THE APPLICATION OF SOIL MICRO-ORGANISMS FOR TREE AND CROP IMPROVEMENT

ITE (AMSAL)/ICRAF WORKSHOP
MARCH 4TH - 10TH 1991

ODA/NERC CONTRACT NO. R4319
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Institute of Terrestrial Ecology, Bush Estate, Penicuik EH26 OQB, Midlothian

ITE is a component of Edinburgh Centre for Tropical Forests

ITE (AMSAL)/ICRAF WORKSHOP - MARCH 4TH-10TH, 1991

(All at National Museums except Day 1)

Programme

Day 0 (Sunday 3rd March)

am -	Delegates travel to Nairobi
pm -	Welcome by Janet Sprent, Philip Mason and Bob Munro at Boulevard Hotel

Day 1 (Monday 4th March)

ICRAF, Gilgiri

9.00-10.30	Registration/Coffee
10.30-11.00	Welcome and introduction by ICRAF - (B. Lundgren or P. Cooper).
	Course structure/aims/admin. - P. Mason/R.C. Munro.
11.30-12.30	Principles of N_2 fixation (J. Sprent)
12.30-2.00	LUNCH
2.00-2.45	Biology of N fixing organisms (J. Sprent)
2.45-3.30	Biology of Endomycorrhizas (P.A. Mason)
3.30-4.00	TEA
4.00-4.45	Biology of Ectomycorrhizas (P.A. Mason)
	Reception for Workshop delegates at B. Scott's residence

Day 2 (Tuesday 5th March) - Field visits

am - Visit KEFRI

- laboratories)
- Polythene houses) Myco and Rhizobium demos
- Nursery)

pm - Visit Machakos

- Introduction to Machakos
- See around demo plots
- Take samples for practical classes
- Examine and sample a *Leucaena*-crop - *Cassia* pit.

Day 3 (Wednesday 6th March)

Practical - N_2 fixation - J. Sprent

am - Examination of Nodules

- different nodule types (Frankia and Rhiz) including false nodules
- microscopy/taxonomy of N fixers

pm - Isolation and culture procedures

- agar and liquid media

Inoculation procedures

- assessment of nodule development

Day 4 (Thursday 7th March)

am - Measurement of N_2 fixation

- Benefits of N_2 fixation

This session will be mainly by lecture/discussion with on-going practical from previous sessions.

pm - Practical - Endomycorrhizas - P.A. Mason and R.C. Munro

General Introduction - lecture on organisms involved etc.

- Isolation of Va mycorrhizal fungi from soils collected at Marchakos.
- Microscopic examination of isolated spore types (including demonstration using TV equipment).

Day 5 (Friday 8th March)

am - Quantitative estimation of spores from different soil.

Production and maintenance of endomycorrhizal cultures

- Inoculation procedures

pm - Assessment of VA infection (on roots sampled at Machakos)

(including clearing/staining of mycorrhizal roots).

Day 6 (Saturday 9th March)

Practical - Ectomycorrhizas - P.A. Mason and R.C. Munro

am - General introduction on fungi involved etc.

- Description of ectomycos (including microscopic examintion; mainly as a demo via TV equipment).
- Culture production.
- Benefits of inoculation.

pm - Concluding lectures/discussion

a) N_2 Fixation

b) Mycorrhizas

- to include feedback from delegates as to applicability of course information

Day 7 (Sunday 10th March)

am - Delegates travel home

1. Techniques for nodulating plants

J.I. Sprent and D.W. Odee[1]

Department of Biological Sciences
University of Dundee
Dundee DD1 4HN
Scotland

[1] On secondment from the Kenyan Forestry Research Institute, Nairobi, Kenya.

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Introduction: Nodulated plants

There are two broad classes of nodulated flowering plants, the legumes and the non-legumes. Legumes are nodulated by single-celled eubacteria collectively known as rhizobia. The non-legumes or actinorhizal plants are nodulated by the filamentous actinomycete genus *Frankia*. We shall briefly consider each of these in turn. For further information and more detailed references see Sprent & Sprent (1990) and Schwintzer and Tjepkema (1990).

Legumes. The family Leguminosae is generally divided into three sub-families, the Caesalpinioideae, Mimosoideae and Papilionoideae (Polhill & Raven (1981). Some taxonomists raise the sub-families to family status and and use the terms Caesalpiniaceae, Mimosaceae and Papilionaceae respectively. Nodulation is rare in the Caesalpiniodeae, which includes important non-nodulating genera such as *Cassia* and *Parkinsonia*. Most of the Mimosoideae nodulate, but there are some genera and species (including a few *Acacia* spp) which appear unable to do so. Nearly all of the Papilionoideae have the potential to nodulate. For a recent synopsis of nodulation at the generic level see Faria, Lewis, Sprent & Sutherland (1989).

It is particularly important to stress that not all legumes CAN nodulate and that of those that can, not all DO nodulate in all environments. To equate legumes with nitrogen fixation is wrong, particularly for many tropical woody species. Most herbaceous crop legumes can and do nodulate. We need much more information on tropical species and all participants in this workshop are invited to help extend the record.

Rhizobia. The organisms which can nodulate legumes are a rather mixed group of at least four genera, which probably separated rather early in evolution. These genera are listed in Table 1. Two of the four (*Azorhizobium*, *Photorhizobium*) have been discovered recently in the essentially tropical genera *Sesbania* and *Aeschynomene* respectively, where they form nodules on stems. Many tropical rhizobia do not fall easily into the genera listed in Table 1, so it is possible that new types will be described.

Non-legumes. A very heterogeneous group of genera can nodulate with *Frankia*. They are listed in Table 2. Only one non-legume genus, *Parasponia*, is known to nodulate with rhizobia.

Legume and non-legume nodules differ in a number of ways, apart from their microbial components. Some of these differences are listed in Table 3.

Frankia. Apart from being a filamentous Gram positive genus, the taxonomy of *Frankia* is not well worked out. It has not yet been possible to grow *Frankia* from all nodulated plants in sterile culture. Thus it may not be possible to make inocula in the ways used for rhizobia. Frequently it is sufficient to use crushed, active nodules as inocula.

Casuarina. In an African context, *Casuarina* is probably the most important genus of actinorhizal plant. Its species grow on dry, saline, high pH and other hostile soils. Nodules on *Casuarina* will be examined during this course. For an excellent practical account of *Casuarina* in the tropics, see the chapter by Diem and Dommergue in the book edited by Schwintzer and Tjepkema (1990). This book also contains much practical information about the growth of *Frankia* and the plants it nodulates.

How to identify legume nodules from the field

Outgrowths on roots can be induced by many agents apart from rhizobia and *Frankia*; for example nematodes and fungi. Bear in mind that the most widely studied nodulated plants, with the exception of some grain legumes such as cowpea, are *not* tropical. It is dangerous to assume that tropical legumes are similar to temperate ones. Some tropical legumes produce nodules that do not look like 'typical' nodules (see Plate 1): others produce out-growths which look like nodules but are not (Plate 2). It is very easy to make mistakes! In writing this section we have drawn on

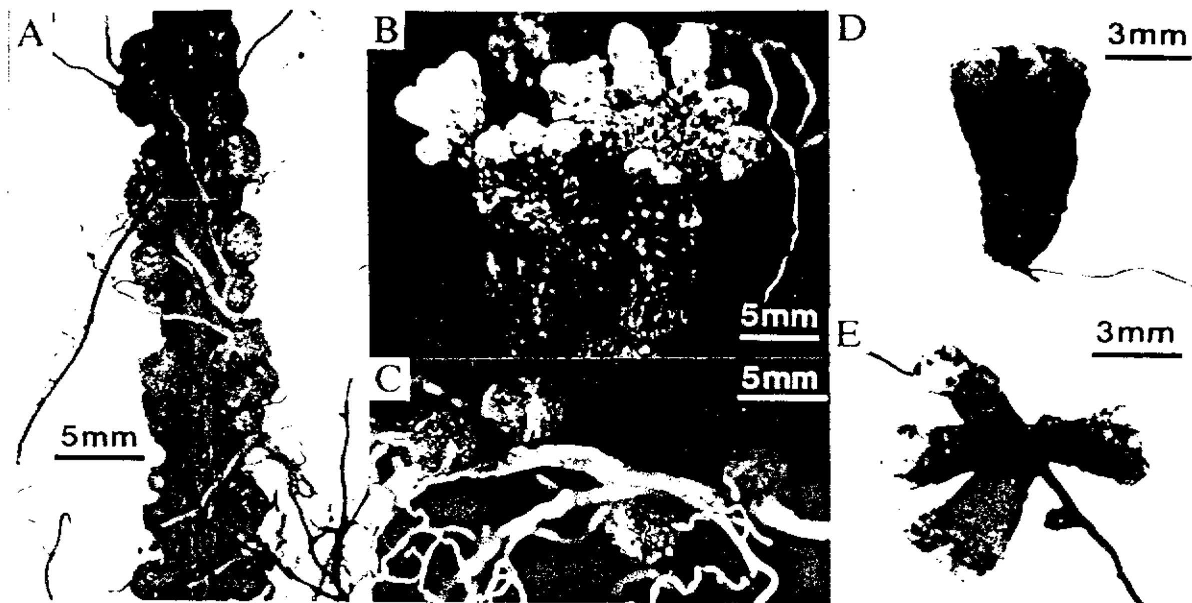

Plate 1. Some examples of legume nodules

A *Dalbergia nigra*: nodules are associated with lateral roots, as they are in several other tropical genera such as *Stylosanthes*, *Aeschynomene*.

B *Daviesia horrida*: nodules are perennial, those shown probably being in their third season, with the paler branch tips containing active nitrogen-fixing tissue.

C *Desmodium* sp.: these are similar to nodules of soybean, bean, cowpea. The pale stripes are lenticels to permit gaseous exchange.

D *Acacia auriculiformis*: these are typical of many mimosoid legumes; they begin unbranched, but may later branch repeatedly. The connection to the subtending root is very slight and nodules very easily become detached during harvesting.

E *Melanoxylon brauna*: a caesalpinioid species; nodules are dark in colour and irregularly branched; in some species they are not easy to distinguish from roots or ectomycorrhizas.

Table 1 Genera and species of rhizobia

GENUS	SPECIES	HOST(S)[1]
Azorhizobium	*caulinodans*	*Sesbania rostrata*
Photorhizobium	?	*Aeschyomene* spp.
Rhizobium	*fredii*[2]	*Glycine max* (Chinese genotypes)
	galegae	*Galega* spp.
	leguminosarum	
	bv *phaseoli*	*Phaseolus* spp.
	bv *trifolii*	*Trifolium* spp.
	bv *viceae*	*Vicia*, *Pisum*
	loti	*Lotus* spp.
	meliloti	*Medicago*
Bradyrhizobium	*japonicum*	*Glycine max*
	sp. (lupin)	*Lupinus*
	sp. (cowpea)	*Vigna* and many others

[1]In the case of *Rhizobium* and *Bradyrhizobium* the host list is not exhaustive. These two genera broadly correspond to the earlier classes of fast and slow-growing rhizobia.

[2]This has been suggested by some to be a distinct genus, called *Sinorhizobium*.

From Sprent & Sprent (1990)

Table 2 Genera of plants known to be nodulated by *Frankia*. Not all species of a genus have been examined and of those that have, not all are necessarily nodulated

Family Genus	Comments
Betulaceae	
Alnus	Hair infection
Casuarinaceae	
Allocasuarina	
Casuarina	Hair infection: nodule roots formed. Vesicles not formed
Ceuthostoma	
Gymnostoma	Nodule roots formed
Coriariaceae	
Coriaria	No uninfected cells amongst infected cells: latter multinucleate
Datiscaceae	
Datisca	Nodule roots formed: no uninfected cells amongst infected cells: latter multinucleate
Elaeagnaceae	
Elaeagnus	Epidermal infection, no pre-nodule
Hippophäe	Epidermal infection, no pre-nodule
Shepherdia	
Myricaceae	
Comptonia	Hair infection
Myrica	Hair infection: nodule roots formed
Rhamnaceae	
Ceanothus, *Colletia*, *Discaria*, *Kentrothamnus*, *Retanilla*, *Talguenea*, *Trevoa*	
Rosaceae	
Cercocarpus, *Chamaebatia*, *Cowania*, *Dryas*, *Purshia*	

From Sprent & Sprent (1990)

Table 3 A comparison of some features of symbioses between dicotyledonous plants and either rhizobia or *Frankia*

Feature	Rhizobia	*Frankia*
Host plants	Many legumes: *Parasponia*	A few genera (usually woody)
Reaction to presence of microsymbiont in rhizosphere	Root hairs may curl or branch: cortical cells may divide	Root hairs may curl or branch
Method of invasion	Through root hairs, wounds, or between epidermal cells	Through root hairs or between epidermal cells
Site of nodule initiation	Root cortex but exceptions possibly occur in woody spp.	Pre-nodule may form in cortex: nitrogen-fixing nodules originate in pericycle
Location of vascular tissue	Cortical(except in *Parasponia*)	Central
Location of infected tissue	Central (except in *Parasponia*)	Cortical
Division of infected cells	Present in most spp. with wound infection and those with determinate nodules	Rare
Infection threads	Absent in some spp. with wound infection, where rhizobia are surrounded by matrix of unknown origin	Absent as such, although *Frankia* always surrounded by host cell wall material
Nitrogen fixing structure	Bacteroid, commonly but not always released from infection thread	Usually a vesicle
Oxygen tension in infected region	Low in those few spp. studied	Low or high, depending on host genus and nodule anatomy
Control/transport of oxygen	Variable diffusion resistance in some spp. coupled with haemoglobin. Perhaps infection thread wall in some primitive spp.	By nodule structure, structure of vesicle wall, sometimes with haemoglobin
Longevity of nodules	Few weeks to perennial	Perennial

From Sprent & Sprent (1990)

problems we have encountered in working in Africa and South America. There may be other problems which we have not yet encountered! We suggest you try and answer the following questions, as a beginning.

1. Is the outgrowth on a root which you have positively identified as being from a legume? Is the legume from a genus known to be nodulated? (check with Faria *etal*, 1989). Remember that many genera have not been examined for nodules – you may have a new record: if you think you have you should isolate rhizobia, reinoculate and check for nitrogenase activity, seeking help from us or others if you wish.

2. Cut the nodule in half. Examine it under a low power microscope. Is the vascular tissue central or peripheral? *All* legume nodules so far examined have a peripheral vascular system (Figure 1). Is it pink in the centre? This is only a guide – some legume nodules are not very pink, some nematode galls are pink!

3. Take a piece of central tissue, squash it, mount in water and examine under high power, Can you see bacteria? Note that the *shape* of rhizobia in their active, nitrogen fixing state (bacteroids) may be spherical, rod-shaped or branched, but never filamentous.

