



## **Guide to handling of tropical and subtropical forest seed**

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*Publication date:*  
2000

*Document Version*  
Publisher's PDF, also known as Version of record

*Citation for published version (APA):*  
Schmidt, L. H. (2000). Guide to handling of tropical and subtropical forest seed. Danida Forest Seed Centre.

# MICROSYMBIONT MANAGEMENT

13

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<b>Contents</b>	<b>13.1 Introduction</b>	<b>1</b>
	<b>13.2 Terminology and Classification</b>	<b>2</b>
	<b>13.3 Biology of Microsymbionts</b>	<b>3</b>
	13.3.1 Mycorrhiza	3
	<b>13.3.2 Rhizobia</b>	<b>5</b>
	13.3.3 Frankia	7
	<b>13.4 Collection and Handling Environment</b>	<b>8</b>
	<b>13.5 Inoculant Types and Inoculation Technique</b>	<b>9</b>
	13.5.1 Soil inoculant	10
	13.5.2 Infected roots and nodules	11
	13.5.3 'Nurse' seedlings	12
	13.5.4 Spores and sporocarps	13
	13.5.5 VAM pot culture	16
	13.5.6 Cultured inoculants	16
13.5.7 Coating and pelleting	15	
13.5.8 Inoculant granulates, tablets and capsules	16	
<b>13.6 Inoculation Conditions</b>	<b>16</b>	
<b>13.7 Storage of Microsymbionts</b>	<b>17</b>	
<b>13.8 Fumigation, Pesticides and Pretreatment</b>	<b>20</b>	
	<b>REFERENCES</b>	

Other Chapters of the book Guide to Handling of Tropical and Sub-Tropical Forest Seed by Lars Schmidt soon available on [www.dfsc.dk](http://www.dfsc.dk)

- Chapter 1: Introduction
- Chapter 2: Seed Biology, Development and Ecology
- Chapter 3: Planning and Preparation of Seed Collections
- Chapter 4: Seed Collection
- Chapter 5: Fruit and Seed Handling between Collection and Processing
- Chapter 6: Seed Processing
- Chapter 7: Phytosanitary Problems and Seed Treatment
- Chapter 8: Seed Storage
- Chapter 9: Dormancy and Pretreatment
- Chapter 10: Germination and Seedling Establishment
- Chapter 11: Seed Testing
- Chapter 12: Genetic Implications of Seed Handling
- Chapter 13: Microsymbiont Management
- Chapter 14: Seed Documentation
- Chapter 15: Trade and Transfer of Forest Seed

## 13.1 Introduction

Microsymbionts encompass soil-living organisms that form symbiosis with plant roots. There are three types of organism that are important for cultivated plants *viz.* mycorrhizas, rhizobia, and frankiae. Mycorrhiza (gr. 'fungus-root') is formed by virtually all forest trees, and many are obligate or facultative mycotrophs meaning that the trees grow poorly, especially under infertile soil conditions, without the mycorrhizal symbiont. A large group of important forest and agroforestry trees of the family Leguminosae depends on the bacterial symbionts, rhizobia (largest genus *Rhizobium*), which cause the formation of nitrogen-fixing root nodules. Some trees like *Alnus* and *Casuarina* species form nitrogen-fixing symbiosis with the bacteria *Frankia*. The bacterial associations rhizobia and *Frankia* are exclusively linked to nitrogen fixation while mycorrhiza play multiple roles in nutrient uptake (mainly phosphorus) and in protecting roots from infection and stress. Many leguminous and actinorhizal (associated with *Frankia*) trees depend on an association with both mycorrhiza and rhizobia or *Frankia* and must consequently be inoculated with both.

Microsymbionts are often present in the soil at the planting site if the site has borne trees of the same or a closely related species within a fairly recent past. In these cases seedlings will normally be infected and form symbiosis with the organism soon after outplanting. Where forest soil is used as sowing or potting medium, seedlings may easily be inoculated via the soil, and some types of microsymbiont may be naturally dispersed to the nursery plants from other host plants or from a closely located forest. However, in modern nursery and planting practices, microsymbionts are often absent, and must consequently be applied by active inoculation, e.g.:

1. Where species are grown on a site for the first time, and the species need specific types of symbiont not likely to be naturally present.
2. Where seedlings are raised on sterile medium such as vermiculite or fumigated soil.
3. Where planting is undertaken on denuded and eroded land, poor in nutrients and depleted of natural soil microsymbionts. Generally the survival of symbionts is short when their host species has disappeared.

