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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*



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

Editors:



Prof. Fátima Maria de Souza Moreira

Universidade Federal de Lavras, C.P 3037, Lavras, MG, Brazil,

CEP 37 200-000

Email: fmoreira@ufla.br

and

Prof. David Edward Bignell*

School of Biological Sciences, Queen Mary, University of London,
London, U.K. E1 4 NS.

E-mail: d.bignell@qmul.ac.uk



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CONTRIBUTORS

General and overview

Mike Swift. IRD Centre de Montpellier, 165 Rue du Perdigal, 34630 Clapiers, France. (Mike.Swift@wanadoo.fr)

General and macrofauna

David Bignell. Institute of Tropical Biology and Conservation, University Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia. Current address: School of Biological Sciences, Queen Mary, University of London, U.K. E1 4NS. (D.Bignell@qmul.ac.uk).

Macrofauna

Suleymane Konaté, University of Abobo-Adjame, B.P. 801 Abidjan, 02 Côte D'Ivoire (skonate2@yahoo.fr)

Jerome Tondoh, UFR SN/CRE, University of Abobo-Adjame, B.P. 801 Abidjan, 02 Côte D'Ivoire (tondoh@yahoo.fr)

Reginaldo Constantino, Departamento de Zoologia, Universidade de Brasilia, 70910-900 Brasilia, DF, Brasil (constant@unb.br)

Ronaldo Zanette, Universidade Federal de Lavras, CP 37, Lavras, MG, Brasil (zanetti@ufla.br)

Anne Akol, Department of Zoology, Makerere University, PO Box 7062, Kampala, Uganda. (aakol@sci.mak.ac.ug)

Julio Louzada, Universidade Federal de Lavras, CP 37, Lavras, MG, Brasil (jlouzada@ufla.br)

Bikram Senapati, Ecology and Environmental Biotechnology Section, School of Life Sciences, Sambalpur University, Jyoti Vihar 768019, Orissa, India. (bikramsenapati@yahoo.com)

Mesofauna

Felix Susilo, Department of Plant Protection, Universitas Lampung, Jalan Sumantri Brojonegoro No 1, Bandar Lampung, 35145 Sumatra, Indonesia. (fxsusilo@telkom.net)

Elizabeth Franklin, INPA, Universidade do Amazonas, CP 478, 69011-970, Manaus-AM, Brasil (beth@inpa.gov.br)

Wellington Morais, INPA, Universidade do Amazonas, CP 478, 69011-970, Manaus-AM, Brasil (morais@inpa.gov.br)

Nematodes

Shiou Ping Huang† and Juvenil Cares. Departamento de Fitopatologia, Universidade de Brasilia, Brasilia, DF CEP 70919-970, Brasil.(cares@unb.br).

Legume nodulating bacteria

Fatima M.S. Moreira and **Eliane G. Pereira**, Departamento de Ciencia do Solo, Universidade Federal de Lavras, CP 37, Lavras, MG, Brasil. (fmoreira@ufla.br).

Esperanza Martinez-Romero, Centro de Ciências Genômicas, UNAM, Av. Universidad s/n, Col. Chamilpa, CP 62210, Ap. 565-A, Cuernavaca, Morelos, México. (emartine@cifn.unam.mx)

David Odee, Kenya Forestry Research Institute, (dodee@africaonline.co.ke)

Arbuscular mycorrhiza

Joseph Bagyaraj, Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK Campus, Bangalore- 560 065, India (djbagyaraj@vsnl.co.in)

Sidney Stürmer, Departamento de Ciências Naturais, Universidade Regionale de Blumenau, C.P. 1507, 89010-971 Blumenau, SC, Brasil (sturmer@furb.br)

Dora Trejo, Universidad Veracruzana, Xalapa, 91000 Veracruz. Mexico (doratrejo59@hotmail.com)

Phytopathogenic, saprophytic and antagonistic fungi

Ludwig Phенning, Laboratório de Citogenética e Biologia Celular, Departamento de Biologia, Caixa Postal 37, CEP 37200-000, Lavras, MG, Brasil. (ludwig@ufla.br)

Sheila Okoth, Department of Botany, University of Nairobi, P.O. Box 30197, Nairobi, Kenya. (dorisokoth@yahoo.com)

Richard Mibey, College of Biological and Physical Sciences, University of Nairobi, P.O. Box 30197, 00100, Nairobi. Kenya (bgbd@kenyaweb.com)

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 decomposers, but the term litter transformers 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 CO₂ or CH₄, 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 soil, 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).

4. Suppression of soil-borne diseases and pests. In natural ecosystems, outbreaks of soil-borne diseases and pests are relatively rare, whereas they are common in agriculture. It is widely assumed that low plant species diversity renders agroecosystems vulnerable to harmful soil organisms by reducing overall antagonisms.

A large number of soil animals, in all size categories, are predators which feed on other animals within the same general size category or are consumers of fungal tissue. Protozoans, nematodes and some mites are **micropredators** ingesting individual microorganisms or microbial metabolites. All these predatory activities will have a regulatory role contributing to population stability.

The soil biota (and hence soils as a whole) are responsive to human-induced disturbance like agricultural practices, deforestation, pollution and global environmental change, with many negative consequences including loss of primary productivity, loss of cleansing potential for wastes and pollutants, disruption of global elemental cycles, and feedbacks on greenhouse gas fluxes and erosion. At the same time, global food supply depends on intensive agriculture. As intensification proceeds, above-ground biodiversity is reduced, one consequence of which is that the biological regulation of soil processes is altered and often substituted by the use of mechanical tillage, chemical fertilizers and pesticides. This is assumed to reduce below-ground diversity as well, which if accompanied by the extinction of species may cause losses of function and reduce the ability of agricultural systems to withstand unexpected periods of stress, bringing about undesirable effects. Scientists have begun to quantify the causal relationship between i) the composition, diversity and abundance of soil organisms ii) sustained soil fertility and iii) environmental effects such as greenhouse gas emission and soil carbon sequestration.

Large numbers of farmers in the tropics have limited access to inputs but are nonetheless forced by circumstances to drastically reduce the complexity of their agroecosystems in an attempt to intensify production. An alternative solution is to intensify while at the same time retaining a greater degree of above-ground diversity. The maintenance of diversity of crops and other plants in cropping systems is widely accepted as a management practice which buffers farmers against short-term risk. Enhanced biodiversity and complexity above-ground contributes to the re-establishment or protection of the multiplicity of organisms below-ground able to carry out essential biological functions. This can be considered at both the field and the landscape level to enhance structural complexity and functional diversity, especially in degraded lands.

It is as yet an unresolved question what relationship exists between species diversity, functional diversity (the number of functional groups), functional composition (the nature of the functional groups) and the occurrence and intensity of ecological processes. More precisely, what is the minimum number of functional groups, and species within functional groups, to ensure soil resilience against natural and anthropogenic stress? Circumstantial evidence and intuition suggest that stress and disturbance affecting functional groups that are composed of relatively few species are the most likely to cause loss or reduction of ecosystem services. To the best of our knowledge this holds for shredders of organic matter, nitrifying and denitrifying bacteria, bacteria involved with C1 compound and hydrogen transformations, iron and sulphur chemolithotrophs, mycorrhizal fungi and bioturbators.

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 slash-and-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 mitigation 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 land-uses 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 below-ground 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.

The objectives were developed to test the following hypotheses:

- Land-use intensification results in a reduction of soil biodiversity leading to a loss of ecosystem services detrimental to sustained productivity.
- Above-ground and below-ground biodiversity are interdependent across scales of resolution from individual plant communities to the landscape.
- Agricultural diversification (at several scales) promotes soil biodiversity and enhances sustained productivity.
- Sustainable agricultural production in tropical forest margins is significantly improved by enhancement of soil biodiversity.

The data sought

After a number of actual field campaigns under previous projects, it is possible to give a more specific idea of the information required from sampling each land-use:

- What are the following biodiversity parameters:
 - taxonomic richness at species and strain (microsymbiont) level
 - abundance and biomass of taxa
 - abundance and biomass of functional groups (FGs)
 - relative proportions of FGs
 - overall quantitative indices of biodiversity and evenness
- How is the land-use defined in terms of:
 - apparent cropping or fallow usage
 - basic physical and chemical soil properties; slope and aspect
 - above-ground vegetation character
 - climatic averages and actual rainfall to sampling date
 - precise history of use and management since undisturbed forest
- Compared with other land-use systems in the regional/local chronosequence, and measured against the best available natural forest:
 - what taxa and/or functional groups are particularly affected?
 - what trends do they show in relation to land-use type?
 - are there trends in the data related to factors other than land use?
 - what is the significance of the changes for soil fertility and other ecosystem services?
 - how will crop productivity be affected in this or future land-uses?

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 spatial 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 of 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) **Mesofauna**, 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 N₂ 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.