4. Can you isolate rhizobia and will they nodulate plants of the same species? This may not be easy – some rhizobia are difficult to isolate, some grow *very* slowly, some are difficult to use as an inoculant in otherwise sterile systems. Be patient.

5. Can you detect nitrogenase activity? If you have access to suitable equipment, carry out an acetylene reduction assay (see section on methods of measuring nitrogen fixation) using roots from the same plant, but lacking outgrowths, as a control.

Measurement of nitrogen fixation by nodulated plants

If you wish to attempt this in your own country, you are strongly advised to obtain a copy of the book by Peoples *et al* (1989) (see references), obtainable on request from The Director, Australian Centre for International Agricultural Research, G.P.O. Box 1571, Canberra, ACT 2601, Australia.

During the lecture/discussion period on this topic, the following techniques will be briefly considered.

1. The acetylene reduction assay. This is based on the fact that the nitrogenase enzyme complex, which reduces N_2 to NH_3, also reduces acetylene (C_2H_2) to ethylene (C_2H_4). These gases are easily separated on a simple gas chromatograph fitted with a flame ionization detector. It is *very* sensitive but *not* quantitative for legume nodules from the field.

2. Total nitrogen and nitrogen balance methods. For this you need to be able to carry out standard Kjeldahl digestions described in most textbooks on soil analysis.

3. Methods based on xylem sap composition. These are really only for those species (cowpea, bean, soybean) which export the ureides allantoin and allantoic acid as products of nitrogen fixation. Most legumes export glutamine and asparagine, the same compounds which are exported when plants are grown on mineral N.

4. Methods based on the stable isotope ^{15}N. There are two types of such assay:

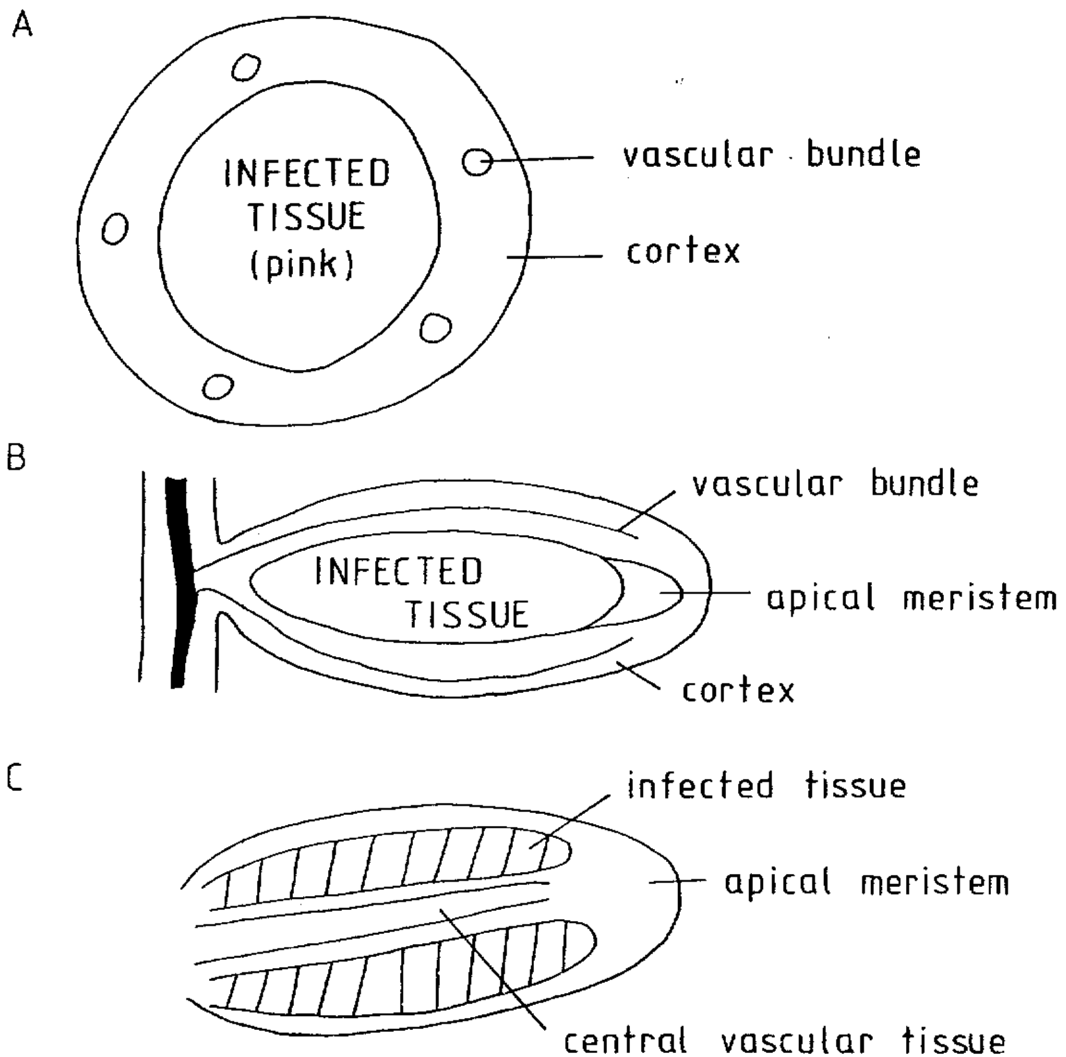

Figure 1. Some structural characters of nodules

A Diagram of a section through a nodule such as soybean, cowpea or bean: such nodules are more-or-less spcherical. They range from 1 to about 7 mm in diameter. They are of limited (determinate) growth.

B Section through an indeterminate nodule such as clover, lucerne (= alfalfa) or *Acacia*. The length ranges from 2 to 15 mm. The infected tissue at the base (nearest the root) may be senescent. The active nitrogen fixing tissue may be pink (clover) or more brownish in colour (some *Acacia* spp). Nodules are often branched.

C Section through a young active lobe of a nodule on an actinorhizal plant such as *Casuarina*. The active tissue is not pink and much of the nodule may be brownish due to phenolic compounds. The length of a single lobe is usually 1–3 mm. Whole nodules, which are perennial, often reach 70 mm in diameter.

(a) based on addition of ^{15}N salts to the soil. For tree crops this is generally impracticable except for some pot experiments with young plants.

(b) based on the natural abundance of ^{15}N. The atmosphere usually contains *less* ^{15}N than soil nitrogen. Thus plants fixing all their own nitrogen will contain less ^{15}N than those entirely reliant on soil N. Plants both fixing nitrogen and using soil N will have intermediate values.

Both (a) and (b) require access to mass spectrometers. For trees in the field (b) is the only potentially feasible method to date. How quantitative it is is still being evaluated.

Apart from (a) and (b), use of $^{15}N_2$ gas is necessary for unambiguous confirmation that a species fixes nitrogen. The radioactive isotope ^{13}N is not a realistic alternative as it has a half life of about 10 minutes!

Collection and preservation of nodules

A *Collection from the field: small plants.*

a) Break the ground around the plant with a suitable digging implement (e.g. spade, pick-axe, hoe, etc.) to cover as much as possible of the area of the root system.

b) Lift out the plant and remove loose soil taking care not to sever the roots and place the whole plant into a plastic bag.

c) Take the plant back to the laboratory as soon as possible and use a gentle stream of water from a hose and wash off the soil adhering to the roots to expose the nodules as shown in Plate 2.

d) Carefully detach the nodules with a piece of root/rootlet bearing bearing the point of attachment. Do not cause any superficial wounds or damage to the surface of the nodule.

e) If nodules cannot be isolated imediately, they may be stored overnight in the refrigerator at temperatures $\geq 4^{o}C$ or between damp paper towels or blotting paper.

f) Nodules are stored for longer periods (about 6 months) in airtight containers (e.g. vials, Macartney bottles, polythene tubes, etc.) containing a desiccant as shown in Figure 2. A label written in pencil containing name of host plant. site and date of collection is also inserted inside the container for future reference during isolation.

B. *Collection from the field: larger plants and trees.*

Recovery of nodules, especially from legume trees and shrubs growing in stressed sites is often very frustrating (see Plate 3). Even if there is some success in nodule recovery, the nodules may not contain all possible rhizobial types or strains that might have been associated with that species during its growth.

Nodules from trees are thus best collected indirectly from grasshouse grown seedlings of the same species in soils collected under the tree (see Table 4 to show nodulation data by this method).

a) Collect sufficient soil (about 10 Kg) from around the treee to a depth of 30 cm or more and transport it back to the laboratory immediately. Collect seeds from the trees if in season.

A & B Washing of soil from a root system to expose nodules.

C Variation in nodulation and root architecture of *Acacia xanthophloea* grown in soils collected from different sites.

D A root system of *Sesbania grandiflora* showing 'false nodules'.

Plate 2

A & B Washing of soil from a root system to expose nodules.

C Variation in nodulation and root architecture of *Acacia xanthophloea* grown in soils collected from different sites.

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Plate 2

A

B

C

Plate 3

A Trunk and partly exposed root system of *Acacia tortilis* growing in a volcanic site at Kibwezi.

B A root system of *Acacia nubica* devoid of nodules.

C A root system of a 7-yr old *Casuarina equisetifolia* showing *Frankia* nodules.

Table 4. A comparison of nodulation and growth of *Faidherbia albida* (provenance Kainuk) grown for 12 weeks in soils collected from two Kenyan sites.

	site	
	Marigat	Yala swamp
Mean annual rainfall (mm)	450 - 900	800 - 1400
Soil classification	Solonchaks	Fluvisols
" N (%)	0.1	0.2
" C (%)	0.5	1.6
" O.M (%)	0.9	2.7
" P (ppm)	8.2	5.2
" pH (in $CaCl_2$)	6.8	5.2
Height per plant[a] (cm)	26.9 ± 5.6[b]	22.1 ± 2.0
Shoot dry wt. per plant (mg)	434 ± 63	586 ± 68
Root " " " " "	618 ± 70	680 ± 26
Nodule " " " " "	5 ± 3	53 ± 21
Nodule number " "	6 ± 2	16 ± 2

[a]Mean of 8 plants
[b]Standard deviation
(Data courtesy of Kenya Forestry Research Institute/Dundee University Nitrogen Fixation collaborative research project funded by the EEC).

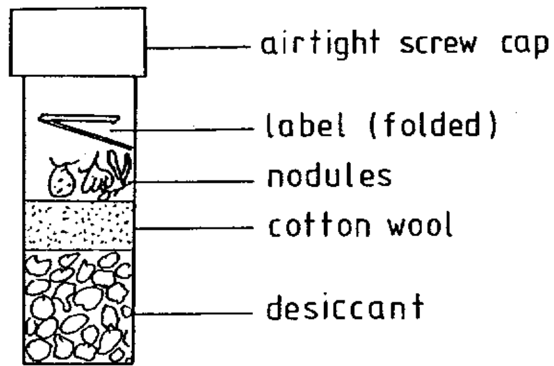

Fig. 2 Nodule preservation vial

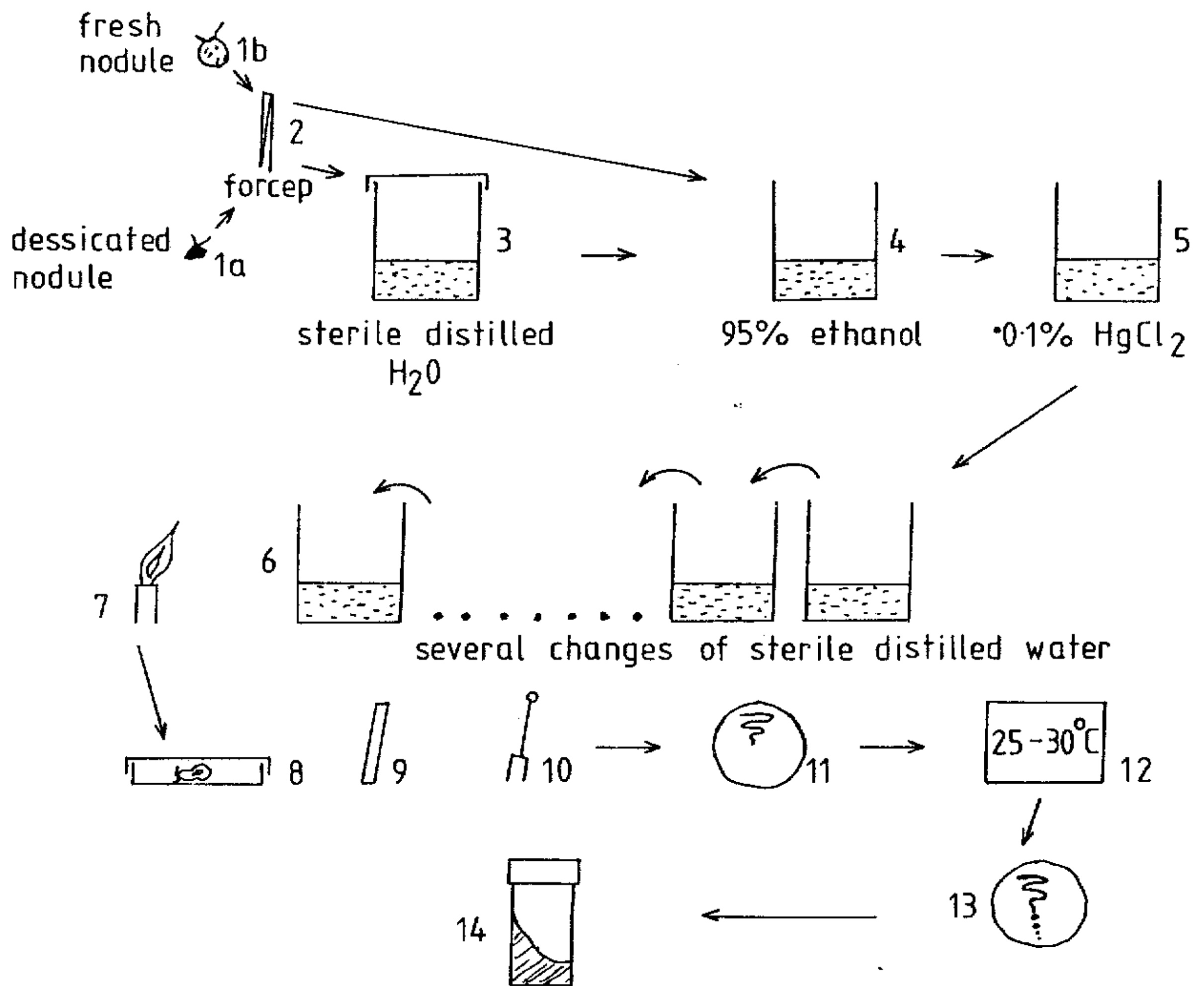

Fig. 3 An illustration of rhizobia isolation (see text for details)

b) Mix up the soil thoroughly and put in 1 kg seedling polythene bags or tubes.

c) Sow three seeds in the seedling polythene bag and thin to two uniform seedlings after germination.

d) Grow the seedlings under glasshouse conditions for a period of 8–12 weeks; watering as necessary, taking precautions not to splash and keep away from other soil sources by appropriate screening.

e) Remove the seedling bag from the soil and wash off the soil to expose nodules as described above.