## 13.2 Terminology and Classification

Failure to establish appropriate symbiosis may cause complete crop failure, or production may be very low, especially on poor soil. On the other hand, productivity may increase significantly by using selected inoculant species or strains instead of naturally occurring ones. For example, in *Pseudotsuga menziesii*, wood production in trees inoculated with a superior strain was more than 100% above the naturally inoculated control after an 8-year study period (Le Tacon *et al.* 1992). In *Paraserianthes* (former *Albizia*) *falcataria*, the best *Rhizobium* strain gave 48% better height growth than the poorest strain (Umali-Garcia *et al.* 1988). Smaller, yet significant differences have been found between different strains of *Frankia* on inoculation of *Casuarina* (Rosbrook and Bowen 1987) and *Alnus* species (Prat 1989).

Because microsymbionts are associated with established trees and often species specific, they are often conveniently collected at the same time as the seed. Since application ('inoculation') is normally undertaken in connection with propagation (whether vegetatively or by seeds), microsymbiont management forms a natural extension of seed handling, and often runs parallel with seed handling. Many forest seed centres, seed banks and other seed and propagule suppliers, who collect, store and distribute seeds and propagules also supply inoculants. Effective management of microsymbionts implies the technical skill of and facilities for identification, collection, extraction, propagation, storage, distribution and inoculation. Detailed descriptions and guidelines have been elaborated for many temperate species, for mycorrhizae especially on pines, for rhizobia especially for agricultural crops. Many of these methods can be generalised to other species and conditions. Some research has been carried out and experience achieved for the most important tropical trees. Unfortunately, a major drawback of microsymbiont management and handling is the requirement for technical facilities. Whereas the actual inoculation is relatively simple, several methods of inoculant production require facilities for aseptic (sterile) operation. This is especially true for rhizobia identification and cultivation, which requires a laboratory equipped for microbiology. If these facilities are not available, one must either rely on inoculants purchased from other places or use simpler methods where less advanced equipment is required.

Microsymbionts are either bacteria or fungi that form a close association with a host plant. The association is denoted a **symbiosis**, which strictly means 'living together', but often implicitly means: to mutual benefit. Microsymbionts infect the feeder root of the host. However, unlike pathogenic infection there are no disease symptoms, and in contrast to a parasitic infection there is a two-way benefit, a nutrient exchange: the plant provides the infecting organism with photosynthates (e.g. sugar); the microsymbiont in turn provides nitrogen or phosphorus depending on infection type. In the two types of bacterial symbiosis the infection is concentrated in special parts of the root, where the host plant forms root nodules which are bacterial colonies surrounded by host tissue. The symbioses exist both in herbal and woody plants, and many plant species have both bacterial and fungal symbiosis.

**Fungal symbionts** are the mycorrhizas, which form the most widespread symbiosis between plants and microorganisms. The whole group of host plants for mycorrhiza is called **mycothrophi**.

There are two types of **bacterial symbionts** viz. rhizobia, named after the most important genus, *Rhizobium*, which forms symbiosis with host species of the family Leguminosae, and frankiae with the one genus, *Frankia*, which lives in association with a number of tree species from different families. Frankiae are actinomycetic bacteria which infect roots (gr. 'rhiza') of their host plants; therefore the hosts are collectively called **actinorhizal** plants.

### 13.3 Biology of Microsymbionts

#### 13.3.1 Mycorrhiza

Mycorrhizal symbiosis functionally forms an extension of the plant root system. A fine net of fungal hyphae in close contact with the plant roots extend their threads into a large volume of soil where they explore and extract nutrients from the soil beyond the reach of the plant roots. The nutrients are translocated through the fungal hyphae, hence bringing them to the plant roots, where they can be assimilated and used by the plant. The fungus, in return, is provided with simple sugars and possibly other compounds from the plants' photosynthesis. Some mycorrhizal fungi produce plant hormones, which stimulate root development, e.g. *Pisolithus tinctorius* on poplars (Navratil and Rachon 1981).

Mycorrhiza is known to protect the roots of the host plant against pathogens and certain toxins, and mycorrhizal plants generally have a higher resistance to drought, soil acidity, and high soil temperatures (Redhead 1982). The fungal sheath surrounding the feeder roots of ectomycorrhiza often has a higher resistance to toxins, acids etc. than the plant root and can consequently form a physical barrier to the soil. Further, soil will adhere to the mycorrhizal net thereby decreasing 'shock' when the seedlings are exposed to field conditions; that is especially important for bare-root seedlings, where mycorrhiza may also reduce the risk of desiccation of the roots during transportation. Mycorrhizal symbionts are grouped into two main types according to the symbiotic structure of the root system: ectomycorrhiza and vesicular-arbuscular mycorrhiza (VAM). A transition group, ectendomycorrhiza, has similarities with both groups but is much less common and only mentioned superficially in this book. Some characteristics of the two main groups are enumerated here:

**Ectomycorrhiza.** The fungal hyphae form a distinct mantle surrounding the root and penetrating between, but not into, the root cortical cells. Macroscopically the ectomycorrhizae are recognised as a white or brightly coloured cover of the roots. They are known to produce plant hormones that stimulate root branching and growth, hence increasing the absorptive surface of the root. Feeder roots infected with ectomycorrhiza are usually short, branched, swollen and lacking root hairs. Many of the common mushrooms, puffballs and truffles form ectomycorrhizal associations with higher plants; the 'mushroom', as we see it, is the reproductive structure ('fruiting body') of the fungus. Ectomycorrhizal fungi usually form large amounts of minute spores, which are dispersed by wind.

