinjiangense	(Chen <i>et al.</i> , 1988)
S. saheli	(de Lajudie et al., 1994)
S. teranga	(de Lajudie et al., 1994)
S. medicae	(Rome et al., 1996)

Table 2 Genera and species of *Leguminosae* nodulating bacteria with

 respective host species mentioned in the original publications.

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Although some host plants are considered highly promiscuous (i.e. show low LNB specificity) no one promiscuous host can be nodulated by all existing LNB species/strains, and conversely, there is no existing LNB strain sufficiently promiscuous to nodulate all legume species. Thus, to evaluate LNB diversity in soil it is desirable to make use of a variety of candidate host plant species, and the more that are employed, the greater will be the variety of LNB strains recognized. The bioassay for LNB can make use of promiscuous hosts (e.g. Odee et al., 1997), grown on field soil samples or inoculated with soil suspensions in the lab (Pereira, 2000) and then compared with species nodulating naturally in the site. Although some of the latter associations may be relatively specific, the comparison of LNB isolated from naturally formed nodules with those sampled via the bioassay provides a useful check on the accuracy of the laboratory procedure. For instance, Macroptilium atropurpureum is one of the widely accepted promiscuous hosts (Vincent, 1970), but in most cases it is reported as predominantly nodulated by Bradyrhizobium species (Woomer et al., 1988), although it can also be nodulated by Rhizobium spp. (Pereira, 2000). Similarly, Lewin et al. (1987) demonstrated that Vigna unguicalata, usually considered a Bradyrhizobium host, in fact has very low specificity and can be nodulated by fast-growing Rhizobium spp. Preliminary experiments are therefore recommended for each soil environment before the final choice of host plants is made for the bioassay.

Nitrogenase is the enzyme responsible for the reduction of nitrogen gas to ammonia. It alternatively reduces, among other substrates, acetylene to ethylene (Dilworth, 1966; Schollhorn & Burris, 1966). This reaction is used as a technique for the measurement of nitrogenase activity. The great advantage of acetylene reduction assay (ARA) is its great sensitivity and speed. It is also not expensive and relatively simple to carry out, even under field conditions. Although the use of ARA for quantitative estimates of N₂ fixation contribution to plant nutrition has been widely criticized (Boddey, 1987; Giller, 1987), it is very useful for the simple detection of N₂-fixers. For instance, as nodule anatomy varies widely in shape and size, the less experienced may confuse them with structures not induced by LNB. It can be also used for confirmation of new LNB symbiosis or other symbiosis forming structures like nodules (Moreira et al., 1992). Nitrogenase activity constitutes valuable information because in many cases it is impossible to verify if nodules are still viable and effective (red colour inside) as for isolation they must be intact. However, it must be considered that nodules without nitrogenase activity can be senescent or ineffective.

Methodology applied in CSM-BGBD project

All the steps of the methodologies applied in CSM-BGBD project are summarized in Fig. 9 (Figures a to f included in Figure 9 for illustration).

Within the BGBD project the conventional method of using promiscuous trap species to isolate LNB from soil samples was employed. However, within the BGBD project using many trap species was not feasible considering the large number of sampling points (around 100) and considering the laborious methodology of using trap species. Therefore, it was agreed that at least one promiscuous trap species would be used by all countries. The chosen species was *Macroptilium atropurpureum* (siratro), due to the small seeds that are easy to manipulate under controlled conditions as plastic pouches and Leonard Jars (with nutrient solution) at growth chambers and greenhouse. An agreement was made that CIAT would provide the seeds

complementary note how to keep nodules for later additional isolations if this analysis shows it is necessary.

5- Include cultural characteristics for screening the whole collection of isolates (i.e. to get clusters) and REP-PCR can be optional or complementary to this. Representatives of clusters {cultural characteristics and (if it is the case) REP-PCR} must be sequenced for 16SrRNA (other than *Bradyrhizobium*) and *dna*K (for *Bradyrhizobium*)

6- See at 4

10- To be considered in a latter phase of the project. However, necessary for isolates authentication.