N.B. If seeds of the required species cannot be obtained, us the nearest related one for which you have seeds. Then when seeds of the required species can be obtained, test your isolate on these.

Isolating and preserving rhizobial cultures

Rhizobia are primarily categorized into two groups depending on their growth rates on yeast maniutol agar (YMA) mineral salts. At optimum temperature (25–30°C) and pH (6.8–7.0) fast growing types develop colonies 2–4 mm in diameter within 3–5 days on YMA plates and the slow growing types do not exceed 1 mm within 5–7 days of incubation (Jordan, 1984). Incorporation of Bromothymol Blue in the media at 25 ppm indicates the pH reaction of isolate. A blue colour (alkaline reaction) is normally obtained with slow growers. A yellow colour (acid) is usually produced by the fast growers. There are exceptions for this test because some strains may behave differently or remain neutral (green), i.e. no discernible pH change (see also Vincent, 1970, Somasegeran & Hoben, 1985).

An isolate is obtained from a colony growing on YMA prepared from a single nodule. However, a nodule may be occupied by more than one type or strain and it is essential that streaking is done in such a way that well isolated colonies appear from which typical ones may be prepared as pure individual cultures.

Isolation procedure

Isolation is done aseptically in a laminar flow cabinet or on a clean laboratory bench top swabbed with 95% ethanol and a burning Bunsen burner to sterilize the air around the operational area.

Follow the steps illustrated in Figure 3.

a) Desiccated nodule (1a) is held by the root attachment with a pair of forceps (2) and rehydrated overnight in sterile distilled water (3). For fresh nodules by-pass step 3.

b) Transfer nodule into 95% ethanol (4) for 5–10 s and then into 0.1% $HgCl_2$, for 15 s up to 2 min depending on the age and size of nodule.

c) Successively rinse nodule in several changes of sterile distilled water (6); at least six changes to remove traces of $HgCl_2$, each time sterilizing the forceps by dipping it into 95% ethanol and flaming with the Bunsen burner (7).

d) The surface sterilized nodule is placed in a sterile petri-dish (8). Crush the nodule in a drop of sterile distilled water with the forceps (if blunt tipped) or with a flamed flat end of a glass rod (9).

e) Flame the inoculation loop (10) and pick a loopful of the crushed nodule and then streak across the surface of the plate containing YMA (11)

f) The plate is then incubated in an inverted position at 25–30°C (12) until colonies of rhizobia appear (13). This will take about 3–5 days for fast growers and 7–14 days for slow growers.

g) A typical colony is picked off the agar surface and transferred to a slant of YMA in a Macartney bottle (14). Colonies may be differentiated by shape, colour and texture; e.g. flat to convex, white opaque to translucent and dry, or dull to shiny and extracellular polysaccharice slime production. The isolates are now considered as presumptive rhizobia.

Authentication

Presumptive rhizobia are authenticated on an appropriate host under aseptic conditions. This is usually done by infecting a pregerminated seed of the plant species from which it was originally isolated or a convenient substitute with similar rhizobial affinities.

Authentication is performed in any growth unit and substrate which is amenable to sterilization and capable of supporting the growth of the plant for the desired duration. There are several types of growth units used, e.g. growth pouches, growth tubes, Leonard jars, etc. The choice of a particular growth unit depends on resources; namely space, time and costs. Among these growth units Leonard jars are widely used because they can be modified to permit use of cheap locally available material and can sustain the growth of plants for longer durations (see Figure 4).

Authentication procedure

a) Prepare 10 ml of yeast extract mannitol broth (YMB = YMA minus agar) in Macartney bottles or test tubes with tops and autoclave at 121°C for 15 min (or use a pressure cooker),

b) Inoculate the broth with a loopful of rhizobia from the slant. If multiple rhizobia with different growth rates are to be authenticated the slow growers are inoculated at least 3 days in advance of the fast growers.

c) Grow the inoculated broth cultures on a shaker at room temperature or more suitably in a shaking incubator at 28°C until visible turbidity develops.

d) Set up 2 Leonard jars for each culture as shown in Figure 4 and fill the jar to two-thirds with N-free nutrient solution (see Somasegaren & Hoben, 1985 for nutrient solution composition) and autoclave the entire growth assembly with contents at 121°C for 1–2 hours.

e) Pregerminate surface sterilized seeds in 0.75% (w/v) water agar plates in the incubator at 28°C, ensuring that hard-coated seeds are pretreated or scarified appropriately (for surface sterilization and scarification details see Somasegaren & Hoben, 1985).

f) Make three holes into the surface of the rooting medium with flamed forceps and select seeds with same radicle length, approximately 1–2 cm; place the seeds with radicle entering first into the hole and cover the hole with the rooting medium.

g) After 5–7 days thin plants to two per Leonard jar by excising the shoot of the unwanted plant aseptically with a sharp blade.

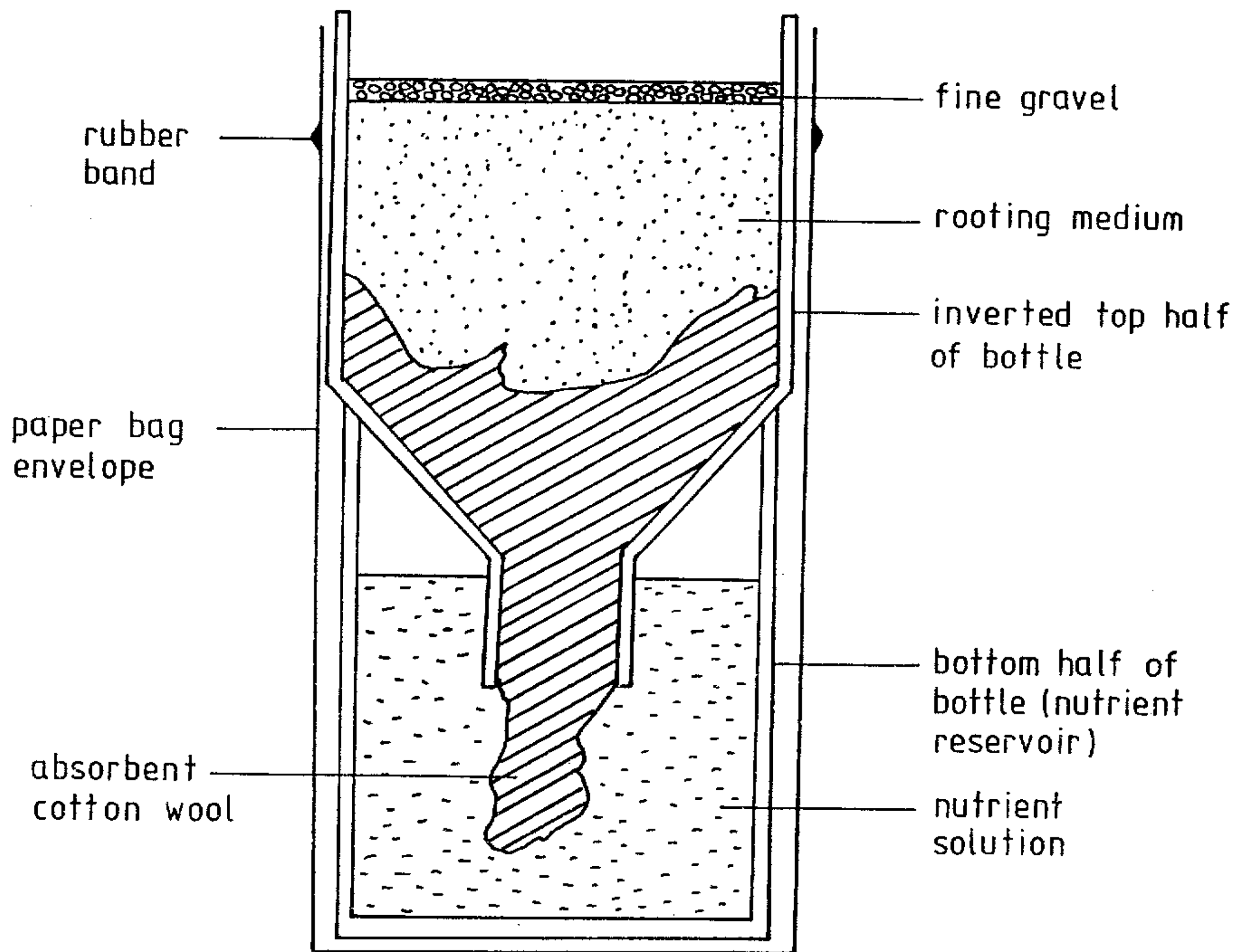

Fig. 4 A simplified Leonard jar

h) Clear area around the root to a depth of about 1 cm and dispense about 1 ml of the fully grown culture (as exemplified by turbidity) to the root with a sterile pipette and fill back the cleared area.

i) Include at least 2 uninoculated control jar plants for every 10 Leonard jars bearing the same species and place them in a growth cabinet or greenhouse.

j) Grow plants for a period of 4–8 weeks depending on the species, and replenish the nutrient solution and water as necessary, observing the same aseptic conditions.

k) After the growth duration, remove the plants and rooting medium by re-inverting the top half of the Leonard jar, then wash off the rooting medium with a gentle stream of water to check for the presence of nodules.

Nodulation of inoculated plants authenticates the presumptive rhizobia and the latter can be stored as a valid culture. However, nodulation of uninoculated plants suggests presence of exogenous rhizobia in the working area and therefore invalidates the whole authentication experiment. It is thus absolutely necessary that sterile handling of the experiment is observed throughout to avoid the disappointment of nodulating controls.

If successfully authenticated, the originasl culture on the slant may be stored at 4°C for a period not exceeding 6 months or under 20% glycerol (v/v) at −20°C for a period of 1 year or more without transfer. There are several methods suitable for long term storage, e.g. lyophilization and liquid N storage (see Vincent, 1970; Bergersen, 1980: Somasegeram & Hoben, 1985).

Natural rhizobial populations in soils

The size and type of soil resident rhizobia are often influenced by the natural distribution of the host plants or continuous cultivation of legume crops (Bergersen, 1980) and have also been recently shown to be dependent on the taxonomic group of the host plant and environmental factors prevalent at the sites (Yousef *et al*, 1987; Woomer *et al*, 1988).

Knowledge of the size of population or the existence of native soil and rhizobia at site are therefore essential in the assessment of the need to inoculate a legume crop new to the site. Estimation of microbial populations is done by plant infection tests. The method consists of inoculating test plants with aliquots from a dilution series of the soil sample. The number of rhizobia are then estimated from the proportion of test plants forming nodules at each dilution. The most probale number (MPN) of rhizobia in the soil is then calculated from standard tables (see Vincent, 1970; Brockwell *et al*, 1975; Bergersen, 1980). Growth of plants is carried out under aseptic conditions as described for authentication. (See Table 5 for data derived from a typical MPN experiment).

References

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Table 5. Most Probable Number(MPN) of rhizobia in two Kenyan soils using *Acacia spp.* as host trap.

Site	Host species	Provenance	Estimated no.of rhizobia (log_{10}/g of soil)
Marigat	*A. tortilis*	Marigat	1.18
Marigat	*A. polyacantha*	Kitui	5.66
Marigat	*A. reficiens*	Katilu	4.63
Loruk	*A. polyacantha*	Kitui	4.63
Loruk	*A. mellifera*	Wamba	4.63

(Data courtesy of Kenya Forestry Research Institute/Dundee University Nitrogen Fixation collaborative research project funded by the EEC).

2. Techniques for Endomycorrhizal Research

P.A. Mason, K. Ingleby and R.C. Munro

Institute of Terrestrial Ecology
Bush Estate
Penicuik EH26 OQB
Midlothian
Scotland

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Techniques for Endomycorrhizal Research

1. GENERAL FEATURES

In 1885, Frank coined the term "mycorrhiza" to describe the symbiotic association of plant roots and fungi. Mycorrhizas which literally means "fungus-roots" are the norm for most vascular plants. Many plants depend on their mycorrhizal structures for adequate uptake of nutrients and survival in natural ecosystems.

Two types of mycorrhizas can be found in nature. In the tropics, the least common are the ectomycorrhizas which are associations in which the fungus forms a sheath around each root but does not penetrate the root cells or move beyond the cortex. Their occurrence in the families Caesalpinaceae and Dipterocarpaceae may help to explain why these families dominate two of the world's most extensive plant formations in the tropics, the miombo woodland of Central Africa and the tropical rain forest of Malaysia and South East Asia.

In contrast, the more ubiquitous endomycorrhizas are symbiotic associations between certain fungi and plant roots, in which the fungal partner grows mainly inside the root cortex and penetrates the cells of the host root. Endomycorrhizas include three groups; ericoid mycorrhizas, orchidaceous mycorrhizas and vesicular-arbuscular (VA) mycorrhizas. The latter group which produces characteristic fungal structures (vesicles and arbuscules) in the cortex region of the root are found in most plant families so far examined, although it may be rare or absent in families such as the Cruciferae, Chenopodiaceae, Caryophyllaceae and Cyperaceae. In addition to the widespread distribution of VA mycorrhizas throughout the plant kingdom, the association is geographically ubiquitous and occurs in plants growing in arctic, temperate and tropical regions. VA mycorrhizas also occur over a broad ecological range, from aquatic to desert environments.

VA mycorrhizas are formed by non-septate phycomycetous fungi belonging to the genera *Acaulospora, Entrophospora, Gigaspora, Glomus, Sclerocystis* and *Scutellospora* in the family Endogonaceae. These fungi are obligate symbionts and have not been cultured on nutrient media. They are not host-specific, although evidence is growing that certain endophytes may form preferential associations with certain host plants.

It is well established that VA mycorrhizas favour plant growth. This they usually achieve solely by enhancing nutrient uptake. In particular VA mycorrhizas are very efficient at taking up soil phosphorous as shown by the dramatic benefits VA mycorrhizas inoculation brings to plants growing in P-deficient soils. However, VA mycorrhizas also enhance the uptake of other nutrients including calcium, zinc, copper and sulphur. Like phosphate, these nutrients are translocated through the hyphal network which extends from the mycorrhizal surface into the surrounding soil far beyond the zone accessible to the non-mycorrhizal roots.

Low mineral nutrient availability, especially that of phosphorus, in most tropical soils has led to obligate mycotrophy of many forest tree species and perennial crops. Such dependence has been correlated with the root morphology of these tropical trees which tend to be coarse with sparse root hairs in sharp contrast to other plants with fibrous root systems and long root hairs which are much less dependent on mycorrhizas. For example, *Leucaena leucocephala* and many species of *Acacia* which have few root hairs are strongly mycorrhiza-dependent in P-deficient soils.