Fungi forming ectomycorrhizae are not obligately associated with roots but can live as saprophytes e.g. on rotten plant material such as decaying wood. This implies that the fungi can be cultivated and propagated separately from the host plant, and that they may survive in the soil for some time without a host. Ectomycorrhizae are often relatively host specific, i.e. a particular fungus only lives in symbiosis with one or a few, often related, host plants, and some trees form mycorrhizal symbiosis with few fungi only. Other ectomycorrhizae have a broad host range (Castellano and Molina 1989, Trappe 1977).

Ectomycorrhizae are not as widespread in the plant kingdom as VAM (see below) but a number of important tree species form ectomycorrhizae. The type is especially important in Pinaceae (*Pinus*, *Abies*, *Larix*, *Pseudotsuga*, *Tsuga*), and Betulaceae (*Betula* and *Alnus*) (Molina and Trappe 1984). The dipterocarps in SE Asia and the Pacific form exclusively ectomycorrhizae. Some of the main families forming ectomycorrhizae are listed in table 13.1.

**Vesicular-Arbuscular mycorrhiza (VAM)** is the main subgroup of the endomycorrhizae in which the hyphae penetrate into the root cells as well as between the cells. VAM can only be recognised microscopically since there are no morphological difference between infected and non-infected roots. Like ectomycorrhizal fungi the major part of the fungus is outside the roots, exploring and extracting nutrients from the surrounding soil, and translocating these compounds to the roots through the fungal hyphae. VAM form vesicles and arbuscules within the root cells. Vesicles are storage organs and also serve as reproductive structures. Arbuscules take part in nutrient exchange. VAM are obligately associated with plant roots and can only live and propagate within that symbiosis.

VAM produce relatively large (30-900 µm (Castello and Molina 1989)) globose spores, which are either dispersed mechanically by soil movement or by small soil-living animals, e.g. mice, rats, birds, insects and earth worms, which consume the spores together with other plant material and leave the spores undamaged with the faeces (Bagyaraj 1989). VAM do not produce large mushroom-like reproductive structures. Unlike ectomycorrhizae VAM show very little host specificity; most VAM are able to form symbiosis with a wide range of plant species, including both herbs and trees. VAM are the most widespread mycorrhiza in higher plants. Some important tropical and subtropical families forming VAM are Leguminosae, Combretaceae, Meliaceae, and Bignoniaceae, see table 13.1.

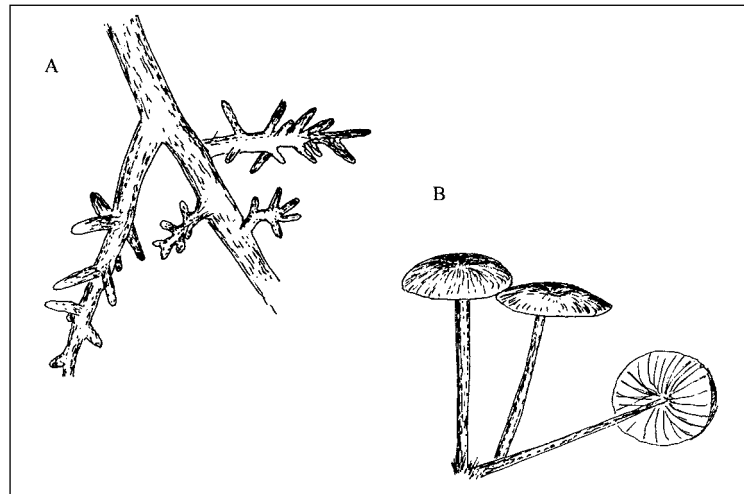
Host plants of mycorrhiza can be grouped into two types:

**Facultative mycotrophs** are plant species which are able to grow without mycorrhiza, but where mycorrhiza greatly improves growth, especially on infertile soils. They are typically early pioneers or 'gap fillers' in the natural forest. Many of the fast growing trees used in forestry belong to this group.

Obligate mycotrophs cannot grow or survive without mycorrhiza. They typically count trees of the climax or mature forest, for example many high-quality hardwood timber trees.

Figure 13.1. Ectomycorrhizal associations.

A. Roots infected with ectomycorrhiza have short, thick and characteristically branching side roots. B. The fruiting bodies of ectomycorrhizae are often large sporocarps ('mushrooms').