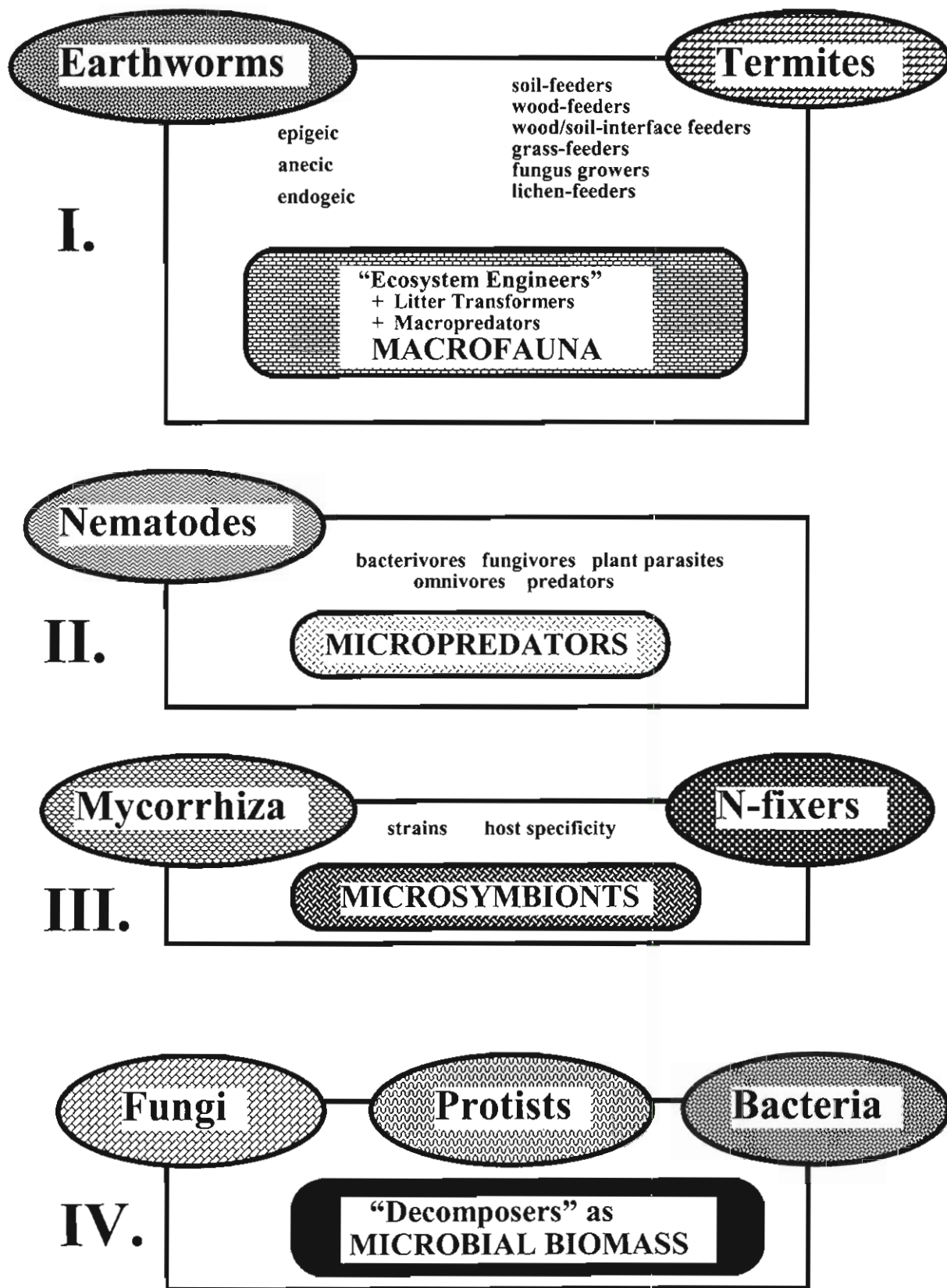


Fig. 1. Main “functional groups” (capitals), subsidiary functional groups and target taxa (ovals) sampled in the ASB project.

However, for the quantitative sampling of termites and for a number of above-ground 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 90° 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.

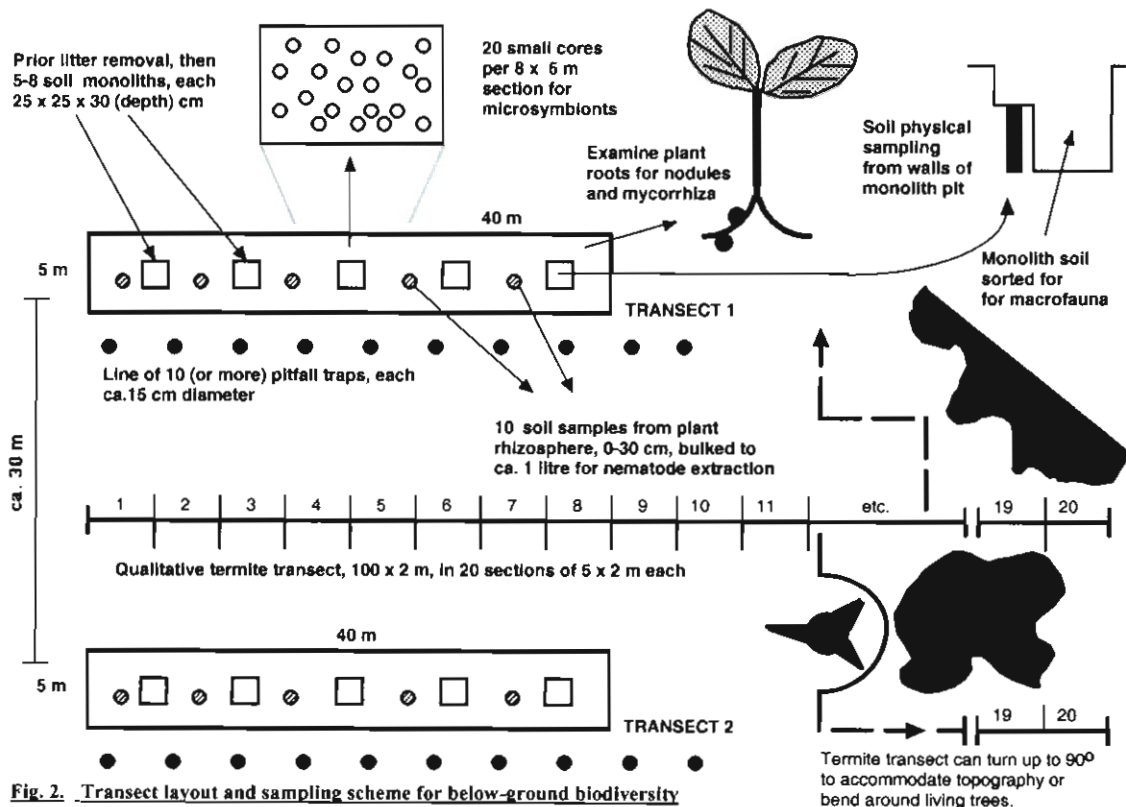


Fig. 2. Transect layout and sampling scheme for below-ground biodiversity

Under CSM-BGBD, emphasis on monoliths is reduced, but the principle of the transect is retained, though modified. The basic approach is as follows (and see Fig. 3):

Macrofauna A single monolith, a set of pitfall traps and at least one transect of 20 m is deployed in each location sampled. The transect sampling is extended to include ants and soil beetles, in addition to termites. Additional monoliths can be dug to improve the sampling of earthworms, and a simple regime of casual sampling for termites is also introduced.

Mesofauna About 12 soil cores arranged in two concentric circles around a single monolith, at 3 and 6 m (radius), respectively, from the monolith.

Microfauna Sampled similarly by about 12 soil cores arranged in two concentric circles around a single monolith, at 3 and 6 m (radius), respectively, from the monolith.

Microsymbionts and soil fungi Sampled similarly by about 12 soil cores arranged in two concentric circles around a single monolith, at 3 and 6 m (radius), respectively, from the monolith.

Soil physics and chemistry 4 soil cores arranged in two concentric circles around a single monolith, 6 m (radius), respectively, from the monolith (analytical methods described in other documentation).

Land-use selection and characterization

Soil biota are expected to vary with land-use and their diversity to broadly diminish along the chronosequence represented by undisturbed forest, logged-over forest, recently cleared and burned forest, cropping systems, derived pastures and recently established fallow. In any locality, therefore, baseline sampling must be carried out in whichever land-use can be identified as the most natural (undisturbed) control site available, preferably closed-canopy forest. However, full site characteristics and classification (and therefore accurate site description) cannot be obtained from apparent land-use alone. Concurrent or prior sampling must therefore be carried out for a suite of basic physical and chemical soil properties, including bulk density, texture (S/S/C ratios), pF, pH, total C, total N, exchangeable cations, available P, CEC, Al³⁺ and H⁺. It is suggested that soil cores taken for these analyses should be from completely undisturbed ground but immediately adjacent to each monolith trench (the outer trench wall is probably the best place), thus providing the opportunity for correlating soil properties with the presence/absence of particular taxa and functional groups. A precise site history is also desirable (though not always obtainable), together with GPS coordinates, altitude, slope, aspect, annual rainfall, mean temperature and humidity, rainy days, length of dry season, and cumulative seasonal rainfall up to the sampling date. Description of sites can be completed by the above-ground vegetation character. Features such as mean canopy height, crown cover percent, basal area, dominant cover/abundance scores for ground flora, litter accumulation and abundance, plant species and generic richness may assist in arranging sites along botanical diversity gradients which have some relationship to their actual positions in the chronosequences and disturbance intensifications.

Site (location) selection is described in other project documentation. The basic principle is that one or more benchmark localities are selected in each

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.