There is also substantial evidence that plant-water relations may be enhanced by mycorrhizal colonisation. Host benefits include increased drought tolerance, decreased drought recovery lag and improved soil water extraction. VA mycorrhizas thus show the potential to enhance crop and tree production in arid and semi-arid regions. The most common mechanism proposed to explain the role of VA mycorrhizas in plant-water relations is improved host phosphorus nutrition. Similarly, mycorrhizal plants appear to be more tolerant of some plant

diseases than non-mycorrhizal plants because of differences in nutrient status.

The widespread occurrence of VA mycorrhizas in tropical legumes has led to considerable attention being given to the tripartite association between legumes, rhizobia and VA fungi. Legumes are relatively poor foragers of phosphorous and generally very responsive to VA infection. As an adequate supply of phosphorous is necessary, not only for plant growth but also for satisfactory nodulation and N_2 fixation, VA mycorrhizas, in increasing phosphorous uptake by the plant, are obviously an important factor in the tripartite association. Recent evidence that mycorrhizal infection can lead to increased nitrogen contents of legume shoots and seeds is of fundamental significance for human nutrition and underlines the need for further studies on the effect of mycorrhizas on nodulation and N_2-fixation in both tree and forage legumes - aspects of which are being studied within the AMSAL project funded by the British Overseas Development Administration.

2. PRACTICAL ASPECTS

2.1 Isolation of VA mycorrhizal fungi from soil

Essentially the starting point for practical studies on VA mycorrhizas is the isolation of mycorrhizal fungi from soil. As VA fungi generally form resting spores in soil either singly or in sporocarps most of the isolation techniques in common use have centred around their separation from soil. These techniques subsequently enable one to either (a) investigate the species distribution, specificity and ecology of VA mycorrhizal fungi or (b) to obtain infective VA mycorrhizal propagules from soil for establishing cultures.

Of the many techniques used to recover VA mycorrhizal spores from soil the most basic of these is wet sieving and decanting. Although this technique is relatively fast, the number of spores extracted are relatively low. Density gradient centrifugation and sucrose centrifugation are now the most commonly used techniques for VA mycorrhizal spore extraction. Their success, however, require that a swinging bucket, horizontal head be used on the centrifuge. Nevertheless large or small quantities of soil may be processed rapidly with reasonable efficiency and little debris with the spores.

2.1.1 Sucrose centrifugation

a) At least 200 g fr. wt. of soil should be removed on each occasion. Where spore assessments are being made at field sites, each sample should be removed from the upper 20-30 cm of soil, either with a corer or a small trowel. Samples should be returned to the laboratory as soon as possible and stored in plastic bags at 5°C until processed.

b) On removal from the cold, each sample should be thoroughly mixed. As spore populations are usually expressed as number of spores per grm dry wt., two 50 g fr. wt. samples of soil should first be weighed out. One should then be dried and weighed while the other should be mixed thoroughly with tap water in a litre plastic beaker, breaking up any lumps in order to ensure that all spores are released into suspension.

c) Allow stirred soil to stand for 15 seconds. Decant through sieve of mesh size 710 µm. This sieve helps to remove large organic matter, roots and sporocarps of some endomycorrhizal fungi. Collect the liquid which passes through this sieve. Wash the sieve in a stream of water to ensure all small particles have passed through.

d) Resuspend the particles in the liquid which passed through the 710 µm sieve and pass the suspension through a sieve fine enough to retain the desired spores, generally 45 µm - 250 µm. If the total population is to be collected and/or assessed only the 45 µm sieve should be employed.

e) Transfer the residue collected by the 45 µm sieve into two 50 ml centrifuge tubes with tap water, balance the tubes and centrifuge for 5 minutes at 1750 rpm in a horizontal rotor.

f) Decant the supernatant liquid carefully and resuspend pellet in a 48% (w/v) sucrose solution (227 g sucrose in 500 ml water).

g) Centrifuge at 1750 rpm for 15 seconds.

h) Pour the supernatant containing the spores through the 45 µm sieve and quickly rinse with water to remove the sugar and the high osmotic pressure which might destroy the spores.

i) The spore sample can then be transferred into a small petri dish and counted.

The sucrose centrifugation method has been outlined in detail above as it will be the method demonstrated within the workshop and the method which we have found most successful with the soils we have been working with. However, the major drawback of this procedure is that spores are suspended in sucrose solution in the final centrifugation which can osmotically stress mycorrhizal spores. Furlan *et al* (1980) solved this problem by substituting various radiopaque media or Percoll for sucrose. These media, however, are cost prohibitive which led Verkade (1988) to use a low cost colloidal silica solution instead as the centrifugation solution.

In addition a further source of error commonly associated with most methods, especially when used to extract spores from organic soils, is the lack of regard for:-

a) spores that float
b) spores that adhere to the wall of the centrifuge tube and
c) spores that become entrapped in or adhere to soil aggregates.

This was largely corrected by McKenney and Lindsey (1987) who used a surfactant, sodium hexametaphosphate to disperse VA mycorrhizal spores and reduce soil aggregates to much smaller particles. This technique was particularly successful with heavier soils. The increase in spores recovered as a result of using the dispersing agent was 15%, 24% and 48% from sandy loam, sandy clay loam and clay soil respectively.

A full range of techniques for recovering endomycorrhizal spores from soil are described in Schenck's Methods and Principles of Mycorrhizal Research (published in 1982 by The American Phytopathological Society, 3340 Pilot Knob Road, St Paul, Minnesota 55121, USA). They include the wet sieving and decanting method which although not ideal for population studies does give rise to spores in a suitable state for setting up pure cultures.

2.2 Quantitative estimation of spores from soil

There are several methods by which the spores in soil can be measured.

2.2.1 Slide method

Extracted spores in water can be pipetted into an eelworm counting slide (Hawksley and Sons Ltd., 12 Peter Road, Lancing, West Sussex, England) which is similar to a haemocytometer but has a 1 ml capacity. The slide is etched into rectangles or can be specially ordered with a rectangle divided into 30 parallel lines. The spores/ml can be calculated by counting the spores contained in a portion of the slide under the high power microscope and multiplying by the appropriate factor.

For large samples this is perhaps the most practical and easiest method yet described.

2.2.2 Petri dish method

An alternative to the above method and that adopted by the AMSAL project uses a small petri dish (5 cm diameter). Parallel lines are etched 2 mm apart onto the base of a small petri dish. The entire sample of spores in a minimal amount of water (to reduce spore movement while counting) is transferred to the petri dish. The etched parallel lines then make it easy to count the total number of spores in the sample under the stereo microscope.

2.2.3 Characterisation of spore types

Both the above methods provide total counts of the number of live and dead spores per g. dry wt. present in a sample. To distinguish between and thereby quantify the different types of VA fungi within a sample requires considerable effort. However, it is a worthwhile exercise and has already brought the AMSAL project some interesting results. In the first instance it is of critical importance to prepare permanent slides of each spore type. The following procedure should be followed.

* 1. Separate different spore types in the petri dish and remove using a pasteur pipette and pipette pump (2 ml). Use freshly extracted spores and collect as many as possible of each type.

2. Transfer to a 1 ml cavity plate. This enables several types in the dish to be separated at the same time.

3. Prepare a twinfrost slide as shown below.

	uncrushed	crushed	crushed
SPORE TYPE SOURCE DATE	↓ ↑	↓ ↑	↓ ↑
Mountants:-	PVLG	PVLG	PVLG/Melzers

Use 1-2 drops of mountant per cover glass depending on the number and size of spores being mounted. Mountant recipes are given in appendix.

*4. Remove all the spores from one cavity with a pipette and allow to drain onto a clean filter paper.

*5. Transfer the spores from the filter paper to the mountant using a mounted needle. Where possible, mount at least 10-25 spores in each drop of mountant.

*6. Allow 1-2 minutes for mountant to become more viscous and then gently add a clean (avoid fingerprints!) 13 mm diameter cover slip. Lower the cover slip gently to ensure that the spores remain central to the cover slip and avoid air bubbles.

*7. Crushing spores: apply gentle pressure on the cover slip above each spore using a clean mounted needle. Avoid squeezing spores out from under the cover slip.

*These operations should be performed under a stereodissecting microscope (x 25 x 50).

Immediately after mounting, spores can be examined at low magnifications under the compound microscope and measurements of length x breadth made on the uncrushed spores. After 2-3 days the mountant will have set and more detailed observations can be made under higher power, oil immersion objectives. In some cases spore wall determinations are difficult and a further slide should be prepared in order to perform progressively heavier crushing of the spores.

Each spore type must be examined critically, especially as few criteria are at present available to differentiate between the taxa of VA mycorrhizal fungi. Spore characteristics, particularly measurements of the spore wall components, need to be recorded. Although interpreting walls is not always simple, don't despair if your first attempts are confusing or frustrating. It takes considerable practice and experience before wall structures can be seen clearly or distinguished. It is therefore recommended that before any spore characterisation is undertaken, one should acquire and become familiar with Schenck and Perez 'Manual for the Identification of VA Mycorrhizal Fungi (2nd edition)', as well as undergoing a period of training, if at all possible, in an appropriate mycorrhizal laboratory.

2.3 Production and conservation of VA mycorrhizal inoculum

2.3.1 Production of 'starter' cultures

Unlike ectomycorrhizal fungi, VA mycorrhizal fungi have not been successfully reproduced in axenic culture. Thus VA mycorrhizal fungi reproduce only in the presence of living roots and inoculum production requires a host plant, and a growth medium, usually soil, with optimum conditions for growth and reproduction of the fungus.

The most common and most dependable method for producing inoculum is the soil culture method. Mycorrhizal fungi must first be isolated from the field. Individual spores, soil sievings, infected roots or soil itself can be used as inoculum. However, whatever the inoculum source it is usually used to inoculate the roots of 'bait plants' growing in a sterilised growth medium. The latter is necessary in order to ensure the complete elimination of all

unwanted native VA mycorrhizal fungi. Suitable methods for sterilisation include
a) autoclaving at 121°C, 15/lb in^2 for a minimum of 1 hour
b) gamma irradiation (0.8-1.0 Mrad)
c) fumigation with biocides such as methyl bromide
(0.45-1.00 kg/m^3) or
d) uniform steaming (83 to 100°C for two separate 1 hr periods)

The growth medium in which the 'starter cultures' are produced should be chosen carefully. Fertilisation is often critically important in producing VA mycorrhizal inoculum. The medium must contain sufficient phosphorous to allow the host plant to grow readily yet not inhibit colonization. While nutrient solution or nutrient-sand culture media are used, the most common growth medium is soil. Ideally, soils should be fertile enough so that only nitrogen need be applied during the 3 to 4 month period leading to successful infection.

Being rather promiscuous, VA mycorrhizal fungi can be 'started' on a wide range of host plants including maize, cowpea, millet, sorghum, onion, clover, alfalfa, sudan grass and bahia grass. Grass species like maize are ideal as they produce rapidly developing fibrous root systems. The starter inoculum should be placed in close contact with actively growing roots. When seeds, ideally pre-soaked in 0.1% thiram to remove surface contaminants, are sown in clean pots, the inoculum should be placed as a 1-2 cm layer in a pot half filled with sterilised growth medium. This ensures that the roots of the transplanted seedlings are put in direct contact with the inoculum before the pot is topped up with the remainder of the growth medium.

Initially the pots should be well watered and covered to reduce evaporative loss and prevent wilting.

In addition it is necessary to ensure that cultures are not contaminated by adjacent cultures containing different mycorrhizal species. General glasshouse/nursery sanitation involves overall cleanliness, control of weed, insect, fungal and bacterial pests. All tools and pots should be sterilised before use by steaming, autoclaving or a disinfectant. Benches should also be periodically washed down with a disinfectant.

Because of their vigorous growth habit roots of 'starter' culture hosts such as maize may grow out of the bottom of the pot leading to cross-contamination of cultures. This can be prevented by using double pots, one inside the other, by placing pots in saucers or by placing pots on open benches.

The method described above leads to the production of mixed 'starter' cultures, ie cultures which consist of a range of species. To produce pure 'starter' cultures of each component fungal species associated with a particular site or plant, spores need to be first extracted by the wet sieving and decanting method (see above) and then, if possible, 50 identical spores of each fungal species should be placed in water in separate petri dishes. Each collection of spores can then be used as starter inoculum and placed in close contact with roots of a young seedling. Both types of culture should be considered as it appears that whereas pure cultures of highly efficient fungi may be appropriate when inoculating annual crops, an inoculum containing several fungal species could be more advantageous for perennials and trees.

2.3.2 Procedure for maintenance of 'starter' cultures

With proper precautions, it will be possible within 3-4 months to have set up a bank of VA mycorrhizal 'starter' cultures originating perhaps from soil taken from under different tree or crop species. Once a pot culture is achieved, it must be maintained by setting up stock cultures in exactly the same way as the starter cultures except that:-

a) they should be set in larger pots in order to reduce the need to renew them to perhaps every 6-12 months, and
b) the source of inoculum will be the infected root material from the 'starter' culture.

Infected 'starter' culture plants should be carefully removed from their pots and their roots thoroughly washed. The roots should then be cut into 1 cm segments and thoroughly mixed together. Extreme care should be taken during this operation in order to ensure that the root inoculum does not become contaminated with any other cultures.

Depending on the quantity of inoculum available a minimum of
1-2 g fresh weight is spread as before as a layer in a clean pot filled with the same sterilised growth medium used for setting up the 'starter' culture. Either a pre-soaked seed (in 0.1% thiram) or preferably pre-germinated seedlings reared in a sterilised growth medium are transplanted as before in order to ensure that the seedlings roots are in contact with the inoculum.

If inoculum is required of a particular culture for future experiments either a large number of stock plants could be set up as above or more simply, several seedlings could be grown together in a larger container, eg a plastic basin.

As before proper glasshouse/nursery maintenance is important in order to prevent contamination by adjacent cultures.

2.4 Procedure for inoculating experimental tree/crop seedlings

Having established stock cultures, the next important step will be to test whether they provide any benefit to their host plant under nursery or field conditions.

Pot experiments affected either in a glasshouse or nursery can be used as a guide to soil-plant-endophyte compatibility in order to reduce the amount of screening necessary in the field. The first screenings should be done in sterilised soil, then the competitive ability of selected endophytes tested in unsterile soil.

However, results from pot experiments do not always match those of field trials although this can be accounted for by obvious differences in environmental factors, e.g. temperature, light, soil moisture. Bearing this in mind, field experiments should be set up to test various plant-soil-endophyte combinations that look promising from initial pot experiments. Field plots should be as uniform as possible, of reasonable size and the experimental design approved statistically. They must be maintained carefully to avoid cross-contamination.

It is likely that benefits from inoculation with VA mycorrhizal fungi are most likely in infertile soils containing few or ineffective endophytes and with plants that have coarse root systems and a high demand for phosphate.