	ECTOMYCORRHIZA	VA-MYCORRHIZA
<b>Morphology</b>	White or brightly coloured sheath (mantel) around the host root. Feeder roots short, branched, and lacking root hairs	No morphological characters on the host root
<b>Anatomy</b>	Hyphae grow between the cortical cells but do not penetrate them	Hyphae grow between and into the cortical cells. Form vesicles and arbuscules in the host root
<b>Reproduction</b>	Minute wind dispersed spores in 'mushroom'-like sporocarps	Large (40-900 µm) spores in small sporocarps. Dispersed by soil movement or small animals
<b>Host specificity</b>	Many are host specific	Little host specificity. Most fungi form VAM with a broad host range
<b>Propagation</b>	Can be propagated separated from the host by spores or mycelium	Can only be propagated in association with a host
<b>Some main families of occurrence</b>	Dipterocarpaceae, Leguminosae, Pinaceae, Betulaceae, Fagaceae, Myrtaceae, Sapindaceae, Salicaceae	Bignoniaceae, Combretaceae, Leguminosae, Myrtaceae, Sterculiaceae, Salicaceae, Ebenaceae, Burseraceae, Euphorbiaceae, Meliaceae

Table 13.1.

Some characteristics of the most common mycorrhiza types in forest trees.

### 13.3.2 Rhizobia

<sup>1</sup> Some taxonomic systems raise the three subfamilies to family level, which then become Caesalpinaceae, Mimosaceae and Papilionaceae. Leguminosae is called Fabaceae, according to some newer taxonomic literature.

Rhizobia are a group of soil-living bacteria, which are able to live in symbiosis with and nodulate members of the plant family Leguminosae. Leguminosae is subdivided into three subfamilies, Caesalpinoideae, Mimosoideae and Papilionoideae<sup>1</sup>. More than 30% of species of the Caesalpinoideae and more than 90% of the species in the other subfamilies form nodules (Brewbaker *et al.* 1982, Dart 1988). Within the subfamilies some genera are characterised by high frequency of nodulated species and others by low. There are also species within an otherwise highly nodulating genus which fail to nodulate. Most acacias, for example, nodulate but there are exceptions (Dommergues 1982). The species-specific capability of nodulation and N-fixation is, however, subject to uncertainty since many species capable of nodulation do not form nodules in some areas, either because of absence of the proper symbiont or because environmental conditions are unfavourable to the symbiosis. There are also differences between provenances in their susceptibility to nodulation by rhizobia (Dart 1988).



Rhizobia are aerobic (require oxygen for their growth) although some species can live under very low O<sub>2</sub> concentration. They can live free in the soil as saprophytes (living on decaying organic material), but they only fix nitrogen when associated with host plants. Infection of the roots takes place through the root hairs or the cortex of actively growing young roots (Dart 1988, Somasegaran and Hoben 1994). The individual root nodules tend to become senescent with age and be replaced by new ones. Usually there is an active growth of nodules together with the most active growth of the host, i.e. in the tropics usually during the rainy season.

Generally, rhizobia prefer a pH close to neutral and most rhizobia are sensitive to acidic soils, high manganese and aluminium content (which are often associated with acidity), and toxins (Alexander 1982, Dommergues 1982). They are sensitive to high temperatures and desiccation (drought) especially in the free-living form. Because they are aerobic, they suffer from oxygen deficit e.g. at waterlogging. Finally, exposure to full sunlight (UV light) is detrimental to rhizobia (Dart 1988). The specific optima and tolerance limits to environmental factors vary enormously between species and strains (Alexander 1982, Somasegaran and Hoben 1994).

The optimal growth conditions and tolerance limits for plant and bacteria respectively are not necessarily the same in all aspects, and nodulation may not take place over the full range of conditions where the two symbionts exist. For example, high combined nitrogen (NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup>) content in the soil generally inhibits nodule formation, without being deleterious to either legume or rhizobium (Dommergues 1982). The symbiotic association often changes over a range of environmental conditions in the sense that different rhizobia species or strains infect the particular legume species according to where they grow. Some rhizobia are strong competitors with other soil-living microbes and can survive for a long time independently of their legume host. Others are much more restricted and tend to decline or disappear from the soil in the absence of the host species.

Some rhizobium - legume associations are very specific and the legume will form nodules only when infected with a specific rhizobium. Others will form nodules with a range of rhizobia. That means in practice that for the first group, inoculants must be collected from the same host species, for the second group a broad range of host species can be used as inoculant sources. Therefore, for practical purposes, legumes have been assembled into cross-inoculation groups. A cross inoculation group consists of species that will form nodules when inoculated with rhizobia obtained from nodules from any member of the group. A cross inoculation group may, in the extreme, consist of one species only. Cross inoculation groups are well established among agricultural crops but only superficially established among tree crops.