Trap species can be used to count and trap LNB or only to trap them. As the first case will be too laborious, it was not mandatory as part of the standard methodology, but countries were free to do so if they wish. To trap LNB, trap species can be cultivated in plastic pouches, Leonard jars or pots with soil samples as described in items below.

It was also recommended that LNB should be isolated from legume species (native or introduced) that nodulate naturally at the various land use systems (LUS). Comparison of LNB isolated from field nodules and from nodules induced on trap species by inoculating soil suspensions, will enable both to increase diversity evaluation and to check efficiency of trap species to evaluate diversity.

After growth and appearance of nodules, LNB must be isolated from trap plants. The isolations from these nodules and those from nodules collected from plants nodulating naturally in the various LUS (Land Use Systems) under consideration (field conditions) will constitute the material that will represent the diversity in each LUS. References in the literature recommend at least 30-50 nodules by treatment to be compared. In Brazil we found between 60 and 110 isolates should be necessary (Jesus et al., in press), considering collection curves to assess whether diversity within a location has been fully characterized. Considering these curves were based on groups obtained by cultural characteristics in which, variability of genetic characteristics can occur, probably a greater number of isolates must be necessary by treatment. So, depending on resources availability (human, lab facilities, etc) chosen number must consider these recommendations. In each situation collection curves can reveal the number of isolates needed. If a plateau in diversity has not been reached then additional samples (nodules isolates) should be analyzed. If nodules collected both in the field and from trap species were kept in silica gel (Fig. 10) then they will be available for new isolations.



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rubber ball (of the type used inside footballs), 1 ml gas-tight syringes, 5 ml vacutainers, 10 ml (or larger) vials with rubber stoppers, calcium carbide (CaC2), Gas chromatograph equipped with flame ionization detector (FID) and Poropak RN column for acetylene/ethylene determinations. NB Nitrogenase assays can be performed in the field.

Methods

Soil sampling Small amounts of soil are cored to a depth of 20 cm from the 12 points distributed in each sampling point according (see Figure X for sampling of all groups of organisms). Each set of 12 samples is bulked to form a composite sample of about 300g and placed inside a sterile plastic bag. Alternatively, if resources permit, three or more composite samples can be collected per sampling point. All sampling materials (corers, spatula, hoe etc.) must be flamed before and after sampling at each sampling point to avoid the introduction of exotic LNB. Steps inside the transect should be limited and litter must be removed just before sampling takes place (LNB sampling should therefore be the first priority before other groups of organisms are addressed). Soil samples should then be transferred to the laboratory in an insulated container (preferably at 4°C) as soon as possible. A second bulk sample of about 200g should be collected in a non-sterile plastic bag for soil physical and chemical analysis.

Nodule sampling Leguminous species inside the transect should be identified and botanic material collected. It is helpful if those which are able to nodulate are known in advance, in which case the collection can be confined to these species. However, it must be taken into consideration that a huge potential for the discovery of new nodulating legume species exist. For herbaceous plants, the whole root system can be removed from the soil (using hoe, spade or mattock as required), with care not to accidentally sever existing nodules. Nodules of woody plants must be discovered by excavation of the roots, taking care to explore the finer ramifications where nodulation is more commonly found. Extreme care must be taken in order to be sure that fine roots belong to the individual identified as a legume. Thus, it is recommended that excavation start by the trunk. The nodules are then excised (leaving a piece of root to facilitate manipulation) and stored individually in screw-cap tubes containing desiccant. At least 50 nodules should be collected per sampling point, and be representative of all nodulating species occurring there. Occasionally, nodules may be too large for the ordinary screw-cap tubes and should be stored in a larger container.

Nitrogenase activity Can be measured in the field on individual nodules, just after sampling, or in the laboratory (Fig. 11). The nodule is put in a 10ml (or larger if needed) vial with a rubber stopper. Acetylene is produced in a Kitasato ehrlenmeyer by the reaction of CaC_2 with water (Fig. 11), and 1ml of this gas injected into the nodule containing vial. After 1 hour (or less) 1ml of headspace gas is removed and transferred to a vacutainer for the analysis of ethylene in the laboratory by gas chromatography.