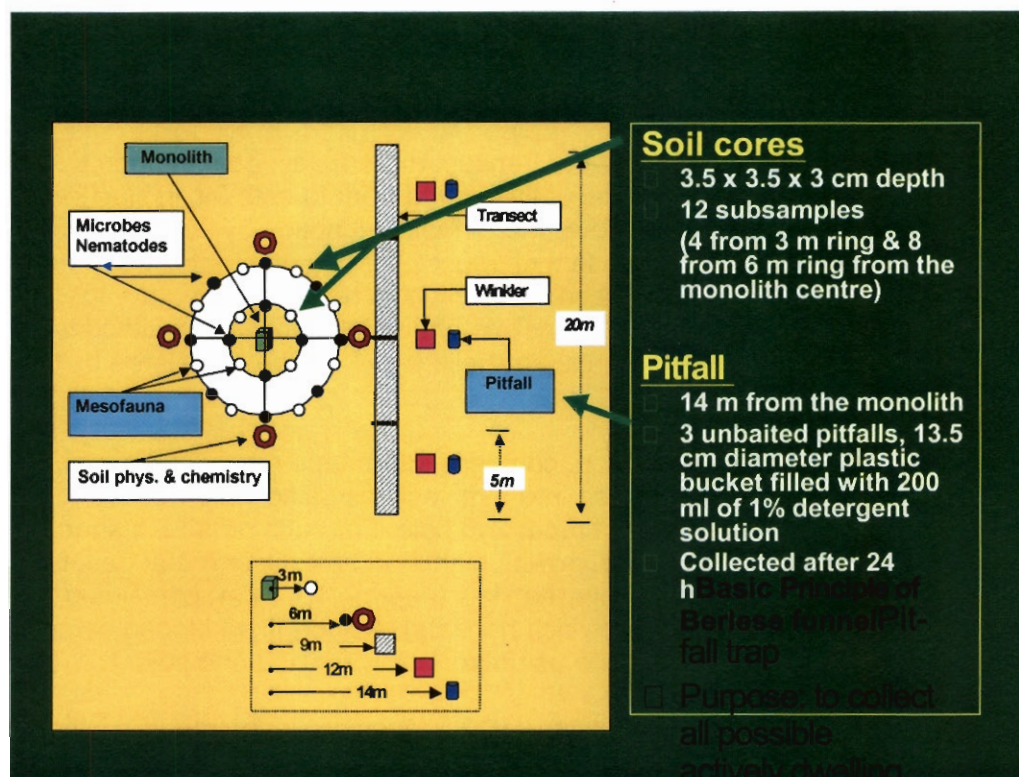


Fig. 3. Minimum point sampling scheme for all soil biota. Sampling can be extended by using one or two additional transects for termites, ants and beetles, by casual sampling for termites (1 hour) and by additional monoliths to capture more earthworms.

MACROFAUNA

Procedures follow Anderson and Ingram (1993), making use of pitfall traps together with the digging of soil monoliths of dimensions 25 x 25 x 30 (depth) cm. An additional 20 x 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

millipedes and earthworms with very low population densities but representing an important biomass. Their abundance and biomass can be calculated on the basis of 0.42m^2 samples, i.e. the width of the block plus two trench widths, squared. Divide the delimited monolith block into three layers, 0-10 cm, 10-20 cm and 20-30 cm. This can be done conveniently using a machette or parang held horizontally and grasped at both ends. Hand-sort each layer separately. If time is short or the light poor (sorting in closed canopy forest is usually difficult as light fades in the late afternoon), bag the soil and remove to a laboratory. Ants can be extracted by gently brushing small (handful) quantities of soil through a coarse (5 mm) sieve into a tray: the sieve retains the ants. Bagged soil should be kept out of direct sunlight and sorted within 24 hours (but preferably sooner).

b. Mark and sample the transect for termites. This method was developed for forests or sites recently derived from forests (as in slash-and-burn agriculture) and follows the formal description by Jones and Eggleton (2000), using a transect of 100 x 2 m. The data obtained are qualitative, but are considered to have relatively high resolution, and are therefore treated separately from the monoliths and pitfall traps. The method is low-impact, is suitable for non-termite specialists and can be completed by two people in two or three days.

i) Equipment required: compass, 30 m tape measure, or (preferably) nylon rope or string marked into 5 m sections using yellow or orange fluorescent ribbon or waterproof tape, 2 m pole. For each person, a sharpened machette or parang, trowel, high-sided plastic or metal tray, two pairs of forceps, ca. 40 stoppered vials (approx. 1 x 5 cm) containing 80% alcohol (more vials in termite-rich habitats). A short fixed-bladed camping knife (ca. 8-10 cm) may also be useful for probing wood and soil.

ii) Lay out a transect line of 20 m adjacent to the monolith, though at sufficient distance to avoid any mutual disturbance. The transect should be placed to run through visually homogeneous surroundings, avoiding such features as large streams, cliff edges or fresh skid trails which are not suitable habitats for termites. However, the transect can incorporate other natural features of the biotic environment which contribute to its physical heterogeneity, such as hill slopes, narrow stream gullies or small canopy gaps. Some subjective judgement is often necessary to decide on the most suitable line, especially where the treatment plots concerned are small. Transect lines do not need to be absolutely linear, and can be turned through angles of up to 90° to avoid natural obstacles, as long as they do not re-intersect with themselves. In small plots a transect can be turned, successively, through two 90° angles to run back towards the starting point, but the two main "arms" of the transect should be at least 15 m distant. Alternatively, two 10 m transects can be run in parallel. If the line runs through the middle of a tree with a very large basal area, the transect can be bent to one side at that point, as long as at least a part of the rooting system falls within the 2 m wide sampling belt. Make a note of the starting point, initial compass bearing of the transect line and any major directional changes. If two transects are employed for replicated monolith and other sampling, the termite transect should run between them (as in Fig. 2), but space all three transects so as to avoid mutual interference and excessive trampling. Keep pitfall lines undisturbed at all times.

iii) The 5 x 2 m sections are sampled sequentially, each for a total of one person hour (30 minutes per section for a team of two experienced

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.

vi) It is helpful if specimen tubes are "cleaned" (i.e. the termites separated from soil and other debris, fresh alcohol added and new neat labels written) on the same day as the collection takes place, or as soon as possible afterwards. This speeds subsequent processing of the material by expert taxonomists, and is another reason why a minimum of two days should be allowed for each transect attempted. The termites should be separated into recognizable taxonomic units, either morphospecies or named species. By treating each 5 m section as an independent sample, a species accumulation curve can be constructed. Ten random sequences of sections are generated by drawing 20 sections at random without replacement. The number of species found in each section is then used to calculate the cumulative number of species for each selection of the ten sequences. Finally, the mean cumulative number of species of the ten sets of 20 sections is calculated for each section and a species accumulation curve drawn. The curve should be asymptotic (i.e. should rise, then flatten out) indicating that few new species would be found by additional sampling in the area. The actual species richness of the site can be obtained by using the first-order jack-knife estimator (Palmer, 1991). The numbers of species and morphospecies encountered per transect can range from just a few to more than 50, depending on the site and the biogeographical region.

vii) Although the basic information generated is **species richness**, other information can be obtained from the same transect. Most important is **functional group diversity**; this can be obtained by noting the numbers of species and morphospecies in the following trophic categories:

Soil-feeders Termites distributed in the soil profile, the organic litter layer and/or epigeal mounds, feeding deliberately on mineral soil, apparently with some degree of selection of silt and clay fractions. Although the ingested material is highly heterogeneous, there are higher proportions of soil organic matter and silica, and lower proportions of recognizable plant tissue than in other groups (Sleaford *et al.*, 1996).

Soil/wood interface-feeders Termites feeding in highly decayed wood which has become friable and soil-like, or predominantly within soil under logs or soil plastered on the surface of rotting logs or mixed with rotting leaves in stilt-root complexes. This group is synonymous with "intermediate feeders", *Sensu* de Souza and Brown (1994), but not the same as the category "rotten wood-feeders" recognized by Collins (1989).

Wood-feeders Termites feeding on wood and excavating galleries in larger items of woody litter, which may become colony centres. This group also includes termites having arboreal, subterranean or epigeal nests but feeding elsewhere, and many Macrotermitinae cultivating fungus gardens. "Wood" includes dead branches still attached to living trees and dead standing trees as well as fallen larger items which are fresh, or in all except the terminal stages of decay.

Litter-foragers Termites that forage for leaf litter, live or dry standing grass stems and small woody items, usually cutting the material before consumption or carrying it to the nest system. This includes some subterranean and mound-building Macrotermitinae, as well as certain Nasutitermitinae that forage on the surface of the ground, and at least one lower termite, *Hodotermes mossambicus*, with a similar habit. Foraging

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 inquiline (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.

Arboreal mounds Nests attached to trees at various heights, usually made of carton. In most cases the nests are connected to the ground by covered runways, which may assist in distinguishing some arboreal termite nests from those of ants.

Some indication of **relative abundance** may be obtained from the frequency of encounter of individual species (number of sections where they were found, out of 20). Also in the sense that the whole transect represents a single large sample, it is arguably possible to assign dominance and therefore to derive indices of species diversity and equitability. It is also possible that the overall number of encounters (i.e. the number of times separate colonies are discovered in the transect as a whole) may be a useful population parameter. For discussion of full range of information obtainable from transects, see the discussions in Eggleton and Bignell (1995), Eggleton *et al.* (1997) and Jones and Eggleton (2000).

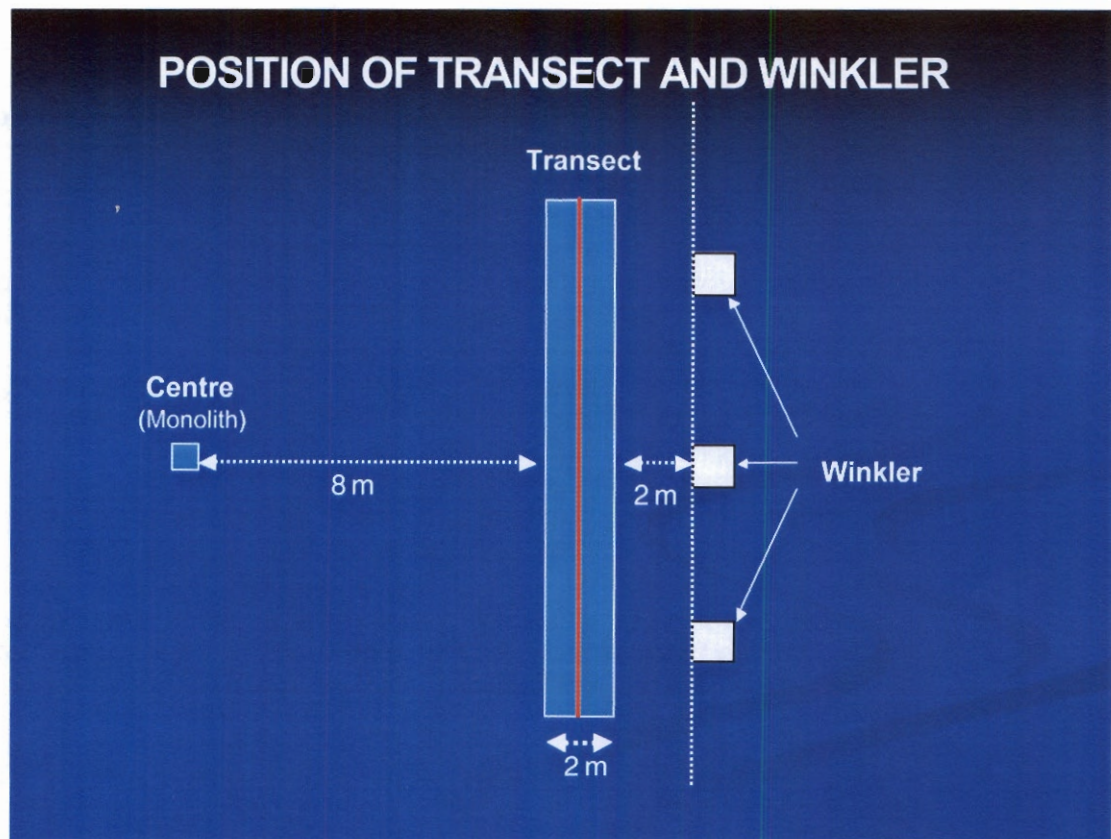


Fig. 4 Positioning of transects for sampling termites, ants and beetles.