Obviously, host-specific rhizobia must be applied as inoculant when the host species is grown on a site for the first time. For other species the requirement depends on the possible available rhizobium in the

soil, i.e. whether other compatible legume hosts have grown on the site within a fairly recent past. Some Australian *Acacia* spp. grown in Africa nodulate freely with the indigenous rhizobia.

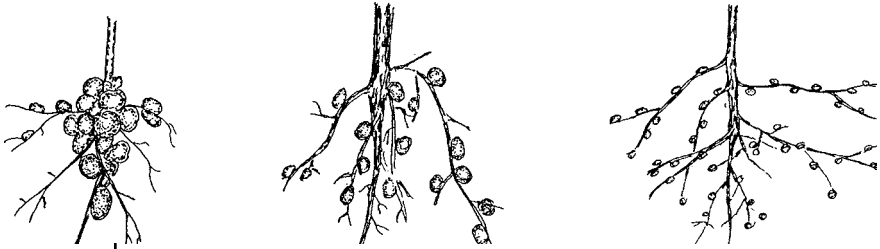


Figure 13.2. Different rhizobia root nodules of Leguminosae.

### 13.3.3

#### Frankia

*Frankia* are actinomycetic bacteria which infect roots (gr. 'rhiza') of their host plants; the hosts are collectively called actinorhizal plants. *Frankia* are filamentous, branching, aerobic, gram-positive bacteria. They differentiate into three different cell types viz. (1) vegetative cells which develop into mycelia almost like mycorrhizal fungi, (2) sporangia forming numerous spores, and (3) vesicles which are the site of nitrogen fixation (Lechevalier and Lechevalier 1990).

*Frankia* may live free in the soil as saprophytes. They are dispersed in the soil via the vegetative hyphae. The long-distance dispersal probably takes place via spores or vegetative cells in moving soil or by wind dispersal of spores; spores are relatively resistant to desiccation (Torrey 1982). Infection occurs via the root hairs or directly through the cortex of the host root, where it causes nodule formation by the host plants (Berry 1994, Berry and Sunell 1990, Lechevalier and Lechevalier 1990). The root nodules are clusters of modified lateral roots. The morphology is mainly determined by the host plant and is typically of a coralloid (like a coral) shape with many nodule lobes of various sizes (fig. 13.3). Root nodules of frankiae are perennial, new lobes being added as the nodule grows. The nodules are for most species up to 3-5 cm in diameter, but may grow even larger e.g. up to 15-20 cm diameter in *Allocasuarina verticillata* (Diem and Dommergues 1990).

*Frankia* form symbiosis with plant species from a number of distinct genera and families, many of which have no close taxonomic relation. So far, around 200 actinorhizal plants are known, distributed over 8 families and 25 genera. The most important forest trees with symbiotic relationship with *Frankia* belong to the plant family Casuarinaceae, a family that comprises almost exclusively actinorhizal plants. Apart from *Casuarina*, the family includes two other actinorhizal genera viz. *Allocasuarina* and *Gymnostoma*. Betulaceae contains only one actinorhizal genus, *Alnus*. The genus *Rubus* contains only one known actinorhizal species viz. *Rubus ellipticus* (Gauthier *et al.* 1984). *Frankia* also form symbiosis with species of the genera *Aelaeagnus* and *Hippophaë*.

Some actinorhizal plants can be inoculated with a range of *Frankia* strains while others are very specific. For example the genus *Allocas-*

*uarina* can be inoculated with strains obtained from that genus only, while *Gymnostoma* are the least specific one and can be inoculated with inoculants even from species outside Casuarinaceae; *Casuarina* spp. are intermediate between those two in terms of specificity (Torrey 1990, Gauthier *et al.* 1984).



Figure 13.3. Actinorrhizal root nodule morphology, left *Ceanothus americanus*, right *Alnus* sp. (redrawn from Berry and Sunell 1990).

### 13.4 Collection and Handling Environment

Mycorrhizae, rhizobia and frankiae are soil-living organisms and spend their entire or the greater part of their life cycle under the soil surface. They are adapted to moist, dark and relatively cool conditions with small temperature fluctuations. These conditions should be maintained during handling. Some microsymbionts form dispersal units, e.g. spores which are relatively resistant to above-soil conditions. They can survive desiccation, higher temperatures and light and have relatively long viability. Generally however, the viability of most microsymbionts is short in comparison to seeds, but there is a great variation between species within the three types. Proper handling and storage conditions can greatly improve the viability of microsymbionts.

Where inoculant material, whether soil, nodules or spores, is collected from the field, a site with mature, healthy and vigorous trees should be selected. Mature trees are likely to support the largest amount of symbionts, healthy and vigorous trees may also be an indication of good inoculation, and the risk of collecting material infected with pathogens, which could be a nuisance later on, is smaller.