Field experiments therefore need to be planned well in advance. Producing sufficient inoculum to inoculate the required number of plants bound for one or more field sites will require a minimum of 3 months and more likely 6 months. Seed of each selected host will also need to be germinated 1-2 weeks in advance of inoculation. When inoculation of the seedlings is effected, each culture being tested should be handled separately and in turn. As before, stock plants of a particular culture should be removed and their roots thoroughly washed. All the infected roots of the particular culture under test should be cut up into 1 cm segments and then mixed together. Depending on the quantity of inoculum produced, a minimum of 1-2 g fresh weight of inoculum should be spread as a layer half way down each pot containaing a sterilised growth medium (remember to use the same amount of inoculum in each pot). A portion of inoculum should be retained in order to determine the level of infection of the stock culture inoculum. In addition the soil in which the stock cultures were grown can also be used as a secondary inoculum as it is likely to be rich in spores. A layer of infected soil (ca 100 ml) can be inserted next to the root inoculum half way down the pot.

After transplanting the required number of seedlings to these pots, they must be removed to the glasshouse or nursery and all benches and equipment thoroughly cleaned with disinfectant to reduce the risk of cross-contamination when the next culture is handled. After all seedlings have been inoculated they should be properly maintained in the glasshouse or nursery to ensure no cross-contamination for approximately 3 months, at which time they should be sufficiently infected to be out-planted to the field sites.

In order to be able to assess the benefit of a particular culture, inoculated seedlings will need to be compared with uninoculated seedlings. However, during the nursery phase, before outplanting, early infection may lead to the inoculated plants growing faster than the control seedlings. Thus in order to ensure that both inoculated and uninoculated (control) seedlings are outplanted with the same height, the latter may need to be fed with an appropriate nutrient solution.

2.5 Assessment of endomycorrhizal infection in tree and crop seedlings

Several studies have shown that plant growth enhancement by VA mycorrhizal fungi is related to the level of colonization. It would therefore be advantageous to determine the level of endomycorrhizal infection preferably at the start of an experiment at least once during the experiment and at the final harvest.

Many different methods exist in the literature for sampling root systems and assessing endomycorrhizal infection. Recently a new method has been developed by the AMSAL project.

All methods basically involve the removal of a root sample, its cleaning and staining to show up the infection within the roots and a measurement of % infection.

2.5.1 Randomised sampling of root fragments

The following technique has been adopted by the AMSAL project for selecting a random sample of root fragments for assessment of mycorrhizal infection.

1. Carefully wash the entire root system, taking care not to damage the fine roots, then cut it up into 1 cm fragments.

2. Place all the fragments in a shallow sampling tray which has been previously marked with 100 dots at random. Mix the fragments well, after adding a small

amount of water to aid dispersal. Then select the sample of 100 fragments by removing the fragment (irrespective of its length) which lies closest to each of the 100 dots. Place the selected roots in a small Petri dish. THIS IS THE SAMPLE.

If less than 100 fragments are present (i.e. if the root system is small), select half the fragments in the dish.

3. For weighing, collect the roots which remain in the tray - pour off excess water through muslin and collect together the roots which remain in the tray and those in the muslin. Place in a covered Petri dish. THIS IS THE REMAINDER.

4. Place both the sample and the remainder of the root system, separately, on filter paper and gently blot to remove excess moisture. Then place in pre-weighed aluminium foil pouches to obtain fresh weights.

5. Then oven dry the remainder of the root system in a labelled envelope (80°C for seven days). Then weigh to obtain the dry weight.

6. Place the root sample in a McCartney bottle with a little water to prevent dehydration and store at 4°C.

7. When a number of samples have accumulated, clear and stain them according to the technique described in 2.5.2 (a modification of the method of Phillilps and Hayman, 1970).

8. After clearing and staining, the fungal tissue appears blue within the cleared plant tissue (which does not retain the stain). Assess total root length and length of infection by the gridline intersect method (see this section), using a 1.2 cm grid.

9. The dry weight of the total root system can be estimated from the ratios of the fresh and dry weights of the remainder of the root system and the fresh weight of the sample.

2.5.2 Staining techniques for assessing mycorrhizal infection in roots

The method used is based on the technique described by Phillips and Haymann (1970) but incorporates modifications from a recent paper by Koske and Gemma (1989).

1. Drain off any water and cover roots with 2.5 % KOH solution and autoclave at 121°C and pressure of 1.03×10^5 N m^{-2} (15 psi) for three minutes to remove the majority of pigmentation and break down the cell walls for easier penetration of the following chemicals.

2. Pour off the KOH and rinse roots well in tap water until no further brown colouring appears in the rinse water.

3. Cover roots with alkaline hydrogen peroxide (10 ml of 30 % H_20_2, 3 ml of 30% ammonia solution and 587 ml H_20) at room temperature for 10-20 minutes or until the roots become bleached. Hydrogen peroxide deteriorates quickly and so must be made up immediately prior to use.

4. Rinse in tap water to remove excess reagent.

5. Cover with 1% HCl for about an hour.

6. Pour off HCl but do not rinse, as speciments must remain acidified to accept the stain in the next stage.

7. Cover with 0.05% Trypan blue in acidic glycerol (500 ml glycerol, 50 ml 1 % HCl and 450 ml H_20) and autoclaved at 121°C and pressure of 1.03 x 10^5 N m^{-2} for three minutes.

8. Do not rinse the specimens immediately after staining as the stain is not fixed. Leave in the stain for at least 12 hours, after that specimens can be stored temporarily in water, or in acidic glycerol if they are to be kept for any length of time.

2.5.3 Assessment of mycorrhizal infection

Studies of endomycorrhizal infection and its relationship with plant growth require an accurate and relatively quick and simple method of assessing the percentage of a root system which is infected. Infection of the root system occurs when spores germinate in the soil, penetrate the root and infect the root tissue, particularly the cortex. When infection levels are low, discrete 'infection units' occur about the point of penetration of the fungus, these can be erratically distributed about the root system. At higher levels of infection, 'infection units' overlap so that considerable lengths of infected root can be seen. Such variations in the location of infection and the lengths of root on which it occurs require careful selection of assessment techniques.

Many of the methods used to assess the percentage of root system infected involve the inspection of 1 cm root lengths (fragments) under a high power microscope. Infection can then be quantified by (i) noting the presence or absence of infection at specific points along a graticule line (Allen and Allen, 1980), (ii) making visual estimates of the infection present (Sanders *et al.*, 1977, (iii) assessing the percentage of the cortex which is infected (Ocampo *et al.*, 1980), or (iv) assessing the degree of arbuscule formation and the presence or absence of vesicles (Ross and Ruttencutter, 1977). All these methods rely on high-power microscopy and therefore limit the quantity of material which can be assessed - an important restriction when the erratic nature of root infection is considered.

A method of assessing percentage of infection at low power magnifications, enabling more material to be examined per unit time is the grid-line intersect method. This is an adaptation of a method devised by Newman (1966) to estimate total root length. Comparison of this method with other techniques (Giovanetti and Mosse, 1980) indicated that the standard errors for this technique were lower than the others. An accuracy of +/- 4% could be obtained when 100 intersections between a grid and root fragments (of undefined length) were counted. When the number of intersections was doubled, accuracy was only improved by a further 1%. Accuracy remained good over a wide range of infection levels, although it deteriorated at levels below 10%. Other studies have checked the overall accuracy of the grid-line intersect method for assessing root length, accuracy seems to vary according to the operator, with +/- 7% recorded by Ambler and Young (1977) and a constant overestimate of 8-10% found by Reicosky *et al.* (1970).

2.5.3.1 Grid Line Intersect Method (Tennant 1975)

This method basically involves spreading out a root sample onto a petri dish marked with a grid so that no root obscures another.

To estimate total root length of the sample both the horizontal and vertical lines are scanned using a stereo microscope and wherever a root crosses a grid line, this is recorded.

Total root length can then be calculated using the formula -

$$R \text{ (total root length)} = \frac{11}{14} \times \text{number of intercepts (N)} \times \text{grid unit}$$

It is perhaps not surprising to find that most researchers use the 0.5 inch x 0.5 inch (ie 1.2 cm x 1.2 cm) grid as when this is incorporated into the above formula.

$$R = \frac{11}{14} \times N \times \frac{14}{11} \text{ cm}$$

$$R = N \text{ cm}$$

the total number of root intercepts equals the total length of root in cm.

The process is now repeated with the same sample in order to estimate the length of infected root. The horizontal and vertical lines are again scanned but note is only made whenever the root crossing a grid line is infected.

If a 0.5 in x 0.5 in grid is again used the number of intercepts recorded then equals the total length of infected root in cm.

The percentage of root infected can then be estimated:-

$$\frac{\text{length of infected root}}{\text{total length of root}} \times 100$$

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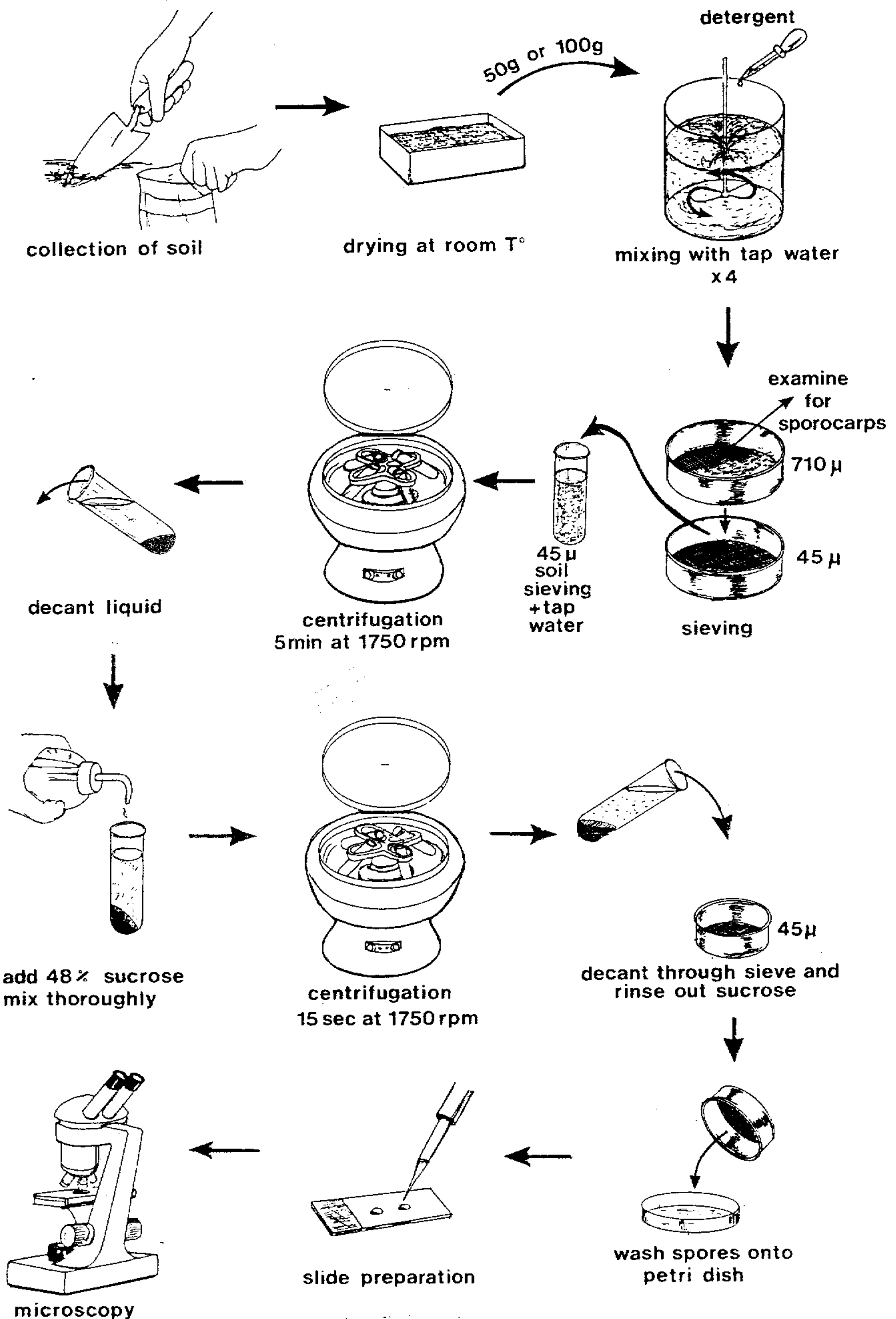
Extraction of VA mycorrhizal spores



3. Techniques of Ectomycorrhizal Research

K. Ingleby and P.A. Mason

Institute of Terrestrial Ecology
Bush Estate
Penicuik
EH26 OQB
Midlothian
Scotland

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Techniques for Ectomycorrhizal Research

1. GENERAL FEATURES

In contrast to the more ubiquitous endomycorrhizas, ectomycorrhizas occur on about 10% of the world flora. However, as ectomycorrhizas are associated with many important forest trees, this type of mycorrhiza is of equal importance to foresters. The dependence of forest trees on ectomycorrhizas has long been recognised. Many trees, in particular pines, will not grow and develop normally without them. Establishment of trees which normally form ectomycorrhizas inevitably fail in the absence of suitable ectomycorrhizal fungal inoculum. For example, unsuccessful attempts were made to introduce *Pinus* spp. into Kenya as long ago as 1902. However, it was not until 1910 that *Pinus radiata* was successfully cultivated following the importation of soil containing mycorrhizal inoculation.

The distinctive features of a typical ectomycorrhiza are the fungal mantle (sheath), which surrounds the host root and the labyrinthine fungal tissue which penetrates between the root epidermal cells to form a network known as the Hartig net. From the mantle surface, hyphae or hyphal strands radiate out into the surrounding soil. In tropical mycorrhizas, strand development can be prolific. As these strands are known to function in the transport of water and dissolved nutrients, it means that ectomycorrhizas are able to efficiently explore the soil substrate around them and transport back sufficient nutrients in order to maintain adequate growth of their associated host, even when growing in soils low in essential nutrients such as phosphorus and nitrogen. Besides being important in nutrient uptake and water absorption ectomycorrhizas can also increase the tolerance of trees to attack by root pathogens to soil toxins, to drought and to extremes of soil temperature and pH.

There are over 5000 species of fungi involved in forming ectomycorrhizas on about 2000 species of woody plants. The major classes of fungi involved are the Basidiomycotina and Ascomycotina. Besides forming mycorrhizas on most conifers including all species of the Pinaceae, this type of symbiosis is also commonly found within temperate regions on members of the Betulaceae, Salicaceae, Juglandaceae, Fagaceae and Myrtaceae. Although endomycorrhizas are most widespread in the tropics, the occurrence of ectomycorrhizas in this region is undoubtedly more widespread than one time thought. For example in the tropical moist savannas, ectomycorrhizal trees dominate both the extensive African miombo woodlands and the Sudanian woodlands.