c. Mark and sample a transect for ants and beetles (Fig. 4). Litter is collected from each of at least three 1 m² quadrats. Each set of litter is sieved to remove coarse items and enclosed within a single Winkler bag, which is then hung up to dry under cover in a well-aired place. Drying time is 72 hours under most conditions. Ants and beetles migrate from the litter as it dries out and are collected in 70% alcohol at the bottom of the bag. As an addition, or an alternative, if Winklers are not available, coarsely sieved litter can be brushed through a 2mm cloth mesh onto a white cloth sheet. This

separates smaller macrofauna and some mesofauna from the organic material, and the animals can then be collected from the sheet using a hand-held 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 μm and 38 μm 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.

Pit-fall trap



- Purpose: to collect all possible actively dwelling meso- (and macro-) faunas on the soil surface within certain period (24 h or ?)
- Requirements: 13.5 cm x 10 cm plastic buckets, small spade, 1% of detergent solution, plastic cover, bamboo sticks.
- Three unbaited pit-fall traps are set at 2 m from the Winkler samples or 14 m from the monolith centre. Five (x 3) per land use.

Fig. 5 Demonstration of the pitfall trap.

Recording and expressing the data.

i) Fully identified species should be listed with the full binomial and descriptive authority:

e.g. *Dorylus laevigatus* Smith

Morphospecies should be listed by letter:

e.g. *Crematogaster* sp. A
Crematogaster sp. B
.....etc.

Species identified only to genus should be listed without numbers:

e.g. *Colobopsis* sp.

Incorporate the species list into a table showing the sites where each occurred.

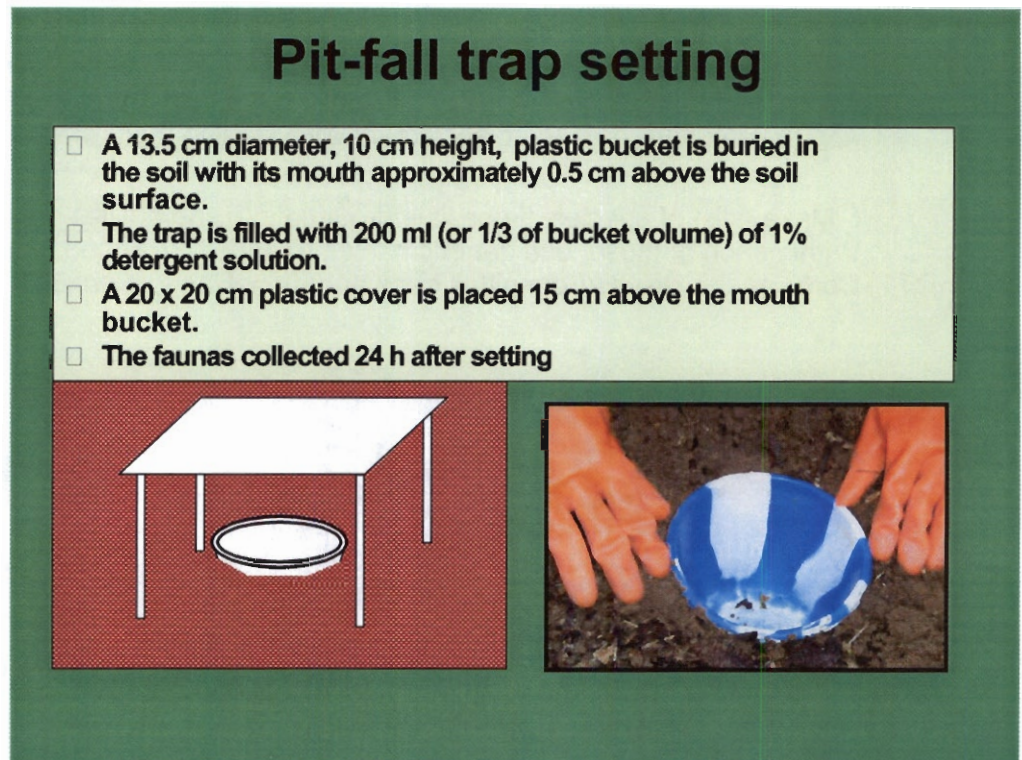


Fig. 6 Construction and protection of the pitfall trap.

Estimate abundance as nos m^{-2} , from each monolith (multiply the raw number per monolith by 16 (except earthworms and millipedes, see above), combining data for all species, including individual taxa. Calculate an arithmetical mean. To estimate the 95% confidence limits the primary data should be transformed as $\log_{10}(x+1)$. If there are not too many zeros (samples with no animals), this should roughly normalize the data and produce homogeneous variances from group to group. In difficult cases a loglog transformation can be tried. Apply descriptive statistics to the transformed dataset, including 95% confidence limits, and back transform to obtain a geometric mean. Quote

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, <i>Paraserianthes</i>	512	47	5-16445
BS8, Rubber	128	11	2-1046
BS10, Jungle rubber	211	25	2-9772
BS12, Alang-alang	3	2	0-20
BS14, Cassava	26	10	2-534

Stratum level (all sites averaged)	Arithmetical mean, nos m ⁻² (n=5)	Geometric mean, nos m ⁻² (n=5) [*]	95% confidence limits [*]
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.

Prepare a summary table, as above.

Show species/morphospecies richness, abundance and biomass graphically

Analysis

The following steps should be followed:

Functional group analysis

Soil invertebrates can be classified according to their feeding habits and distribution in the soil profile as follows:

Epigeic species, which live and feed on the soil surface. These invertebrates effect litter comminution and nutrient release, but do not actively redistribute plant materials (though the comminuted material may be more easily transported by wind or water than the material from which it was derived). Mainly a variety of arthropods, for example ants, beetles, cockroaches, centipedes, millipedes, woodlice, orthopterans, together with gastropods and small, entirely pigmented earthworms. Surface-active macrofauna will include those organisms sampled by pitfall traps.

Anecic species, which remove litter from the soil surface through their feeding activities. Considerable amounts of soil, mineral elements and organic matter may be redistributed through these activities, accompanied by physical effects on soil structure and hydraulic properties. Earthworms and non soil-feeding termites are the main groups in this category, but also some arachnids.

Endogeic species, which live in the soil and feed on organic matter and dead roots, also ingesting large quantities of mineral material. The two main groups are earthworms and soil-feeding termites.

Assemblages can be compared by the relative proportions of species or recognizable taxonomic units, which can be allocated to one (or more) of the functional categories.

It is recommended that an additional functional group classification can be attempted for macrofauna, using the following categories:

Ecosystem engineers (usually ants, termites, anecic and endogeic earthworms). Large invertebrates (> 1 cm, but sometimes smaller) ingesting a mixture of organic material and mineral debris, forming stable, long-lived faeces which are organo-mineral complexes.

Litter transformers (usually non-social arthropods, epigeic earthworms and molluscs) ingesting a mixture of organic matter and microbial biomass and forming short-lived holorganic faeces. This group includes large sized (> 1 cm) and medium-sized (0.2 - 1 cm) invertebrates, but some smaller animals (e.g. many mites) may have analogous functions.

Macropredators (predatory species > 0.2 cm, usually from various arthropod groups).

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 soil-feeders))

All earthworms (+ as FG1, FG2, etc.)

All beetles

Other invertebrates

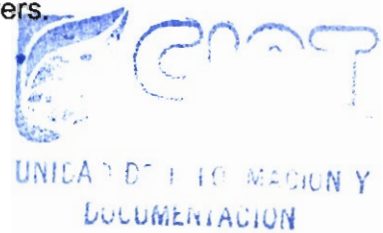
All invertebrates

From the transect

Relative abundance of termites = $\frac{\text{no. of encounters for FG1, FG2, etc.}}{\text{total no. of encounters}}$

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

From the Winkler bags



Total abundance of ants as nos. m^{-2} (+ as FG1, FG2, etc.)
Total abundance of beetles as nos. m^{-2}
(average 3-4 bags)

From the pitfall traps

Itemise taxa not sampled by other means (only).

Per Land Use Sampling (provide land Use intensification score)

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 monoliths

Mean abundance as a) nos. $m^{-2} \pm SD$, b) as geometric mean $\pm 95\%$ confidence interval and c) as arithmetic mean $\pm 95\%$ confidence interval, where n = no. of points per LUS, separately for:

- All ants (+ as functional group (FG) 1, FG2, etc.)
- All termites (+ as FG1, FG2, etc. or by default as wood-feeders and soil-feeders))
- All earthworms (+ as FG1, FG2, etc.)
- All beetles
- Other invertebrates
- All invertebrates

From the transects

Mean relative abundance of termite FG1, FG2, etc. as $\% \pm SD$, where n = no. of points per LUS.

From the Winklers

Mean abundance of ants (+ as FG1, FG2, etc.) as nos. $m^{-2} \pm SD$, where n = no. of points per landuse.