Collection should be made from or under trees of the same species or species with compatible microsymbionts, for rhizobia and *Frankia* within the same cross inoculation group (Baker 1987). Collection should be made from trees growing on typical growth sites; these are likely to contain symbionts adapted to the prevailing soil type. Exceptionally good or poor sites should be avoided unless the trees to be inoculated are supposed to be grown on similar sites (Benoit and Berry 1990).

The best time of collection differs for different types of inoculant material. Soil usually contains a reasonable amount of inoculant and can be collected at almost any time of the year. Sporocarps of

ectomycorrhizal fungi are only available for a short period of the year. The moist season normally supports the greater number of sporocarps, but both duration and season of sporocarp formation vary with species. Inoculant collected from or together with host roots should generally be collected during the most active growth season, which normally is the rainy season. This is also practical as the soil is easier to dig up and there is less risk of damage to both the host tree and the inoculant.

Rhizobium nodules should preferably be collected from young roots. The nodules of older roots are likely to be senescent and contain few infective bacteria. Seedlings or young trees are the best source of nodules. Cutting and examining the interior of a few nodules with a hand lens gives an indication of the condition: fresh and active  $N_2$ -fixing rhizobium nodules are typically pink, red or brown, *Frankia* whitish or yellowish; senescent nodules are typically greyish green (Benoit and Berry 1990).

### 13.5 Inoculant Types and Inoculation Technique

Inoculant types vary from simple forms in which microsymbiont-infected soil is applied to the nursery soil, to sophisticated production of pure culture inoculants, incorporated into carriers and applied to seeds as pellets or beads. Which species and method is used is a result of balanced consideration of various factors:

1. Some commercial pure culture inoculants contain microsymbiont species which promote productivity under particular environmental conditions, but may be less productive than local species under other conditions.
2. Different methods of inoculant production and inoculation apply to different species and situations. Some tree species may only form symbiosis with specific bacteria or fungal species. Sometimes compatibility between the two organisms varies with the environment.
3. Pure culture production is usually both technically complicated and expensive. In many cases, inoculants purchased from specialised manufacturers and dealers may be more economical than starting independent production or using unselected material.

Apart from soil mixtures which may contain all types of organisms, both type of inoculant and application methods vary with type of symbiont. Mycorrhizal inoculants can be applied as spores or mycelium. Mycelium inoculates usually give faster infection but is more sensitive to desiccation and other environmental factors. They have short viability and are relatively bulky as compared to spores. Some ectomycorrhizal fungi can be grown in pure culture on a nutrient medium to obtain a mycelial culture. The spores are often initially germinated on agar prior to cultivation. Some ectomycorrhizal fungi can be multiplied by applying spores directly to the nutrient medium (Marx 1980).

VAM fungi cannot be grown in pure culture on nutrient media and are therefore multiplied by infecting roots of an intermediate host

e.g. sorghum or sweet potato with the spores of VAM. Both rhizobia and *Frankia* can be grown in pure culture but the method is too slow and too expensive for most *Frankia*. Many plants need dual inoculation with mycorrhiza and either rhizobia or *Frankia*. Generally the two types of organism are not antagonistic to each other and can sometimes be mixed. However, in many cases it is difficult to control the application rate if the two inoculants are mixed, and they are therefore usually handled separately throughout.

Inoculation rate, i.e. amount of inoculant used per seedling, varies with application method, and the concentration of infective bacteria, spores or mycelium in the inoculant. Increasing amount of applied inoculum generally speeds up the colonisation process and symbiont formation. Plants are usually inoculated in the nursery rather than during planting in the field. Nursery inoculation has the following rationale:

1. Inoculated seedlings are generally much more competitive and able to withstand the inevitable stress they will be exposed to immediately after outplanting; especially, if the plants are planted under harsh environmental conditions.
2. Early inoculation usually reduces requirement for fertilizer and pesticides in the nursery. In addition to reducing the cost and possible harmful effects of these applicants, mycorrhizal seedlings are known to be generally more resistant to pests, diseases and adverse environments.
3. Nursery inoculation opens the potential for selective inoculation with superior microsymbiont strains or types, specifically adapted to the species and the planting site and is hence potentially more effective. Selection of types and strains will not be discussed in this book; reference is made to Trappe (1977) and Marx *et al.* (1982).

Field inoculation has its main advantage in that the seedlings are exposed to the future environment when inoculated and may consequently preferably form symbiotic association with the species that are better adapted to that particular field condition. It is known for mycorrhiza that even if seedlings are inoculated in the nursery, fungal associations often change when the plants are transplanted into the field, provided that a microsymbiont is present at the planting site (Marx *et al.* 1982). Where seedlings are inoculated with several species or strains, one or few usually become dominant under the prevailing field conditions.