In the tropical rain forests, all Dipterocarpaceae form ectomycorrhizas, as do most legumes in the tribe Amherstieae of the Caesalpiniodeae, *Afzelia*, *Instia*, and *Eperua* in the tribe Detariae and *Aldinia*, *Swartzia* and *Pericopis* in the Papilionoideae. The existence of ectomycorrhizas in these important groups demonstrates the importance of this type of mycorrhizas throughout the rain forests of South-East Asia, Africa and certain parts of South America.

2. PRACTICAL ASPECTS

2.1 Isolation and culture of ectomycorrhizal fungi

Unlike endomycorrhizal fungi, many ectomycorrhizal fungi can be cultured in vitro. Isolation and experimental manipulation of ectomycorrhizal cultures has been critical in developing all phases of ectomycorrhizal research. As a result a large body of literature exists on pure culture studies of ectomycorrhizal fungi.

Ectomycorrhizal fungi are most commonly isolated from fruitbody tissue but can also be isolated from surface sterilised ectomycorrhizas, spores, sclerotia and rhizomorphs/strands.

2.1.1 Isolation from fruitbodies

Fruitbody isolation is generally preferred as many ectomycorrhizal are members of the Basidiomycotina and therefore produce large fruitbodies from which isolation is relatively easy and which also enable the species to be identified. Thus, in most cases these fungi can be isolated by simply breaking open the fruitbody and aseptically transferring clean tissue to an agar plate of the appropriate nutrient medium. For this method collection of young fruitbodies is preferable so that clean, actively growing tissue is cultured. Isolation should be attempted immediately on return to the laboratory. Plates should then be incubated at 20-25 °C checking regularly for contamination and removing as necessary. Contaminants normally encountered are the fast-growing, spore-producing members of the fungi imperfecti and slime-producing yeasts and bacteria

2.1.2 Isolation from spores

When clean fruitbody tissue is not available, as with many small agarics, resupinate fungi and discomycetes, the spores should be collected on agar plates. In this case collection of mature fruitbodies is preferable and it is important to ensure that fruitbodies of different species are placed in separate bags to prevent cross-contamination of spores. Collection of spores can be made by attaching pieces of spore bearing tissue to the inside of a sterile petri dish lid using sterilised lanolin. Make sure that the cleanest tissue is used for this purpose. The lid is then placed over a nutrient agar plate for up to 16 hours so that the spores drop onto the solid nutrient surface. After 16 hours (or sooner if a spore 'print' can be seen on the nutrient agar surface) replace the lid with a clean one (ie one from a clean sterile dish). Spore collection plates should be incubated at 20-25°C for 2 weeks, checking daily for contamination and removing any contamination immediately it is observed. This initial period will ensure that spore collections are clean, and although the spores of some species may germinate readily, it is more than likely that some form of stimulation will now be required. In this case aseptically-produced tree seedlings should be added to the petri dish, making sure that the roots are placed in contact with the spores on the agar surface. If sufficient numbers of spores have been collected then germination should occur readily with all but the most difficult of species. Other studies have shown that mycelia of the same fungus and activated charcoal can also stimulate spore germination.

2.1.3 Isolation from mycorrhizal roots

Many ectomycorrhizal fungi can be isolated from their mycorrhizal roots. Best results are obtained when fresh mycorrhizas, initially free of adhering debris, are treated immediately upon collection. Isolation success from individually treated ectomycorrhizas varies considerably between different fungi, but is usually less than 20% and frequently less than 5%. This isolation should be attempted from a large number of mycorrhizas.

After collection roots should be carefully excised and cleaned with forceps (not in water!) and then taken through a series of rinses in sterile water and an appropriate surface sterilant. Individual mycorrhizas are then cut and placed on suitable isolation medium, usually containing antibiotics and a selective fungicide. Use of diluted media is recommended for root isolation work as it slows the growth of contaminating fungi. Plates should be inspected daily and any slow growing, non-sporing fungi (potentially mycorrhizal) should be transferred to a fresh nutrient agar plate.

2.1.4 Maintenance and growth in pure culture

After 1-2 months growth, cultures established from fruitbody tissue, germinated spores or roots should then be grown on different media to determine their growth preferences. For basidiomycetes and ascomycetes media such as Hagems, MMN and PDA are appropriate (appendix 1). Many fungi respond to different concentrations of nutrients in these media, particularly glucose, so that full, ½ and ¼ strength formulations should be tested. The initial growth which develops on the isolation plate provides an insight into the isolates media preferences. A preponderance of aerial hyphae indicates the fungus is strongly aerobic and should be maintained on a solid medium such as vermiculate/peat (V/P). A preference to grow on or below the surface of the agar indicates that the fungus should be maintained in liquid media. Cultures can also be tested for optimum temperature requirements. Although most fungi will grow best between 20-25 °C, gasteromycetes such as *Pisolithius tinctorius* (Pers.) Coker & Couch and *Scleroderma citrinum* Pers. have been found to tolerate temperature in excess or 30 °C.

V/P or liquid flasks can be prepared following the methods described in appendix 2. Stock cultures maintained in flasks should be subcultured every 6-12 months depending on the rate of growth of the fungus. Many isolates will benefit from alternate culturing in V/P and liquid media. The V/P medium encourages natural, slower growth of the fungus and as a result probably reduces the chance of loss of infectivity. In contrast liquid media promote rapid growth and vigour of the fungus as well as providing a check for contamination.

Further information on the isolation and nutritional requirements of ectomycorrhizal fungi is given by Jackson & Mason (1984).

When large amounts of inoculum is required of one or more isolates, the first stage involves removing a small amount of fungus from the stock culture and spreading it across several (at least 5-10) nutrient agar plates. This produces new, vigorous hyphal growth with which to subsequently inoculate new flasks (it also provides a check for contamination). Allow at least 2-3 months for the fungus to grow throughout the flask substrate thus ensuring that nutrient levels in the flask have been depleted. If necessary, (eg if physiological experiments are being undertaken) V/P inoculum can be gently washed free of surplus nutrients, but care should be taken not to destroy the structure of individual grains of vermiculite. These grains consist of compressed layers of vermiculite between which the hyphae grow and probably gain considerable protection against disturbance.

2.2 Inoculation methodology

Information gained while determining the nutritional requirements of newly established cultures will show that ectomycorrhizal fungi show strong physiological variability both within and between species. This is to a great extent a reflection of the fact that some species appear more ecologically adapted to certain sites than do other species. To gain maximum benefit from mycorrhizal inoculation, seedlings should be inoculated with fungal symbionts which are best suited to specific tree species, and which are well adapted to the environmental conditions of the planting site.

Of all the types of inoculum used, pure cultures have been repeatedly recommended as the most bioliogically sound. Unfortunately large scale nursery applications of pure mycelial cultures have been severely hampered by lack of sufficient quantities of viable inoculum. It is relatively simple to produce sufficient inoculum (eg 30 to 40 litres V/P or liquid inoculum) for research studies in containers, pots and small nursery and field plots. However, it is completely different to produce sufficient inoculum for large-scale nursery inoculation. Nevertheless, if adequate inoculum can be produced, inoculation of nursery seed beds using V/P inoculum has proved most successful when the inoculum has been applied in a layer just below the soil surface, immediately prior to sowing.

Further information on inoculation technology can be gleaned from the voluminous publications on this aspect by D H Marx and colleagues.

2.3 Assessment of ectomycorrhizas

At the completion of an ectomycorrhizal experiment, whether it be in the laboratory, glasshouse, nursery or field, it will be necessary to record the abundance of ectomycorrhizas present in order to relate mycorrhizal development to different parameters of growth.

Whatever the size of plant, the intact root system should be washed clean in gently running water over a 1-2 mm sieve in order to trap any mycorrhizas that become removed. Roots growing in mineral soils can be cleaned readily, but those growing in soils of a more organic nature will require the careful (and tedious!) removal of adhering particles, using fine forceps under a stereo dissecting microscope.

Where seedlings are small, the entire root system can be placed in water in a petri dish and observed for ectomycorrhizal formation.

However, when seedlings with root systems more than 50 cm in length are assessed, the intact root system should be laid across a grid of parallel lines 1 cm apart. With the root collar positioned at zero, transverse cuts should be made at 1, 2, 4, 5, 7, 8, 10, 11 cm and so on across the roots. Subsequently, only the 1 cm lengths (1-2, 4-5, 7-8 etc) need to be transferred to separate dishes for detailed examination. By using this method (Last *et al.*, 1985) for large root systems, the number of roots needing to be counted is considerably reduced.

When sampling of the whole root system is not possible then core sampling should be carried out. As roots are fragmented by the coring it is important to ensure that only roots of the tree species being studied are assessed.

In order to assess mycorrhizal development observations should be made within each batch of 1 cm segments of:-

a) number of mycorrhizas - each mycorrhiza, whether branched or unbranched, is counted as 1 unit, and
b) number of roots

Thus the extent of mycorrhizal development (ie percentage infection) within a particular root system can then be estimated by calculating -

$$\frac{\text{total number of mycorrhizas}}{\text{total number of roots}} \times 100$$

Counts can also be made of mycorrhizal apices (tips) as results suggest that seedling growth can be more strongly correlated with numbers of mycorrhizal tips rather than numbers of complete mycorrhizal units.

2.5 Characterisation of ectomycorrhizas

If an assessment of the performance of a particular inoculant fungus needs to be made, then the number of mycorrhizas and hence degree of infection by each mycorrhizal type needs to be recorded.

Typically, ectomycorrhizal fungi form mantles with associated hyphae that are characteristic for the species. This enables individual mycorrhizas to be characterised and identified so that proportions of different fungi on a root system can be accurately assessed. Although quantitative assessment of different mycorrhizal types is made under the stereo dissecting microscope the validity of these separations should be confirmed by examining individual mycorrhizas microscopically under a compound microscope. At ITE the following methods are employed:

Macroscopic separation (x5Ø - x50 magnification)

Populations of mycorrhizas can initially be separated on features such as colour, form, size, associated hyphae, strands and sclerotia. At the higher level of magnification (x50), individual hyphae and specialised mantle cells, such as setae and cystidia, may just be discernible. These features should be recorded while the mycorrhizas are still fresh.

The validity of the separation should be confirmed by selecting a minimum of five typical members of each population for microscopic examination of a whole root mount. Where there are mixtures of similar mycorrhizas, further samples should be taken.

Microscopic separation (x500 - x1000 magnification)

Mycorrhizas should be mounted on glass slides in both lactophenol cotton blue and toluidine blue for about 10-15 seconds, before being squashed firmly under a cover glass. Several mycorrhizas can be examined under a single cover glass.

Mounting stains used are:

i) 0.1% (w/v) cotton blue in 10% (v/v) lactophenol/H_20

This is a good general-purpose stain to examine associated hyphae, strands and mantle surfaces, staining most fungal tissues blue. We use a small concentration of lactophenol to avoid shrinkage of stained cytoplasm, which makes details of septa and clamp-connections difficult to observe.

ii) 0.1% (w/v) aqueous toluidine blue

This stains cell walls and is effective in highlighting the structure of smooth compacted mantles. It is also useful in looser mantle structures, as it can penetrate to lower tissues without staining the surface hyphae too strongly. Because of the metachromatic properties of toluidine blue, many fungi produce a diagnostically useful, if not distinctive, colour reaction which may range from blue → purple → violet → pink.

Features examined (Figure 1).

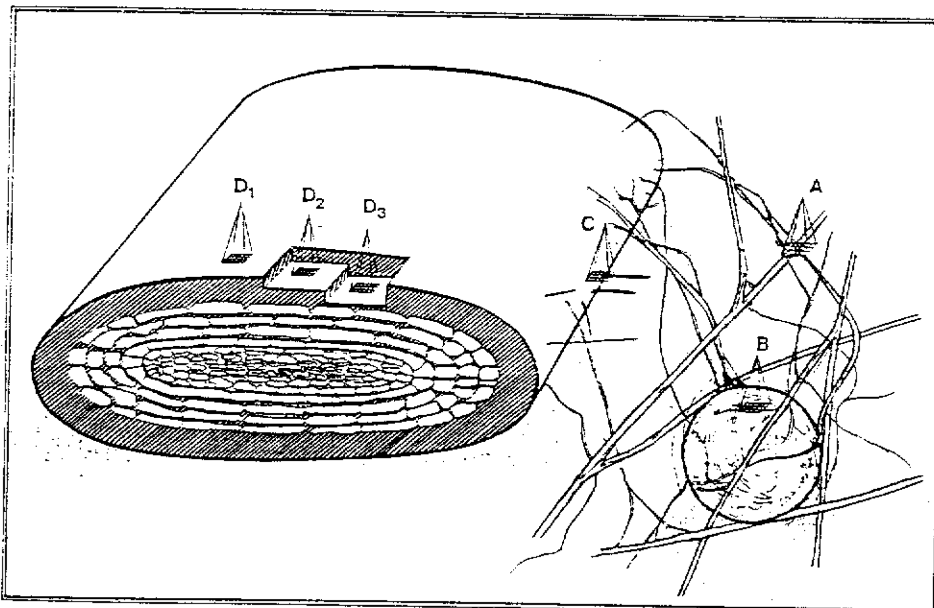
A. Strands and associated hyphae
B. Sclerotia
C. Mantle edge, emanating hyphae, specialised elements. These features are observed by moving to the edge of the mycorrhiza and focusing on the mantle surface, which is then viewed tangentially.
D. Mantle as seen in plan view. One to three layers can be distinguished in different mycorrhizas. Where this is only one distinct layer, it has been designated D1 and the inner D2; where are three layers, the surface, intermediate and inner layers are designated D1, D2 and D3. In some rare instances, it is possible to observe the Hartig net where mantles are very thin or absent.

NB - not all of these features will be found in each mycorrhizal type.

Finally, a sample of each mycorrhizal type should be preserved in 2% glutaraldehyde and stored at 4°C in an herbarium.

Details on characterisation and identification of ectomycorrhizas along with 24 descriptions of fungi frequently found on tree seedlings are given in Ingleby *et al.*, 1990. Further descriptions of ectomycorrhizal types are published by Agerer (1988).

SKETCH OF A SQUASHED MYCORRHIZA SHOWING THE LOCATION OF FEATURES DESCRIBED IN THE MICROSCOPIC EXAMINATION



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Appendix 1. Culture Media for Ectomycorrhizal Fungi

Hagems medium

$MgSO_4$ $7H_20$	0.5 g
K H_2 PO_4	0.5 g
NH_4 Cl	0.5 g
Fe Cl_3 (1% soltn)	0.5 ml
Glucose	5.0 g
Malt extract	5.0 g
Thiamine HCl	50 ug
*Agar	10.0 g
Dist. H_20	1000 ml

Modified Melin Norkrans' medium (MMN)

Ca Cl_2	0.05 g
Na Cl	0.025 g
KH_2 PO_4	0.5 g
$(NH_4)_2$ H PO_4	0.25 g
Mg $S0_4.7H_20$	0.15 g
Fe Cl_3 (1% soltn)	1.2 ml
Thiamine HCl	100 ug
Glucose	10.0 g
*Agar	10.0 g
Dist. H_20	1000 ml

Potato Dextrose Agar (PDA)

Potato extract	200.0 g
Dextrose	20.0 g
*Agar	15.0 g
Dist. H_20	1000 ml

*Omit if solutions are required, ie for vermiculite/peat or liquid culture in flasks.