Mean abundance of beetles as nos. $m^{-2} \pm SD$.

Per Window Sampling

From all sampling methods

Species turnover along the gradient as Whittaker's β , i.e. β -diversity

3.2 For all mean total abundances and mean values for relative abundances

Correlates with individual gradient parameters, including LUS intensification scores.

Date and transect identification number

The section number along the transect where the specimen occurred (1,2,3, etc. 20) should be included.

The microhabitat concerned (new dead wood, rotten wood, mound, soil, litter, soil at tree root etc.) should also be included.

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).

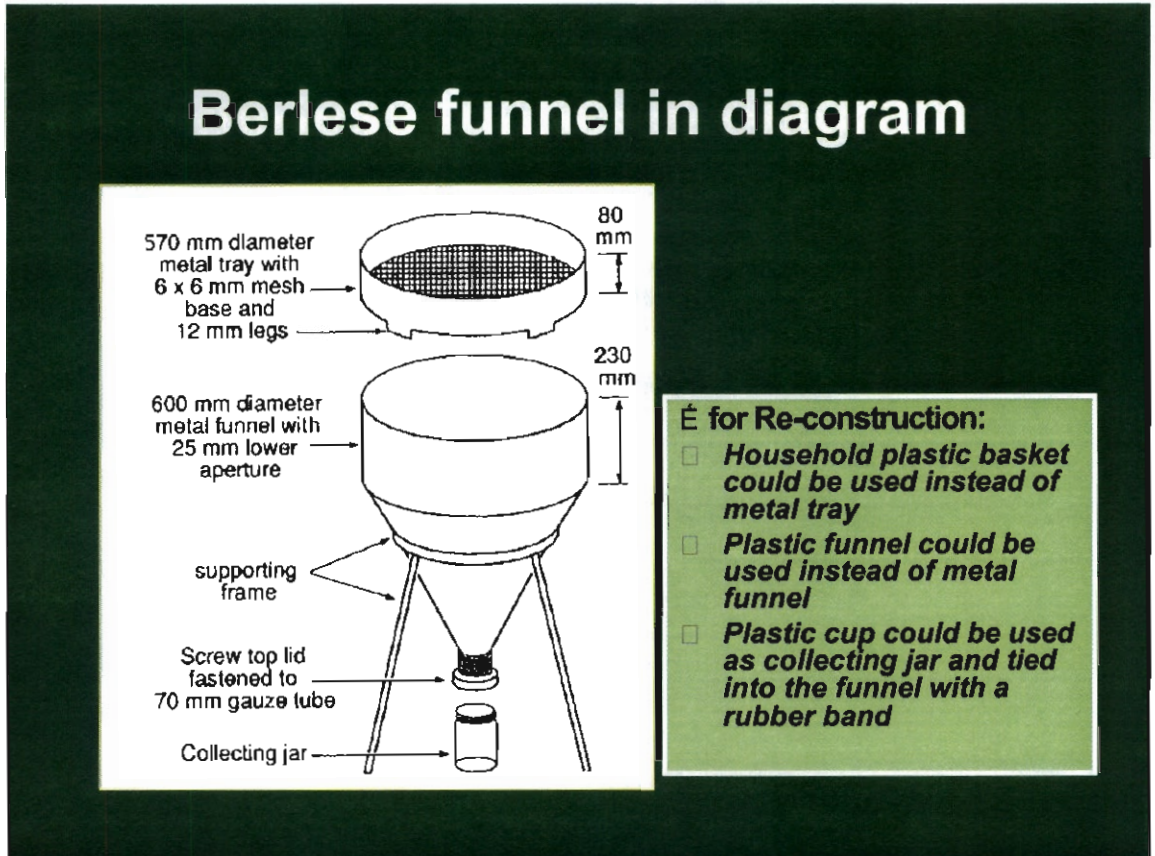


Fig. 7 Construction of the Berlese funnel.

Primary soil samples of 3.5 x 3.5 x 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).

Basic Principle of Berlese funnel

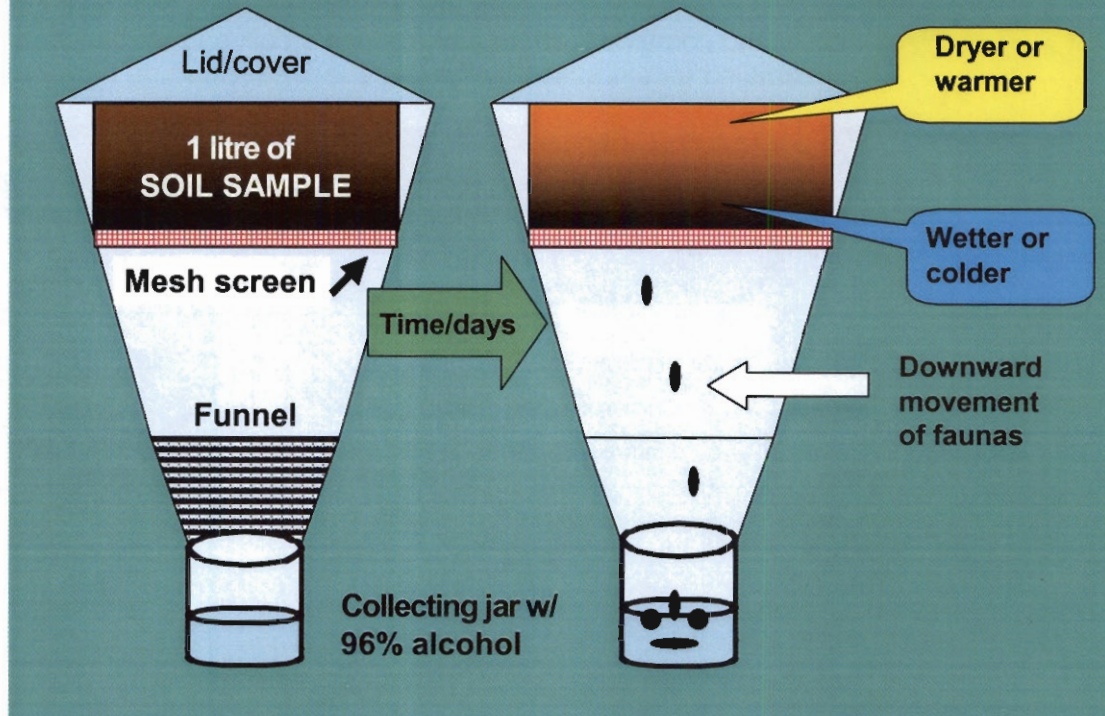


Fig. 8 Principle of the Berlese funnel.

NEMATODES

i) From each site, collect 12 samples in concentric circles as specified above. To make the samples, two parallel zig-zag lines are marked along the transect, with 10 evenly spaced sampling points on each line. For each sample, two cores are taken to 30 cm depth at opposite points on the zig-zag line, using a carbon steel tube, then combined to make a pool of at least 500 g of soil. Samples must be bagged and sealed, to avoid desiccation, but kept out of direct sunlight. Samples can be temporarily stored in an insulated box for transportation to the laboratory, and then at 4°C until extraction, which should be performed as soon as possible.

ii) Nematodes are extracted quantitatively by combining the flotation and sieving methods of Flegg and Hooper (1970). 300 g of soil from each sample is added to 2 litres of water and shaken for 30 seconds. Soil particles are allowed to settle for 2 min., then the suspension is passed through 50-mesh (297 μ m) and 400-mesh (37 μ m) screens. After extraction on the second screen, the specimens are killed by gentle heating in water at 60°C and preserved with Golden fixative (8:2:90 mixture of formalin, glycerin and water) at room temperature. Nematode suspensions are then further clarified by a modification of the centrifugation-sugar flotation method of Jenkins (1964). The suspension is centrifuged at 3500

rpm for 5 minutes and the supernatant discarded. The residue is then re-suspended 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:

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

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

required by natural and agricultural ecosystems (Burns & Hardy, 1975) in an environmentally sound way. Replacement of N-chemical fertilizers by inoculation with LNB strains, selected for high efficiency and for adaptation to environment conditions, is currently applied in some countries for a few legume species. In Brazil, inoculation with *Bradyrhizobium* selected strains totally replaces chemical fertilizers for soybean. The Brazilian yield of 57 million ton in 2004 represented savings of about US\$ 2 billion due to the employment of this biotechnology.

Current taxonomy of LNB

The nitrogen-fixing bacteria, formerly known collectively as rhizobia, form nodules on roots (and exceptionally on stems) of some species of Leguminosae and on roots of *Parasponia* spp. The term "rhizobia" is derived from Rhizobiaceae Conn 1938 the first family to comprise the bacteria able to nodulate Leguminosae. One of the greatest breakthroughs regarding Leguminosae nodulating bacteria (LNB) taxonomy was the discovery that bacteria belonging to the β -Proteobacteria (genera *Burkholderia* and *Ralstonia*) and to other families in the α -Proteobacteria (genera *Methylobacterium*, *Blastobacter* and *Devosia*) were also able to nodulate Leguminosae (Moulin et al., 2001; Chen et al., 2001; Sy et al., 2001; Jourand et al., 2004; van Berkun and Eardly, 2002; Rivas et al., 2002, 2003). That is why the name "rhizobia" is no longer a suitable name to denote this group of bacteria, although some authors still use this name.

Leguminosae comprise about 18,000 species distributed in the sub-families Caesalpinoideae (1,929 spp., mainly woody tropical plants), Mimosoideae (2,727 spp., mainly woody tropical, sub-tropical and temperate) and Papilionoideae (13,000 spp., largely herbaceous, see Sutherland and Sprent, 1993). The extent of the symbiosis with LNB among Leguminosae is variable and still the subject of active research. Until 1989, only 57% of genera and 20% of species had been examined for nodulation, and the proportions of species able to nodulate were 23%, 90% and 97% among Caesalpinoideae, Mimosoideae and Papilionoideae, respectively (Faria et al., 1989). Although extensive searches for new nodulating genera and species have been made, especially in Brazil (Faria et al., 1989; Moreira et al., 1992), it is thus still possible that many new symbioses will be identified in natural ecosystems (e.g. tropical forests). Traditionally, the taxonomy of LNB was based on strains isolated from temperate crop plants. However, now that isolates from other species and regions are available, and new techniques of molecular genetics have been developed, the taxonomy has been revolutionized, with 44 species added to the original four listed in 1984 by Jordan (Table 2) and further revisions are expected.