Plant voucher specimens Voucher specimens of nodulating plant species must be collected with careful attention to labeling (see below) and, if possible, the inclusion of flowers and fruits. The specimens should then be sent to a Herbarium for identification, accompanied by an identification card as in the following example:



Fig. 12. Method for calculation of most-probable-number of rhizobia cells in soil by the plant infection technique. Base dilution rates (DR) can vary from 2 to 14.5 and replicate numbers per dilution (N) from 2 to 5. (Adapted from Woomer (1993) and Vincent (1970) by Moreira and Pereira (2001).

LNB isolation and characterization LNB are isolated from nodules collected in the field and from those obtained under laboratory bioassay. In the latter case, nodules obtained at each dilution, can indicate those strains which are rare in the soil sample, as well as the most common ones. The first step is to surface sterilize the nodules by a brief immersion in 95% alcohol, followed a longer immersion up to 3-4 minutes. in HgCl₂ (Na or Ca hypochlorite, or H₂O₂ can be used to substitute) and washing in several rinses of sterile water (Vincent, 1970). The nodule is then crushed in a few drops of sterile water, using forceps, and a loopful of this suspension is streaked onto an agar medium. In the case of desiccated nodules, they should first be soaked in sterile water to improve their wettability by the sterilants. Immersion times in HgCl₂ (or in other disinfectant) should be adjusted to nodule size (shorter for smaller nodules). Composition of the yeast-mannitol-mineral salts agar medium (Fred and Waksman, 1928) (especially pH and carbohydrate source) can be varied to take account of particular soil conditions (Date and Halliday, 1979; 1987; Souza et al., 1984; be used, essentially as recommended by the vendor. DNA is quantitated at A₂₆₀ nm in a diode array spectrophotometer (Hewlett Packard).

16S rDNA sequencing: For isolates representative of clusters (obtained either by cultural characterization, REP-PCR profiles or other technique), near full-length 16SrRNA genes are amplified with prime pair 27F (pA:5`AGAGTTTGATCCTGGCTCAG) and 1492R (5´GGTTACCTTGTTACGACTT) which correspond to positions 8 to 27 and to 1507-1492, respectively, of *Escherichia coli* 16S rDNA gene (Wilson et al., 1990). The final concentrations in the reaction mixtures (100 uL) are: 1X PCRBuffer, 2.5 mM MgCl2, 0.2 mM of each dNTP, 0,2 μM of each primer and 2 unit of Taq polymerase. The PCR program has an initial denaturing step at 94° Cfor 5 min; followed by 30 cycles of denaturing at 94° C for 40 sec, annealingat 55° C for 40 s, and extension at 72° C for 90 sec. The final extension is performed at 72° C for 7 min. Purification of PCR products is performed with Microcon[™] filters (Millipore) or other purification system. Single pass sequencing of PCR amplified rDNAs is performed with the 27F primer.

dnaK sequencing: 634 nt long dnaK fragment is amplified by using primers dnaK1230F (5'-CACCACGATCCCGACCAA-3') and dnaK1846R (5'-GGTGAACTCYGCGTCGAC-3') which correspond to positions 1230to 1247 and 1846 to 1863, respectively, of the Bradyrhizobium japonicum USDA110 dnaK gene. After removing the primers sequence, 595 nt of usable sequence were got. The final concentrations in the reaction mixtures (50 uL) are: 1X PCRBuffer, 1.5 mM MgCl2, 0.2 mM of each dNTP, 400 nM of each primer and 1 unit of Tag polymerase. The PCR program has an initial denaturing step at 94° C for 3 min; followed by 30 cycles of denaturing at 94° C for 1 min, annealingat 62° C for 45 s, and extension at 72° C for 1 min. The final extension is performed at 72° C for 7 min. Cleaning of the PCR products is performed with the High Pure PCR Product Purification Kitfrom Roche and making the final eluting step with 50 uL of the elution buffer provided in the kit. The purified products are sent to sequencing. In a few cases we have obtained a low yield of PCR product or the amplification of an additional non specific product. In the first cases we repeat the PCR reactions lowering the annealing temperature to 59° C. In the second cases the bands corresponding to the unespecific products are very faint and we have obtained good sequences using the purified PCR product from these reactions.