### 13.5.1 Soil inoculant

Forest soil or litter collected under appropriate tree species often contains a balanced population of adapted microsymbionts. As freshly collected forest soil is often used as planting medium because of its physical properties, seedlings are naturally exposed to inoculation in this way. It may also be used deliberately as inoculation material, e.g. by applying a small amount of inoculated soil or litter (leaves, needles and root fragments) to the planting medium. This method is often used for mycorrhizal inoculation, whereas for rhizobia and

*Frankia* the amount of inoculant provided this way is often too small. Soil collected from nursery beds previously supporting seedlings with good mycorrhiza is appropriate. About 10 - 15% by volume of soil is mixed into the top approximately 10 cm of the nursery bed or mixed in the same ratio into the potting soil (Molina and Trappe 1984). If soil is scarce, a handful of soil can be placed at root level during pot filling.

Problems of using soil as inoculant material are the bulk to be transported and that soil may contain infective pathogens. Fumigation and other soil sterilisation normally kill microsymbionts (see section 13.8).

### 13.5.2 Infected roots and nodules

Problems with bulk and potential pathogens may be reduced by collecting infected mycorrhizal roots and bacterial nodules only. Roots are chopped and nodules crushed before application. However, for mycorrhiza relatively large quantities are needed if the material is used as inoculant directly. Cruz (1983) estimated that at least one kg of ectomycorrhizal roots should be used per cubic meter of nursery soil to assure proper inoculation. VAM is often applied as chopped roots of intermediate hosts after being multiplied in pot culture (section 13.5.5). Crushed nodules are rarely used as inoculant for rhizobia because the number of bacteria that can be applied in this way is too small. The nodules are more often used as a source for cultured inoculants (section 13.5.6). Because the root nodules of *Frankia* are relatively large, and because culture of *Frankia* is slow, crushed nodules are often used directly as inoculant for this type. Both fresh and stored *Frankia* nodules can be used, but dry nodules should be rehydrated before crushing. A method of preparing crushed nodule inoculant of *Frankia* is here referred from Rodriguez-Barrueco *et al.* (1988), and Rosbrook (1990):

The nodules are pulverised in a mortar (possibly with dry sand) or blended in a glass homogeniser with sterilised water or 1% sucrose solution. Alternatively the nodules may be ground in 1-2% polyvinyl pyrrolidone (PVP-40), 10% (w/vol) activated charcoal, or 0.6-1% NaCl; these compounds are believed to absorb possible phenolic compounds from the plant tissue (Torrey and Racette 1989). If the inoculant is to be stored before inoculation, aseptic techniques must be applied. If PVP is used, the inoculant is washed several times with centrifugation and resuspended in 1% NaCl solution until the supernatant is clear. Note that individual salt tolerance may differ between strains. The inoculant is applied by mixing it into the planting soil or, where there is a shortage of inoculant, by banding it at root level. 0.5-2 mg or more nodule fresh weight (= 0.12 - 0.5 mg dry weight assuming that nodules contain appr. 75% water) should be applied to each plant (McCluskey and Fisher 1983, Perinet *et al.* 1985).

### 13.5.3 'Nurse' seedlings

The principle of 'nurse seedlings' is that microsymbionts from already inoculated seedlings will spread naturally to neighbouring seedlings in the nursery. Hence a precondition is that there is likelihood of movement in the soil which is the case with i.a. both ectomycorrhiza and VAM. The inoculated seedlings are planted in the nursery bed at intervals of one to two meters before the seeds are sown. Mycorrhiza will spread from the infected to the newly germinated seedlings

Figure 13.4. Seedbed with *Pinus merkusii*. Nurse seedlings have been planted in order to provide the soil, and later on the seedlings, with mycorrhiza; Indonesia.



The main advantage of nurse seedlings is that fresh inoculant material is always available and that the inoculant is adapted to the prevailing climate and nursery soil. However, the method also has certain drawbacks:

- a. The nurse seedlings may compete with the young established seedlings for nutrients and light
- b. The nurse seedlings may interfere with the preparation and management of the seed bed
- c. Inoculation may be slow and uneven
- d. Fumigation or other soil sterilisation procedures cannot be undertaken after the nurse seedlings have been planted. Therefore there is a higher risk of soil pathogens and competition with naturally disseminated mycorrhizal fungi

### 13.5.4 Spores and sporocarps

This method is limited to species of ectomycorrhiza with abundant sporocarps. Sporocarps may be used directly as inoculant or extracted spores are germinated and used for mycelium culture (see section 13.5.6). Direct use as inoculants is described in Boyle *et al.* (1985), Boyle *et al.* (1987), Castellano and Molina (1989), Cruz (1983), Mikola (1970), Molina and Trappe (1984), Theodorou (1984), and Theodorou and Benson (1983).