Evaluation of LNB diversity in soil

To isolate and enumerate LNB from a diverse microbial population, such as that occurring in the soil, a method is required which clearly separates LNB from other

Table 2 Genera and species of *Leguminosae* nodulating bacteria with respective host species mentioned in the original publications.

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

General/ Species	References
<i>S. arboris</i>	(Nick <i>et al.</i> , 1999)
<i>S. kostiense</i>	(Nick <i>et al.</i> , 1999)
<i>S. adhaerens</i>	(Willems <i>et al.</i> , 2003)
<i>S. morelense</i>	(Wang <i>et al.</i> , 2002)
<i>S. kummerowiae</i>	(Wei <i>et al.</i> , 2002)
<i>S. americanum</i>	(Toledo <i>et al.</i> , 2003)
<i>Mesorhizobium</i>	(Jarvis <i>et al.</i> , 1997)
<i>M. loti</i>	(Jordan <i>et al.</i> , 1984; Jarvis <i>et al.</i> , 1982; Jarvis <i>et al.</i> , 1997)
<i>M. huakuii</i>	(Chen <i>et al.</i> , 1991; Jarvis <i>et al.</i> , 1997)
<i>M. ciceri</i>	(Nour <i>et al.</i> , 1994; Jarvis <i>et al.</i> , 1997)
<i>M. tianshanense</i>	(Chen <i>et al.</i> , 1995; Jarvis <i>et al.</i> , 1997)
<i>M. mediterraneum</i>	(Nour <i>et al.</i> , 1995; Jarvis <i>et al.</i> , 1997)
<i>M. plurifarium</i>	(DeLajudie <i>et al.</i> , 1998)
<i>M. amorphae</i>	(Wang <i>et al.</i> , 1999)
<i>M. chacoense</i>	(Velazquez <i>et al.</i> , 2001)
<i>M. temperatum</i>	(Gao <i>et al.</i> , 2004)
<i>M. spetentrionale</i>	(Gao <i>et al.</i> , 2004)
<i>Allorhizobium</i>	(de Lajudie <i>et al.</i> , 1998)
<i>A. undicola</i>	(de Lajudie <i>et al.</i> , 1998)
<i>Methylobacterium</i>	
<i>M. nodulans</i>	(Sy <i>et al.</i> , 2001, Jourand <i>et al.</i> , 2004)
<i>Burkholderia</i> sp. *	(Moulin <i>et al.</i> , 2001)
<i>Ralstonia</i>	
<i>R. taiwanensis</i> **	(Chen <i>et al.</i> , 2001)
<i>Blastobacter</i>	
<i>B. denitrificans</i> ***	(van Berkun and Eardly, 2002)
<i>Devosia</i>	
<i>D. neptunea</i>	(Rivas <i>et al.</i> , 2002, 2003)

Note: **Agrobacterium* species *A. tumefaciens* (syn. *A. radiobacter*), *A. rhizogenes*, *A. rubi* and *A. vitis* and *Allorhizobium undicola* were proposed to be included in *Rhizobium* (Young *et al.*, 2001). ** Genus *Wautersia* (Vaneechoutte *et al.*, 2004) and later genus *Cupriavidus* (Vandamme and Conye, 2004) were proposed to accommodate this species
 *** These authors did not describe this species but they discovered it is able to nodulate legumes.

Species The plant infection technique makes use of the nodulation process itself to estimate LNB populations in soil. Further culture and characterization of the LNB from the nodules thus formed can provide information on the taxonomic composition of LNB populations and the degrees of specificity between particular strains and candidate hosts.

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 unguiculata*, 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

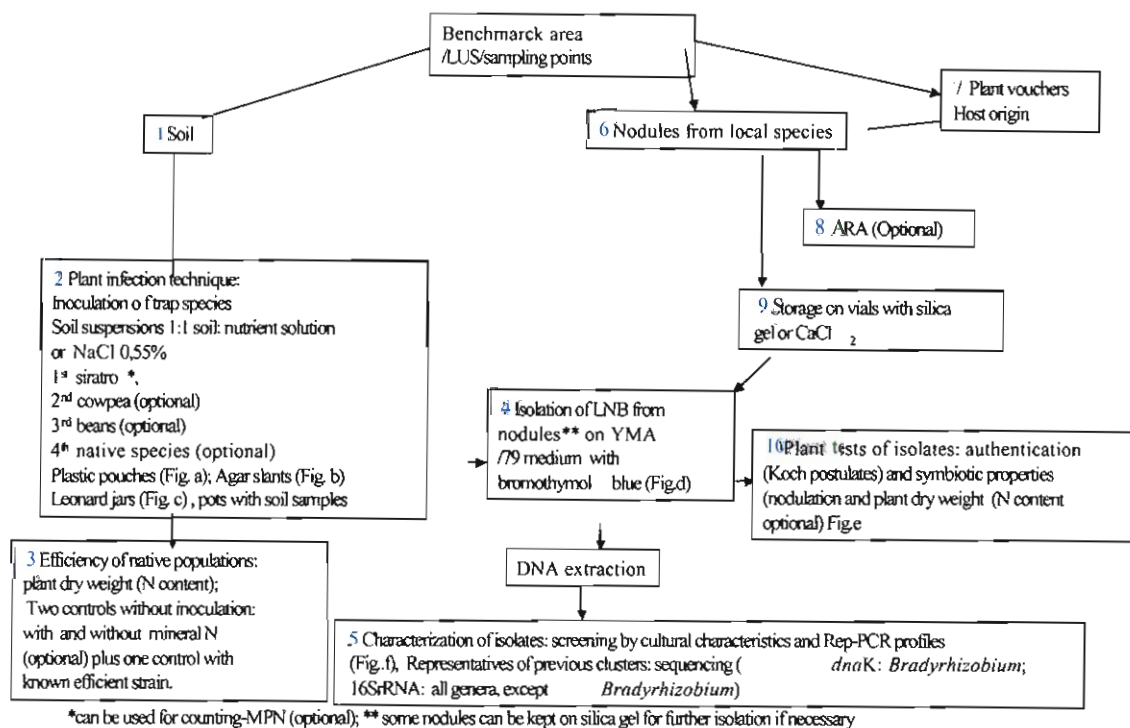
All the steps of the methodologies applied in CSM-BGBD project are summarized in Fig. 9 (Figures a to f included in Figure 9 for illustration).

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

for all countries. This would also avoid any possible influence of plant accession on LNB trapped.

In case each team can handle more than one trap species the other promiscuous trap species recommended were respectively *Vigna unguiculata* (cowpea as the second species) and *Phaseolus vulgaris* (beans as the third species). CIAT could provide the seeds for these species also, but each country might also choose varieties/accessions commonly used in that country. These two species were chosen because they are quite promiscuous and relevant as food crops in many countries. Regarding *Phaseolus vulgaris*, choice was also made because there were some scattered LNB population data which were assessed with the bean as the trap in quite a number of countries which could act as reference. After these three species (siratro, cowpea and beans), other species can be selected, which might be different between countries. Selection should be based on the criterion that they are ecologically and economically relevant to the country. It would be impossible to standardize these choices.

Figure9: Evaluation of Nitrogen Fixing Leguminosae nodulating bacteria at diverse LUS



1- six or 12 (the same for all microbes) -small experiment inside the BGBD inventory testing 6 and other 6 cores in 3 sampling points in the same LUS. LUS should be forest in which we expected higher variability due to the most diverse rhizosphere effect

2-Pots with soil samples are to be used, only if, labs are at the benchmark area, taking care to follow all principles of contamination control used in microbiology.

3-time of harvesting- flowering or when significant differences can be observed among treatments.

4- Small experiment with forest and legume crop soil samples to apply species extinction curves to determine needed isolations number. See in the

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 *dnaK* (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.

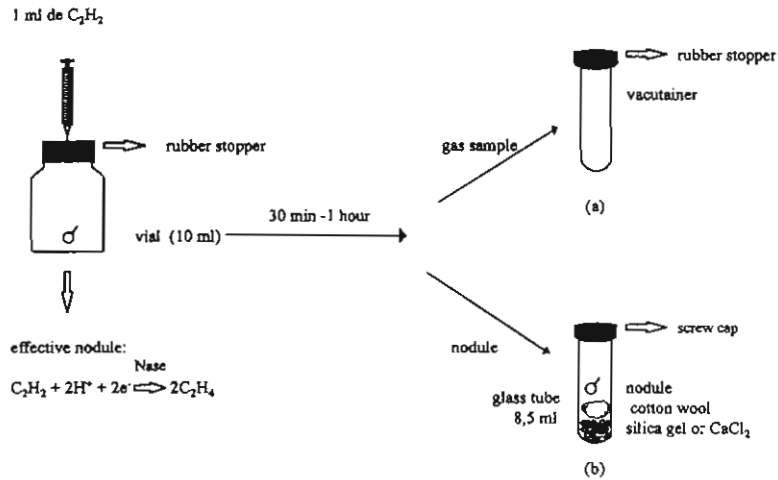


Fig. 10 Field work: a) taking gas samples from nitrogenase-mediated acetylene reduction and b) storage of nodules until isolation in the lab. (extracted from Moreira and Pereira, 2001)

Isolation of LNB from nodules must be performed at YMA with bromothimol blue. This enables cultural characterization (growth rate, pH change, exopolysaccharide production, colony morphology, etc.). Clusters can be obtained based on a screening of these cultural characteristics. Genetic diversity of LNB populations can be also assessed by Rep-PCR (cluster analysis by softwares, e.g. Gelcompar) (Be Brujin et al., 1997) or other technique, however this will be optional depending on resources available in each country. Finally, representatives of cultural clusters and Rep-PCR clusters (if this characterization was also carried out) will be sequenced for 16S rRNA (LNB other than *Bradyrhizobium*) and for *dnaK* (in case of *Bradyrhizobium*) genes, as this last gene discriminates better *Bradyrhizobium* isolates. If resources are available others genes can also be sequenced for *Bradyrhizobium* strains as *atpD*, *rpoB*, *recA*, *glnII* (Parker, 2004; Vinuesa et al., 2005a; Gaunt et al., 2001).