Phylogenetic analysis: Ribosomal RNA or other sequences is aligned in ARB against the RDP database (Release 8.1). Trees were cast first in ARB using both Neighbor Joining and Maximum Likelihood. The sequence alignments were filtered to eliminate all columns that laced data from all taxa and regions of uncertain alignments. The same filtered data set was exported to PAUP and additional trees were cast with bootstrapping to assess the robustness of the inference. Sequences can be compared to GenBank database.

should be mixed together to form a composite sample per sampling point. This sample is used for studying AM fungi.

Methodology for assessing arbuscular mycorrhizal fungi diversity

Background

It is very well documented now that arbuscular mycorrhizal(AM) fungi improve growth of plants that are important in agriculture, horticulture and forestry. Mycorrhizal fungi provide a greater absorptive surface than root hairs and thus help in the absorption of relatively immobile ions in soil such as phosphate, copper, and zinc. In addition, mycorrhizal plants were shown to have greater tolerance to toxic metals, to root pathogens, to drought, to high soil temperature, to saline soils, to adverse soil pH and to transplant shock than non-mycorrhizal plants(Mosse et al., 1981; Bagyaraj, 1990; Bagyaraj and Verma, 1995). In most tropical soils, available phosphorous is very low. Thus mycorrhizae play an important role in plant growth in tropics. Tropical cropping systems are established on areas previously occupied by two main species-rich ecosystems: tropical forests and savanna woodlands, or a degradation stage of either of the two. These ecosystems occur on generally poor soils. The plant species richness of tropical ecosystems is associated with a diversity of symbiotic fungi. The speed at which the natural tropical ecosystems are converted into agriculture or artificial forest plantations is alarming. No converted land reverts to the original situation.

AM fundi occur in the large majority of tropical trees, annual plants and grasses, and in almost all crops. The role of AM in relation to large-scale afforestation has not yet been sufficiently investigated. Studies conducted on agro-forestry trees and important crops have shown that AM increase biomass production (Howler et al., 1987; Bagyaraj, 1992). Increased growth in AM plants is mainly attributed to increased phosphorus uptake. In the past, research on AM in cropping systems has been plant oriented. The major parameter for mycosymboint selection being the amount of growth achieved by inoculated plants over control(Menge et al., 1978; Plenchette et al. 1983). It is well known that AM fungi are not host specific. However, recent studies have led to the concept of host preference. This in turn resulted in selection of efficient AM fungi for inoculating seedlings in the nursery. This method has been successfully used to produce worthwhile and economic growth responses in agronomically important crops like chilli, finger millet, tomatoes and tobacco, horticultural crops like citrus, mango, asters and marigold and forest tree species like Leucaena, Tamarindus indica, Acacia nilotica, Calliandra calothrysus and Casurina equisetifolia(Bagyaraj, 1992).

The conversion of natural forests into industrial forest plantations, subsistence or cash crops brings about changes in which plant species, soil organic matter, soil structure and soil fungi may be affected (Adejuwon and Ekanade, 1987). The site is usually cleared of multispecies, uneven-aged vegetation and normally planted with a single species of one age-class. This constitutes a drastic site disturbance which alters mycorrhizal abundance and species composition in the site. Jasper et al. (1987) observed a drop in spore numbers and a shift in species composition after disturbance of some Australian sites. Similarly, Mason et al. (1992) in a plantation of *Terminalia ivoriensis* in Cameroon, found that the number of spores of AM greatly decreased 3 months after complete clearance. They also noticed a change in species composition.

Procedure

- 1. Wash roots and rinse in several changes of tap water.
- 2. Add 10% KOH at 90°C for 1 hour or autoclave at 120° C for 15 min.
- 3. Decant KOH and rinse with water to remove KOH.
- 4. Acidify roots by adding 1% HCl for 5 min.
- 5. Decant HCI. Do not rinse with water because the specimens must be acidified for proper staining.
- 6. Add 0.05% trypan blue in lacto glycerol and simmer for 10 mins.
- 7. Decant stain and add lacto glycerol.
- 8. Examine under microscope for mycorrhizal colonization.