Appendix 2. Flask culture

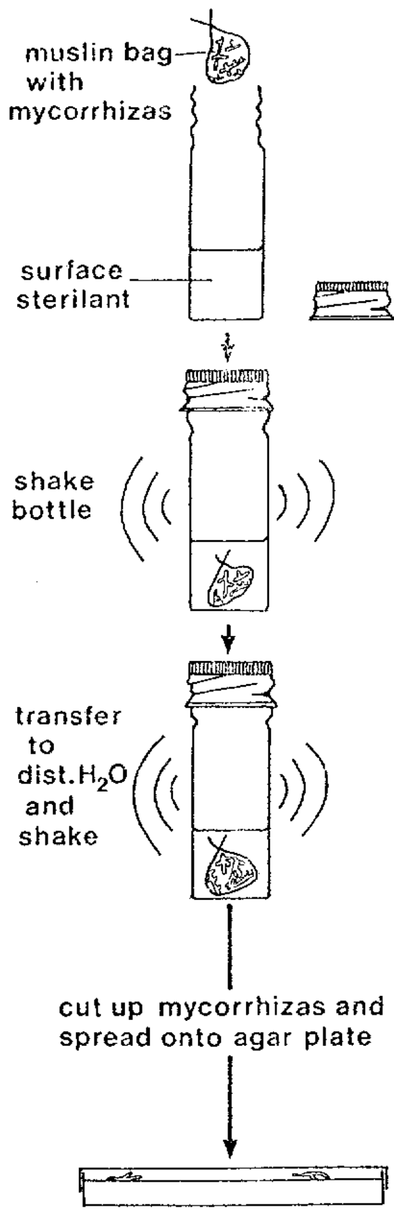
a) Mix vermiculite granules retained by a 2 mm sieve with acid peat at *c* pH 3.5 to give, after adding nutrients and autoclaving, a pH of 4.8-5.2. The ratio of vermiculite to peat varies from batch to batch but is near 9:1 by volume. Unlike vermiculite, the peat used has passed through a 1 mm sieve.

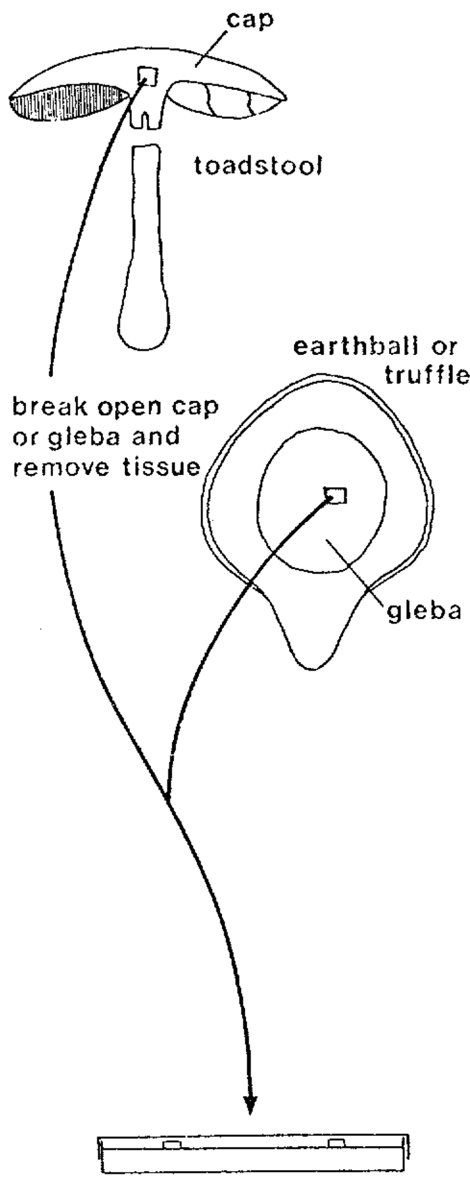
b) Place 250 ml of vermiculite/peat mixture in each 500 ml wide-necked Erlenmeyer flask, add 180 ml of a nutrient solution (eg MMN) and autoclave for 30 minutes at 121°C. For liquid culture, use 200 ml nutrient solution per flask. Add 2-3 pieces of broken glass for later fragmentation of the growing mycelium. Liquid flasks need only be autoclaved for 15 min at 121°C.

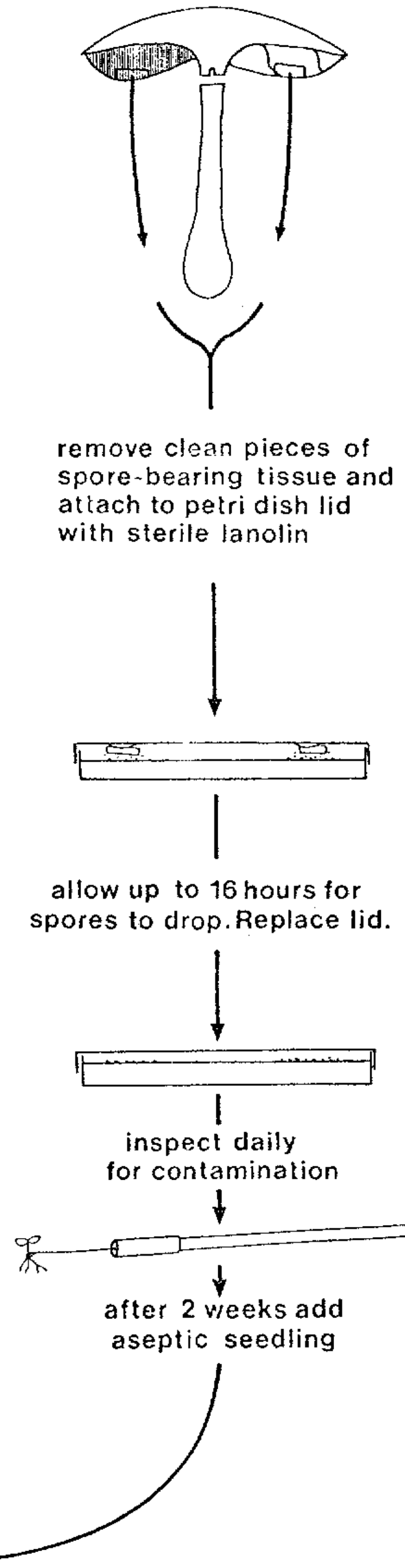
c) Cool and inoculate each flask with four 2-3 mm^3 agar blocks of inoculum.

d) Incubate the inoculated flasks at 20°C for 3-5 months. Inspect regularly for possible contaminants and/or poor mycelial growth. Shake the flasks each month.

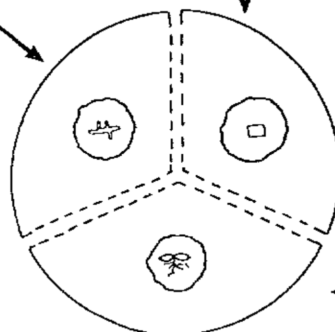
Isolation methods for ectomycorrhizal fungi







inspect daily for contamination



2.1.4 Maintenance and growth in pure culture

After 1-2 months growth, cultures established from fruitbody tissue, germinated spores or roots should then be grown on different media to determine their growth preferences. For basidiomycetes and ascomycetes media such as Hagems, MMN and PDA are appropriate (appendix 1). Many fungi respond to different concentrations of nutrients in these media, particularly glucose, so that full, ½ and ¼ strength formulations should be tested. The initial growth which develops on the isolation plate provides an insight into the isolates media preferences. A preponderance of aerial hyphae indicates the fungus is strongly aerobic and should be maintained on a solid medium such as vermiculate/peat (V/P). A preference to grow on or below the surface of the agar indicates that the fungus should be maintained in liquid media. Cultures can also be tested for optimum temperature requirements. Although most fungi will grow best between 20-25 °C, gasteromycetes such as *Pisolithius tinctorius* (Pers.) Coker & Couch and *Scleroderma citrinum* Pers. have been found to tolerate temperature in excess or 30 °C.

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2.2.1 Pure culture synthesis

Initially pure culture synthesis, although somewhat artificial, will confirm the ability of a particular host-fungus combination to form ectomycorrhizas. The screening of fungal isolates under strictly controlled conditions (eg within the laboratory glasshouse or a growth cabinet) will require a standardised method of inoculation and a uniform dose rate. Clean, aseptic seedlings need to be produced by surface sterilising healthy seed and germinating the seed on sterile water agar. Soon after germination, young seedlings can be transferred to the growing substrate (eg autoclaved V/P) and simultaneously inoculated by placing pieces of mycelium (liquid culture) or small pieces of V/P inoculum adjacent to the lateral roots in the planting hole. Alternatively the V/P inoculum can be mixed with the growing substrate (1 part V.P inoculum plus 3 parts growing substrate) just before 'potting on' of the seedlings.

If the pure culture synthesis is effected in a glasshouse (eg in pots or seed trays), the area should be kept very clean and should experience a minimum of air turbulence to lessen the chance of contamination. Typical ectomcyorrhizal syntheses are ready to assess after 3-4 months, depending on the growth rates of fungus and host.

2.2.2 Ectomycorrhizal inoculation for nursery and field studies

Because of the risk of increased contamination, successful production and testing of ectomycorrhizal seedlings at nursery and field is dependent upon the type and age of inoculum used, timing of inoculation, inoculum density, inoculum placement in the growing medium and a number of host and fungus interactions. Mycorrhizal inocula consisting of soil from natural stands, mycorrhizal roots, fruitbodies, spores and pure cultures have all been used to inoculate seedlings.

The most widely used natural inoculum is soil or humus from established plantations. A major drawback with soil or humus inoculum is the lack of control of species of ectomycorrhizal fungi in the inoculum. There is no assurance that soil inoculum contains the most desirable fungi for the tree species being inoculated. In addition, soil inoculum may contain harmful micro-organisms and noxious weeds.

Spore inoculum is now being used widely to inoculate tree seedlings in nurseries. Recent studies have revealed that spores can be applied in various forms including:-

a) spores mixed with a physical carrier before soil inoculation
b) spores suspended in water and drenched onto soil
c) spores dusted onto soil
d) pelletized spores broadcast onto soil
e) spore encapsulated seeds, and
f) spores in hydrocolloid chip

An advantage of spores is their ability to maintain viability in storage from one season to the next. However, this depends on sufficient fruitbodies being available every year. Nevertheless spore inocula are now commercially available in the United States from International Forest Seed Co. and Southern Pine Inc. in Alabama.

Of all the types of inoculum used, pure cultures have been repeatedly recommended as the most bioliogically sound. Unfortunately large scale nursery applications of pure mycelial cultures have been severely hampered by lack of sufficient quantities of viable inoculum. It is relatively simple to produce sufficient inoculum (eg 30 to 40 litres V/P or liquid inoculum) for research studies in containers, pots and small nursery and field plots. However, it is completely different to produce sufficient inoculum for large-scale nursery inoculation. Nevertheless, if adequate inoculum can be produced, inoculation of nursery seed beds using V/P inoculum has proved most successful when the inoculum has been applied in a layer just below the soil surface, immediately prior to sowing.

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At the completion of an ectomycorrhizal experiment, whether it be in the laboratory, glasshouse, nursery or field, it will be necessary to record the abundance of ectomycorrhizas present in order to relate mycorrhizal development to different parameters of growth.

Whatever the size of plant, the intact root system should be washed clean in gently runningwater over a 1-2 mm sieve in order to trap any mycorrhizas that become removed. Roots growing in mineral soils can be cleaned readily, but those growing in soils of a more organic nature will require the careful (and tedious!) removal of adhering particles, using fine forceps under a stereo dissecting microscope.

Where seedlings are small, the entire root system can be placed in water in a petri dish and observed for ectomycorrhizal formation.

However, when seedlings with root systems more than 50 cm in length are assessed, the intact root system should be laid across a grid of parallel lines 1 cm apart. With the root collar positioned at zero, transverse cuts should be made at 1, 2, 4, 5, 7, 8, 10, 11 cm and so on across the roots. Subsequently, only the 1 cm lengths (1-2, 4-5, 7-8 etc) need to be transferred to separate dishes for detailed examination. By using ythis method (Last *et al.*, 1985) for large root systems, the number of roots needing to be counted is considerably reduced.

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Thus the extent of mycorrhizal development (ie percentage infection) within a particular root system can then be estimated by calculating -

$$\frac{\text{total number of mycorrhizas}}{\text{total number of roots}} \times 100$$

Counts can also be made of mycorrhizal apices (tips) as results suggest that seedling growth can be more strongly correlated with numbers of mycorrhizal tips rather than numbers of complete mycorrhizal units.

2.5 Characterisation of ectomycorrhizas

If an assessment of the performance of a particular inoculant fungus needs to be made, then the number of mycorrhizas and hence degree of infection by each mycorrhizal type needs to be recorded.

Typically, ectomycorrhizal fungi form mantles with associated hyphae that are characteristic for the species. This enables individual mycorrhizas to be characterised and identified so that proportions of different fungi on a root system can be accurately assessed. Although quantitative assessment of different mycorrhizal types is made under the stereo dissecting microscope the validity of these separations should be confirmed by examining individual mycorrhizas microscopically under a compound microscope. At ITE the following methods are employed:

Macroscopic separation (x5 - x50 magnification)

Populations of mycorrhizas can initially be separated on features such as colour, form, size, associated hyphae, strands and sclerotia. At the higher level of magnification (x50), individual hyphae and specialised mantle cells, such as setae and cystidia, may just be discernible. These features should be recorded while the mycorrhizas are still fresh.

The validity of the separation should be confirmed by selecting a minimum of five typical members of each population for microscopic examination of a whole root mount. Where there are mixtures of similar mycorrhizas, further samples should be taken.

Microscopic separation (x500 - x1000 magnification)

Mycorrhizas should be mounted on glass slides in both lactophenol cotton blue and toluidine blue for about 10-15 seconds, before being squashed firmly under a cover glass. Several mycorrhizas can be examined under a single cover glass.

Mounting stains used are:

i) 0.1% (w/v) cotton blue in 10% (v/v) lactophenol/H_20

This is a good general-purpose stain to examine associated hyphae, strands and mantle surfaces, staining most fungal tissues blue. We use a small concentration of lactophenol to avoid shrinkage of stained cytoplasm, which makes details of septa and clamp-connections difficult to observe.

ii) 0.1% (w/v) aqueous toluidine blue

This stains cell walls and is effective in highlighting the structure of smooth compacted mantles. It is also useful in looser mantle structures, as it can penetrate to lower tissues without staining the surface hyphae too strongly. Because of the metachromatic properties of toluidine blue, many fungi produce a diagnostically useful, if not distinctive, colour reaction which may range from blue → purple → violet → pink.