Material Requirements for field and lab work

Fieldwork For *soil sampling*: alcohol, insulated cold-box, sterilized plastic bags (300 ml), spatula, large plastic bags (5 l) and small soil corer; for *nodule sampling*: small scissors, spade, hoe, mattock, forceps, shovel, screw cap tubes with silica gel or anhydrous $CaCl_2$; for *plant vouchers*: alcohol, press, old newspapers.

Laboratory work for LNB trapping and enumeration 1 ml and 5 ml pipettes, diluent solution, 1 l and 125 ml Erlenmeyer flasks, orbital shaker, sterile plastic bags (125 ml, as growth pouches) or glass tubes (150 x 20 mm or 200 x 30 mm), racks for growth pouches or tubes, nutrient solution, seeds of promiscuous host plants, controlled environment room (temperature, light, humidity);.

Laboratory work for LNB isolation and culture characterization Petri dishes, 95% alcohol, 0.1% $HgCl_2$ (acidified with conc. HCl at 5 ml/l), sterilized water, forceps, yeast-mannitol-mineral salts agar medium, pH 6.8; for *nitrogenase activity* (by acetylene reduction) KITASATO erlenmeyers,

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 (CaC_2), 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:

Project: CSM-BGBD.

Collector: Fatima Moreira.

Date: 2 April 2005.

Localitiion: Benjamin Constant.

Altitude: 500 m.

Species vulgar name : faveira.

Species scientific name:

Voucher number: 05.

Nodule characteristics: indeterminate growth, size 0.5 to 1.5 cm.

Site description: pasture, cattle grazing, open with few stumps.

Soil type : sandy loam.

Plant characteristics: herbaceous with mature fruits.

Other comments: seeds collected, yellow flowers.

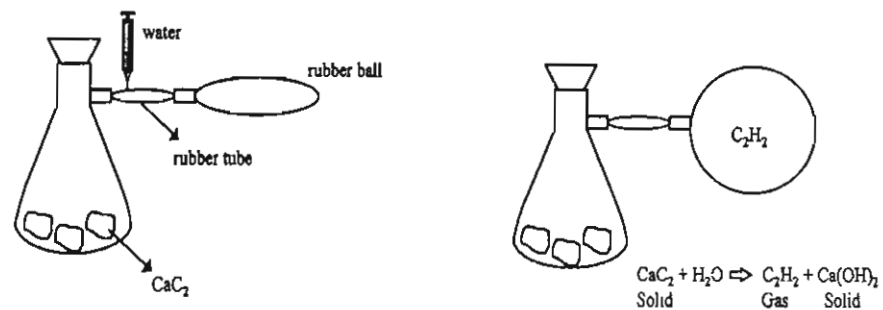


Fig. 11 Acetylene production in the field or laboratory (extracted from Moreira and Pereira, 2001)

LNB counting: Soil samples are submitted to serial dilutions before candidate host plants are inoculated (Figure 12). The dilution ratios vary between 2.0 and 14.5, depending on the expected concentration of cells in the soil sample, i.e. greater dilution for soils with more LNB. However it is still necessary to inoculate host plants at all dilutions (see below). Also, at each dilution used, replication of the bioassay (2-5 times) should be employed. Plants are grown under controlled environment conditions (Vincent, 1970), and examined for nodule formation after 15 days. Populations of LNB are estimated by the Most Probable Number method (Woomer *et al.*, 1990). For inoculating plants growing in plastic pouches and Leonard jars, soil samples must be re-suspended at sterile water or nutrient solution (the same used at the system, see annex 1) at the ratio 1:1.

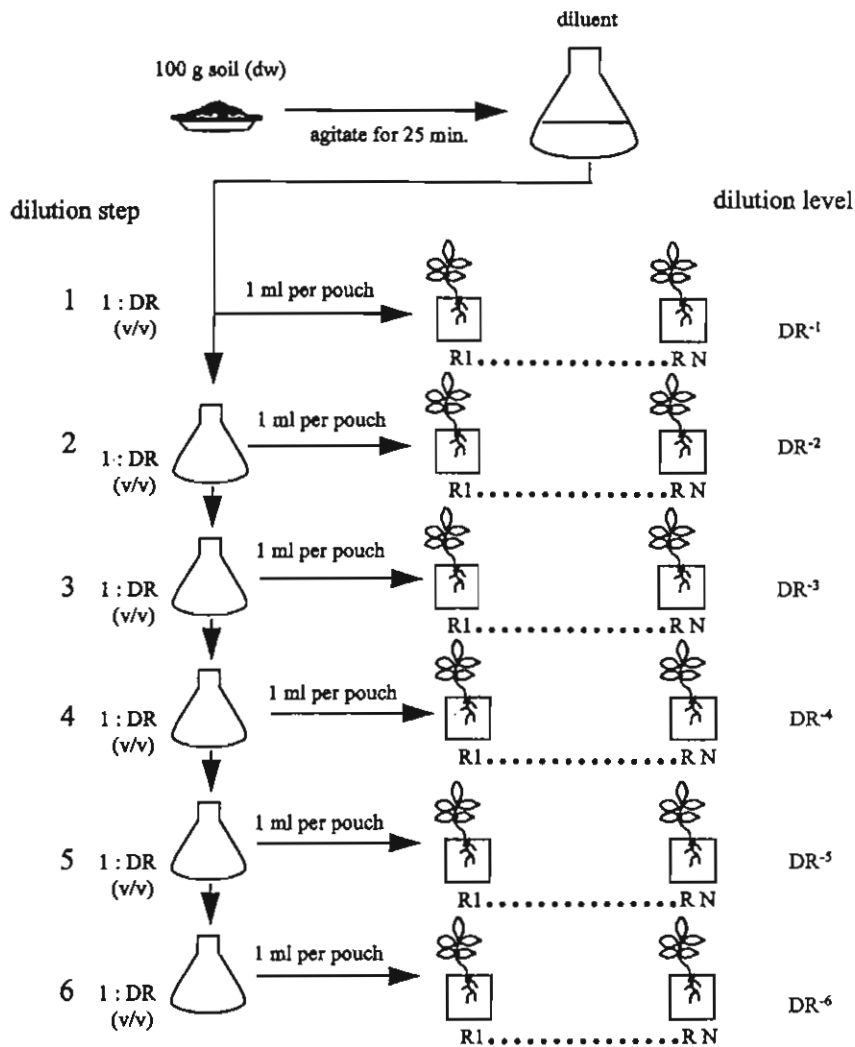


Fig. 12. Method for calculation of most-probable-number of rhizobia cells in soil by the plant infection technique. Base dilution rates (DR) can vary from 2 to 14.5 and replicate numbers per dilution (N) from 2 to 5. (Adapted from Woormer (1993) and Vincent (1970) by Moreira and Pereira (2001).

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 HgCl_2 (Na or Ca hypochlorite, or H_2O_2 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 HgCl_2 (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;

Elkan and Bunn, 1991). Bromothymol blue can be included as an indicator, as pH changes caused by LNB growth may be useful in genus identification. Other characters include growth rate (TAIC-time of appearance of isolated colonies), the extent of extracellular polysaccharide deposition, colony shape and colony color. The main generic descriptors are:

Allorhizobium, *Rhizobium* and *Sinorhizobium*: colonies circular, 2-4mm in diameter, but usually coalesce due to copious extracellular polysaccharide production, convex, semi-translucent, raised and mucilaginous, most with a yellowish center (due to pH indicator), fast growers (TAIC <3 days).

Mesorhizobium : same as *Rhizobium* , but usually intermediate growers (TAIC 4-5 days).

Bradyrhizobium: colonies circular, do not exceed 1 mm in diameter, extracellular polysaccharide production from abundant to little (the latter generally in those strains taking >10 days to grow), opaque, rarely translucent, white and convex, granular in texture, produce an alkaline pH shift, slow or very slow growers (TAIC 6 or more days).

Azorhizobium: colonies circular, 0.5mm in diameter with a creamy colour, very little extracellular polysaccharide production (much less than in *Bradyrhizobium*), produce an alkaline pH shift, fast to intermediate growers (TAIC 3-4 days).

Burkholderia: Growth characteristics similar to fast growers except for pH modification as they can produce acid and alkaline reaction depending on age and sometimes at the same time.