Note

If roots are highly pigmented, after KOH treatment and subsequent washing with tap water, add alkaline H_2O_2 for 10 to 20 minutes or until roots are bleached (Alkaline H_2O_2 is made by adding 3ml of NH₄OH to 30ml of 10% H_2O_2 and 567ml of tap water. This solution shall be prepared afresh and used).

Determination of the percentage mycorrhizal colonization by gridline intersect method (Giovannetti and Mosse, 1980) (Optional).

The stained root samples are spread out evenly on the inner portion of the petridish lid placed on a circular piece of acetate with gridlines marked on it to form 1.0cm squares. The bottom portion of the plate is then placed inside the top. Instead of petridish 2 square glass plates can also be used with a square piece of acetate marked with gridlines. Vertical and horizontal gridlines are scanned under a stereo- microscope(40X). The total number if root intersections with the grid as well as the number of intersects with colonized roots are to be recorded. The percent mycorrhizal colonization is given by the formula:

Percent AM = <u>Total No of intersections positive for AM colonization</u> X 100

Colonization Total No of intersections between root and the gridline

Extraction and Enumeration of AM spores in soil (Optional):

Soils samples will collected in the same way for all microbes (see nematode methodololy). Extramatricular spores produced by the AM fungi in soil is estimated by wet sieving and decanting method (Gerdemann and Nicolson, 1963). Fifty grams of representative soil samples is suspended in 500 ml water and stirred thoroughly. The suspension is allowed to stand undisturbed for one minute and is then passed through a series of sieves of sizes 1 mm,450, 250, 105, and 45 um arranged one below the other in the same order. More washings with water can be given. The spores on the bottom two sieves are transferred on to a nylon mesh with pore size of 40 um which is then placed in a petriplate and spores counted under a stereozoom microscope.

7. For each of the 5 replicates in each of the four dilutions $(10^{-1}, 10^{-2}, 10^{-3})$ and 10^{-4} , one might obtain a combination of numbers such as 5 5 3 2

This means that all the 5 replicate tubes are positive for AM colonization in dilutions 10^{-1} and 10^{-2} ; three are positive tubes in dilution 10^{-3} and 2 positive tubes in dilution 10^{-4} .

For the calculation of MPN of propagules only three numbers of the given combination are required. The first number (N1) is that corresponding to least concentrated dilution in which all the (or the greatest number of) tubes are positive for AM colonization. The two other numbers (N₂ and N₃) are those corresponding to the next two higher dilutions. In our example it would be the combination

 $N_1 N_2 N_3$ 5 3 2

8. The most probable number of AM propagules can then be calculated using MPN table.(Cochran, 1950 or Fisher and Yates, 1963)

Making use of the table with these values of N_1 , N_2 and N_3 , the value given for the combination 5 3 2 is 1.4. To obtain the MPN of infective propagules of AM fungi in the sample the table value has to be multiplied by the middle dilution i.e. 1.4×10^3 l.P/g soil.

Modification based on the training workshop held at Bangalore

In Indonesia where there is a problem of getting onion seeds any other suitable host, preferably a grass can be used.

NoteThe MPN technique gives a more realistic estimate of the number of infective propagules of AM fungi in soil.

PHYTOPATHOGENIC, SAPROPHYTIC AND ANTAGONISTIC FUNGI

The following methods are illustrative of those employed by two countries (Mexico and Brazil). Differences elsewhere will be noted in future project documents.

Field sampling

Eight soil samples, without litter, are obtained by coring to 20 cm depth. Four cores are obtained from a circle with a radius of 3 m around the grid point (monolith) and a further four from a circle of 6 m radius. The soil was bulked and held at 5oC until isolation of fungi is attempted. Chen, W.X., Li, G.S., Qi, Y.L., Wang, E.T., Yuan, H.L. and Li, J.L. (1991) *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. *International Journal of Systematic Bacteriology* **41**, 275-280.

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