Features examined (Figure 1).

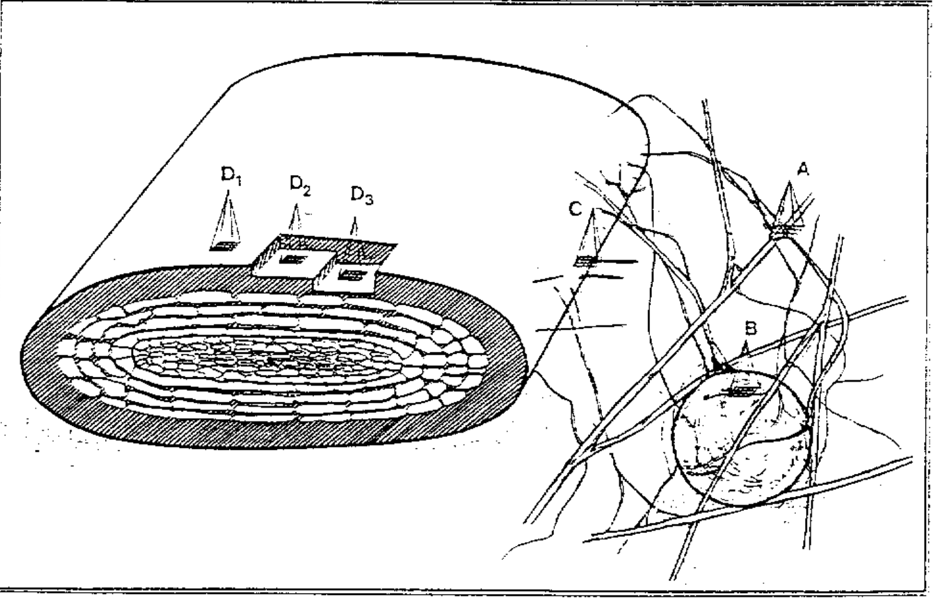
A. Strands and associated hyphae
B. Sclerotia
C. Mantle edge, emanating hyphae, specialised elements. These features are observed by moving to the edge of the mycorrhiza and focusing on the mantle surface, which is then viewed tangentially.
D. Mantle as seen in plan view. One to three layers can be distinguished in different mycorrhizas. Where this is only one distinct layer, it has been designated D1 and the inner D2; where are three layers, the surface, intermediate and inner layers are designated D1, D2 and D3. In some rare instances, it is possible to observe the Hartig net where mantles are very thin or absent.

NB - not all of these features will be found in each mycorrhizal type.

Finally, a sample of each mycorrhizal type should be preserved in 2% glutaraldehyde and stored at 4°C in an herbarium.

Details on characterisation and identification of ectomycorrhizas along with 24 descriptions of fungi frequently found on tree seedlings are given in Ingleby *et al.*, 1990. Further descriptions of ectomycorrhizal types are published by Agerer (1988).

SKETCH OF A SQUASHED MYCORRHIZA SHOWING THE LOCATION OF FEATURES DESCRIBED IN THE MICROSCOPIC EXAMINATION



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Appendix I. Culture Media for Ectomycorrhizal Fungi

Hagems medium

$MgSO_4$ $7H_20$	0.5 g
K H_2 PO_4	0.5 g
NH_4 Cl	0.5 g
Fe Cl_3 (1% soltn)	0.5 ml
Glucose	5.0 g
Malt extract	5.0 g
Thiamine HCl	50 ug
*Agar	10.0 g
Dist. H_20	1000 ml

Modified Melin Norkrans' medium (MMN)

Ca Cl_2	0.05 g
Na Cl	0.025 g
KH_2 PO_4	0.5 g
$(NH_4)_2$ H PO_4	0.25 g
Mg $S0_4.7H_20$	0.15 g
Fe Cl_3 (1% soltn)	1.2 ml
Thiamine HCl	100 ug
Glucose	10.0 g
*Agar	10.0 g
Dist. H_20	1000 ml

Potato Dextrose Agar (PDA)

Potato extract	200.0 g
Dextrose	20.0 g
*Agar	15.0 g
Dist. H_20	1000 ml

*Omit if solutions are required, ie for vermiculite/peat or liquid culture in flasks.

Appendix 2. Flask culture

a) Mix vermiculite granules retained by a 2 mm sieve with acid peat at *c* pH 3.5 to give, after adding nutrients and autoclaving, a pH of 4.8–5.2. The ratio of vermiculite to peat varies from batch to batch but is near 9:1 by volume. Unlike vermiculite, the peat used has passed through a 1 mm sieve.

b) Place 250 ml of vermiculite/peat mixture in each 500 ml wide-necked Erlenmeyer flask, add 180 ml of a nutrient solution (eg MMN) and autoclave for 30 minutes at 121°C. For liquid culture, use 200 ml nutrient solution per flask. Add 2-3 pieces of broken glass for later fragmentation of the growing mycelium. Liquid flasks need only be autoclaved for 15 min at 121°C.

c) Cool and inoculate each flask with four 2-3 mm^3 agar blocks of inoculum.

d) Incubate the inoculated flasks at 20°C for 3-5 months. Inspect regularly for possible contaminants and/or poor mycelial growth. Shake the flasks each month.

Isolation methods for ectomycorrhizal fungi

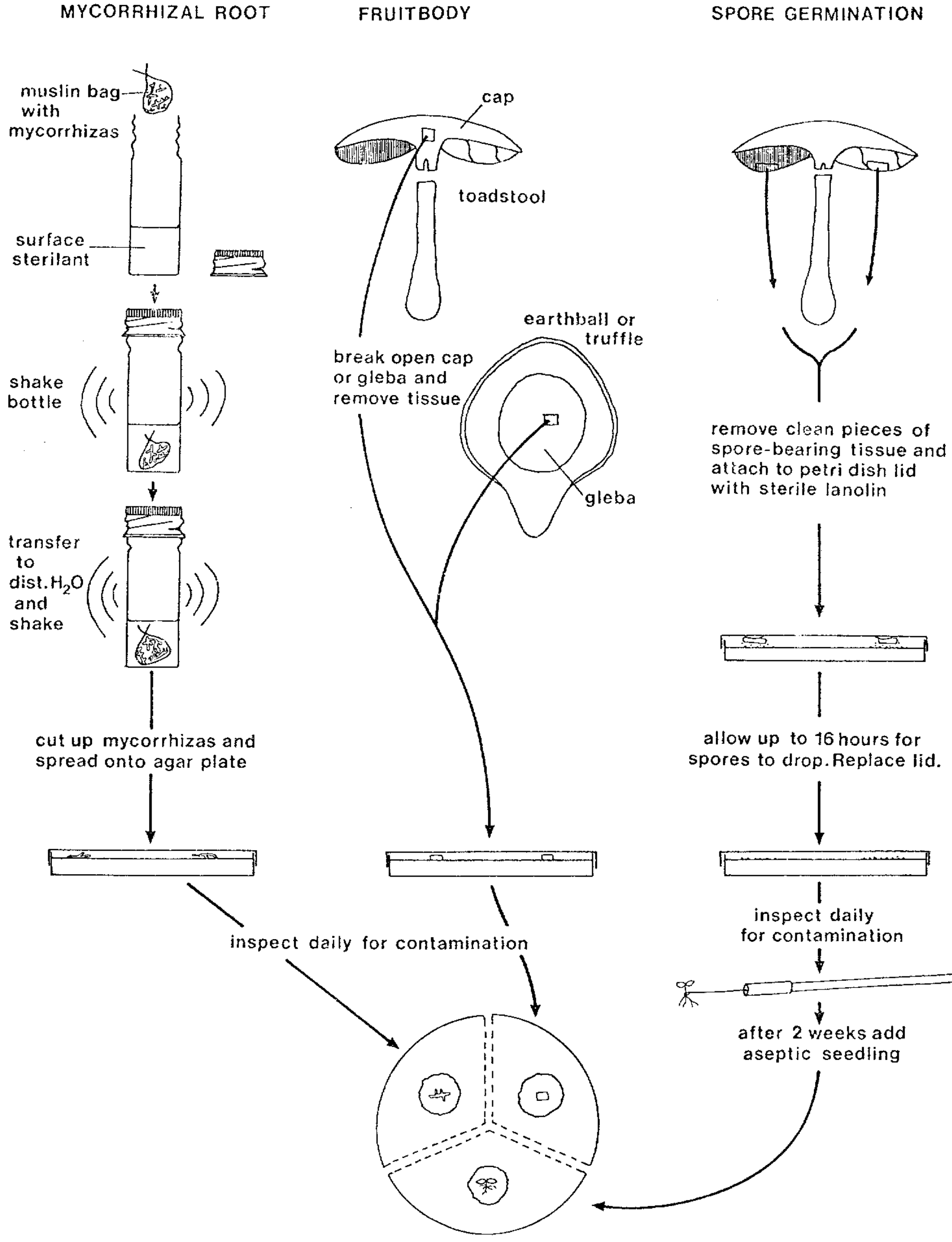
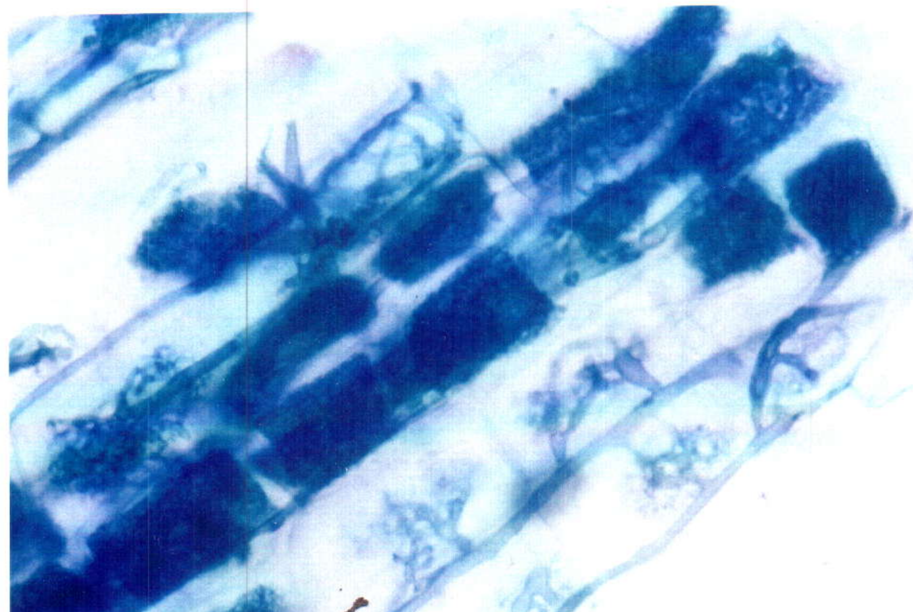
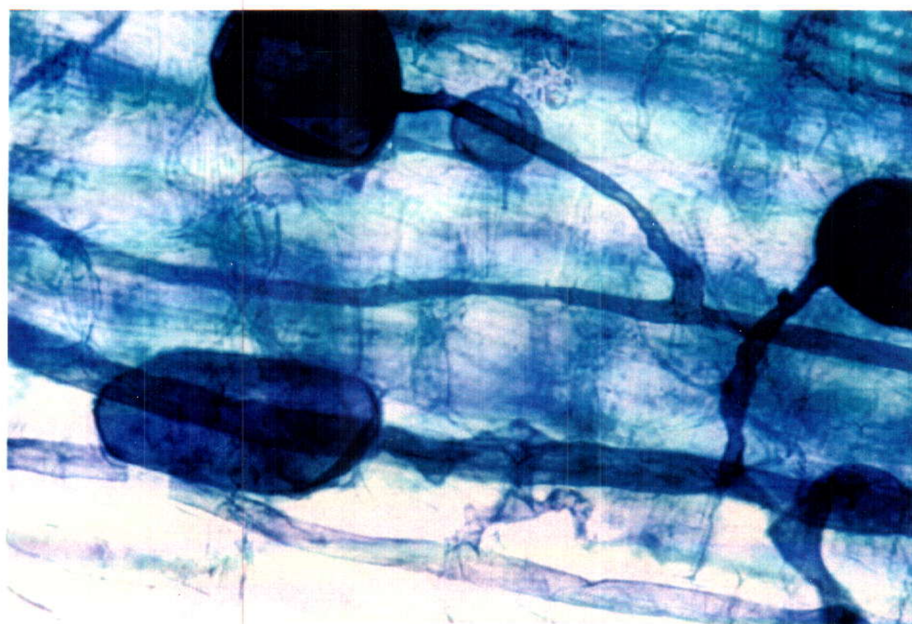


Plate 1



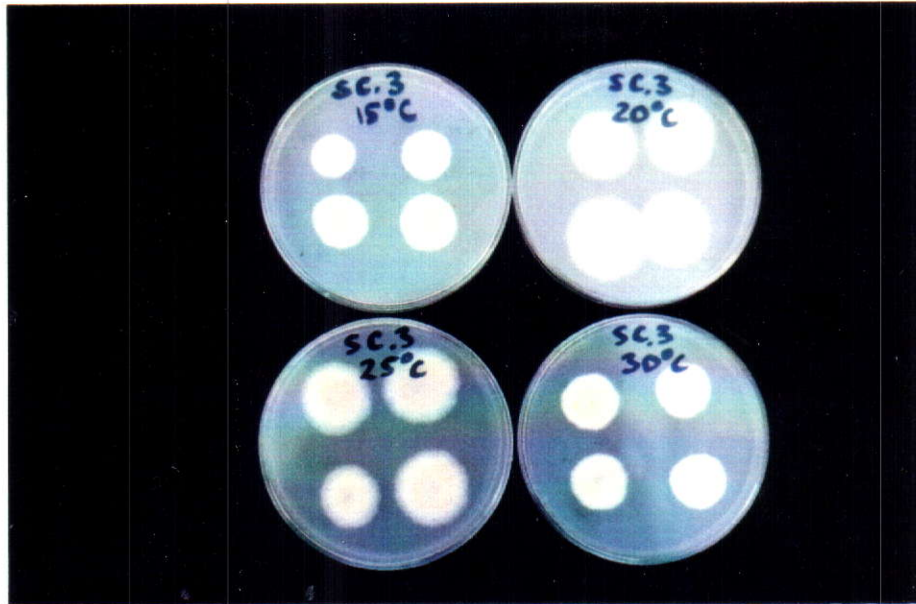
Arbuscular infection within roots



Vesicles and intercellular hyphae within roots

Plate 1

Pure culture of ectomycorrhizal fungi



a) Growth on agar medium at different temperatures

b) Growth in solid (V/P) and liquid media