LNB generally are non-spore forming G- rods, usually containing poly- β -hydroxybutyrate granules refractile under phase contrast microscopy. Isolates must be reconfirmed as LNB by demonstration that they will again nodulate a test host plant under bacteriologically controlled conditions following Koch postulates (Vincent, 1970). Table 1 gives references in which full details of LNB species characteristics are given, in addition to Jordan (1984) and Elkan and Bunn (1991). Strain diversity within species may be high, both genetic and phenotypic (for example symbiotic, cultural morphological and physiological traits) and it is thus necessary to define the level of diversity which is appropriate to characterize particular genera and strains. Additional characters can include serology (Dudman and Belbin, 1988), cell lipopolysaccharides (de Maagd *et al.*, 1988), total protein patterns by SDS-PAGE (Hames and Rickwood, 1985; Nwaga *et al.*, 1990; Moreira *et al.*, 1993), small subunit RNA sequence (Young and Haukka, 1996), plasmid profiles (Giller *et al.*, 1989), intrinsic antibiotic resistance (IAR, Kingsley and Bohool, 1983), multilocus enzyme electrophoresis (Selander *et al.*, 1986) and growth on different C sources (Dreyfus *et al.*, 1983), Rep-PCR (Rademaker and Brujin, 1997; Brujin *et al.*, 1997). Numerical cluster analysis of an adequate number of strains and comparison with LNB type strains can permit the characterization of large populations. Genetic characterization (DNA, 16S rDNA, 23S rDNA or other gene sequencing; DNA:DNA homology) is time-consuming and requires specialized equipment, and is usually confined to representatives of clusters only. Minimum standards for the description of new genera and species are given by Graham *et al.*(1991)

DNA extraction: Genomic DNA is isolated from log phase cultures grown on YMA for varying incubation periods depending on the specific growth rate of each strain (2-10 days). Kits for DNA extraction maybe the ones available in the labs. Ultra-clean Soil DNA isolation kits from MOBIO laboratories can

be used, essentially as recommended by the vendor. DNA is quantitated at A_{260} 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'GGTACCTTGTACGACTT) 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 μ L) are: 1X PCRBuffer, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 0,2 μ M of each primer and 2 unit of Taq polymerase. The PCR program has an initial denaturing step at 94° C for 5 min; followed by 30 cycles of denaturing at 94° C for 40 sec, annealing at 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'-CACACGATCCCGACCAA-3') and *dnaK1846R* (5'-GGTGAAGTCYGCCTCGAC-3') which correspond to positions 1230 to 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 μ L) are: 1X PCRBuffer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 400 nM of each primer and 1 unit of Taq 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, annealing at 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 Kit from Roche and making the final eluting step with 50 μ L 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 lacked 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.

Annex 1: Jensen's nutrient solution for growth of leguminous species in plastic ouches and Leonard jars

Components	
K ₂ HPO ₄ (2%)	10 ml
MgSO ₄ .7H ₂ O (2%) + NaCl (2%)	10 ml
CaHPO ₄ (10%) or Ca(H ₂ PO ₄) ₂ .H ₂ O 8,14 g/l	30 ml
FeCl ₃ .6H ₂ O 1.4 % or FeCl ₃ 1%	10 ml
* Micronutrient solution	1 ml
Distilled water	1.00 0 ml

pH = 6,7, adjustment with KOH

Dilute solution to one fourth

* Micronutrient solution (for 1L of water).

H ₃ BO ₃	2,86 g
MnSO ₄ .4H ₂ O	2,03 g
ZnSO ₄ .7H ₂ O	0,22 g
CuSO ₄ .5H ₂ O	0,08 g
Na ₂ MoO ₄ .H ₂ O	0,09 g

Observation This is the same protocol for seedling agar (p. 75). Vincent (1970) recommends this same protocol to nutrient solution but diluted like here (p.82-84, 88). Then we use this solution to siratro in plastic bags, too.

Controls

N-controls are provided to a final concentration of approx. 70 ppm N (0,05% KNO₃ or NH₄ NO₃). This may be added to the nutrient solution at the beginning of the experiment or latter, i.e. 7-10 days after planting. If this is insufficient for the sustained growth and green color of N-control plants towards the end of the experiment, it can be supplemented. However, higher concentrations (>0,07% KNO₃) can be toxic. P. 76. Another important control, not mentioned by Vincent is the use of an efficient strain as a positive control for nodulation. If strains recommended as inoculants are available they should be preferred.

Reference – Vincent, J. M. A Manual for the Practical Study of Root-Nodule Bacteria. 1970. 164p. (Pages 75, 76, 82-84).

MICROSYMBIONTS: ARBUSCULAR MYCORRHIZAS

Sampling protocol for AM fungi

A triangle of 50 x 50 x 50 m is to be laid at each sampling point. The center point of the triangle is marked. From the center point at a distance of 3m three soil cores of 0-20 cm depth, without litter, should be collected. Similarly at a distance of 6m from the center, three soil cores of 0-20 cm depth, without litter, should be collected (see fig. below) Thus at each sampling point six soil cores should be collected. The six soil core samples

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 phosphorus 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 fungi 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 mycosymbiont 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 calothyrsus* 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.

It has been observed that in tropical soils, mixed cropping and application of organic amendments encourage proliferation of AM fungi while application of heavy doses of fertilizers, especially P, leaving the land fallow and growing non-mycorrhizal crop plants reduce the population of AM fungi in soil. Monocropping has been shown to reduce the AM fungal diversity in soil. It can be generalized that modern high-input agricultural practices generally are detrimental to AM fungi, while the low-input sustainable agriculture methods enhance the population of AM fungi in soil. More studies are needed, especially in the tropics, to understand the effects of various land use intensification on AM fungi in the soil.

Principally there could be two strategies for managing AM fungi. The first strategy is to develop inoculation techniques with efficient AM fungi, adapted to the crop plant and the environment. Considerable work is being undertaken at present on this aspect. The second strategy is to manage the indigenous AM fungi by agricultural practices in such a way that efficient native fungi are enhanced and inefficient fungi are depressed. A good deal of research is necessary before we formulate some general recommendations for this management method for each soil and crop. Therefore, manipulation of this symbiotic association to attain its full ecological and economic potential should be the goal for future studies in this project.

Methods

Method for staining roots with trypan-blue for assessing mycorrhizal colonization. (Method of Philips and Hayman, 1970., with modifications by Kormanik et al; 1980)

The roots within the soil sampled using 20cm depth soil is recommended for staining and determination of % root colonization.

Justification

- In all the ecosystems, the root system of a number of plant species apart from specified crop is invariably present.
- Differentiating/seperating roots of individual species is very time consuming, laborious and often may lead to misconstrued conclusions.
- Since the approach is ecosystem based, overall association of above ground plant species and different mycorrhizal species (to be identified later) occurring below ground, will suffice for comparing different LUS.

Materials needed

1. 10% KOH solution
2. 1% HCl solution
3. Lacto glycerol (lactic acid-20ml; glycerol-40ml; distilled water-40ml)
4. 0.05% trypan blue in lacto glycerol (0.5 g/litre)

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 HCl. 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 H₂O₂ for 10 to 20 minutes or until roots are bleached (Alkaline H₂O₂ is made by adding 3ml of NH₄OH to 30ml of 10% H₂O₂ 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:

$$\text{Percent AM Colonization} = \frac{\text{Total No of intersections positive for AM colonization}}{\text{Total No of intersections between root and the gridline}} \times 100$$

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.

Note: If the soil is clay that yields a suspension which blocks the sieve, precipitate the particles in 0.1M sodium pyrophosphate (Fugel and Hunt, 1979).

3. Identification of the diversity of AM fungi using trap plants (mandatory)

Although spores extracted direct from soil can be used for identification. Better identification of AMF is after "host baiting technique" or "trap pot culturing", because not all AM fungal species sporulate at the time of sampling, or the spore number can be very low in soil. Also, spores from baits are in a better state (e.g. more percentage viable) to be analyzed. The composite samples obtained from the sampling points are brought to the laboratory. The test soil sample can be mixed with sterile sand soil (1:1) mix [50 % test soil sample + 50 % sterile sand soil mix] and planted with a suitable trap plants. A mixture of sorghum and cowpea is preferable. After 3-4 months, the potting mix can be wet sieved and the spores are observed under a compound microscope. Identification of the spores can be done by referring to the "Manual for the Identification of VAM Fungi" by Schenck and Perez(1988) and the INVAM website by Joe Morton. <http://invam.caf.wvu.edu>

Modification based on the training workshop held at Bangalore

In localities where sorghum and cowpea do not grow well, any suitable host plants, preferably a legume and a grass mix can be used for this purpose.

Note: The percent mycorrhizal colonization in the trap plants may be determined if desired (Moorman and Reeves, 1979, Amer. J. Bot. 66: 14-18). This is optional.

Most probable number method for estimating number of infective propagules of AM fungi in soil (Porter, 1979) (Optional)

1. Materials needed:
2. Plant tubes (150 cm X 2.5 cm)
3. Stands for keeping plant tubes
4. Polythene bags (30 cm X 20 cm)
5. Sterilized sand: soil (1:1) mix
6. Onion seeds
7. Physical balance

Procedure

1. Take 30 g of test soil in a polythene bag. Add 270 g of autoclaved sand: soil (1:1) mix. Shake thoroughly to get 10^{-1} dilution.
2. Make a ten fold series of soil dilutions up to 10^{-4} dilution.(go for higher dilution if needed)
3. Place diluted soils in plant tubes, using five replicate tubes per dilution.
4. Sow seeds of onion in to each tube.
5. Grow plants in green house or growth chamber for six weeks.
6. Wash, clear and stain roots with trypan blue and examine microscopically. Determine whether colonization is present or absent.

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 (N_1) 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 (N_2 and N_3) 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 I.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.

Note The 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 50C until isolation of fungi is attempted.

Isolating fungi

For *Aspergillus*, *Penicillium*, *Trichoderma* and *Fusarium*, a soil dilution plate technique is used, with PDA as the medium. 1g of soil and roots are added to 9ml of 2% agar water and vortexed, then diluted a further 10 times. 1ml of the final dilution was used to make each spread plate, which was incubated at 28°C for 72 hours. For *Phytophthora* and *Pythium*, PARHP and 3P media were employed, with incubation for 10-20 days. For isolation from roots, roots were first washed in 1% Na hypochlorite for 30 seconds and then rinsed in sterile distilled water, dried on sterile paper towels, and further cultured as above. Selective media are also employed, for example Kerr medium and SNA medium for *Fusarium*, Komada and Hora medium for *Rhizoctonia* and PDA supplemented with lactic acid or Rose Bengal for *Trichoderma*.

Alternative isolations can also be carried out using soil washing (particle filtration) with a sequence of sieves of mesh sizes 1.0 mm, 0.7 mm, 0.5 mm and 0.2 mm. Baiting of lupine radicels and grass leaf blades in sterile water is also available.

Fungi were identified by reference to keys which are identified in other project documents (see Bangalore Workshop report).

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