Sporocarps are collected and rinsed with tap water and surface sterilised. To minimise bulk the 'stem' of mushrooms can be removed in the field as it contains no spores. The sporocarps are then treated differently according to application method and whether fresh or stored material is used:

1. Chopped into pieces and applied as such.
2. Macerated and blended into a homogeneous material and suspended in sterile water.

3. Dried (initially in air, later in a desiccator at approx. 30°C) until they are completely dehydrated, then crushed into fine powder, stored in sealed polythene bags or mixed in water and applied.

Chopped sporocarps are mixed with the nursery soil, either in the seedbed or in the transplanting or potting soil. Dry or suspended macerated or blended fresh material may be applied by several methods:

1. Spraying on the surface with a watering can prior to sowing, and mixing with the soil by raking. Spraying may also be delayed to 6-12 weeks after sowing when the roots of the seedlings are susceptible to inoculation. Excess watering prevents desiccation and facilitates incorporation into the soil. Applying spores twice, 2-3 weeks apart, gives a good result (Castellano and Molina 1989).
2. Injecting the inoculum into the soil at root level of each seedling.
3. Dusting the dry mixture onto the soil and mixing it into the soil by raking, or mixing it with the potting soil.
4. Dipping the roots of bare-root seedlings into a slurry of suspended material.
5. Applied to seeds by coating, pelleting, capsules or tablets (see section 13.5.7 and 13.5.8).

Inoculation with spores or sporocarps is very efficient where abundant sporocarps are available. Both dry sporocarps and suspended material can be stored at least from one season to the next. However, compared to mycelia cultures, spore inoculants give relatively slow mycorrhiza formation (several weeks to a few months).

### 13.5.5 VAM pot culture

Since VAM do not form sporocarps, spores must be multiplied on a cultured host plant. Spores of VAM, either single or in lumps are extracted from the soil by wet sifting, i.e. washing the roots with a strong stream of water. The spores are large enough to be subsequently retrieved manually under a stereo microscope (Hepper 1984). They are then surface sterilised and applied to an aseptic growth medium in which a host plant is grown, e.g. clover, sorghum or sweet potato. The spores colonise the host plant roots, form VA mycorrhiza and produce new spores. The spores can be retrieved from the growth medium and used as inoculant or the entire medium with spores and VAM are chopped and used as inoculum. Spores or spores + infected roots are mixed with soil or banded 3-5 cm below the soil surface. Application rate appr. 200-500 spores per seedlings or 20 ml of pot cultured inoculum (spores + roots) per seedling.

Once the culture has been started, host plants can be maintained as a resource base for inoculants (nurse seedlings). It should be noticed that because VAM has a wide host range, including herbal plants, inoculation with VAM is usually not necessary unless sterile growth media are used.

### 13.5.6 Cultured inoculants

In this context cultured inoculants include all types of inoculants multiplied on or in a nutrient medium away from host plants. This covers mainly ectomycorrhiza and rhizobia, and to a lesser extent *Frankia*.



VAM cannot be cultured away from host plants. Pure fungal and bacterial culture implies working under aseptic (sterile) conditions.

**Mycorrhiza.** Mycelium can be grown either from spores germinated on agar, or extracted from infected roots. Agar culture is prepared by adding 15 g agar per litre nutrient solution before autoclavation. When it has been cooled down to 60°C, the culture is poured into petri dishes and cooled to solidify. Spores are placed on the medium and incubated at 30°C. When spores have germinated and mycelium started to form, it can be transferred to the liquid culture (see below), or when abundant mycelium has formed, pieces of agar with mycelium are applied to each seedling. In some cases the agar culture can be omitted if the spores will germinate in liquid culture.

Mycelium extracted from infected roots or grown from spores germinated on agar nutrient culture can be multiplied in a nutrient solution, e.g. cooked and sterilised cereal grains as wheat or white millet, possibly with particular nutrients added as required for the species in question. The mycelium is grown in small flasks for some days, then transferred to larger containers (e.g. 10 l tanks) containing the same nutrient solution and aerated two to three hours daily for 3-4 months. The liquid with mycelium is poured into e.g. 5 l containers or sterile polythene bags containing sterilised substrate (e.g. peat moss, sterile soil, sawdust or vermiculite) and fresh nutrient solution. The mycelium continues to grow in the substrate and is ready to use after another few months.

If the culture is to be stored before use, it is recommended to remove non-assimilated nutrients in the solution by leaching. This is done by placing a few litres of inoculum in a double layer of cheesecloth and rinsing it for several minutes under tap water. Excess water is removed by squeezing the inoculum in the cheesecloth by hand (Mikola 1970). In addition to preventing saprophytic contamination of the inoculant by removing excessive nutrients the procedure also reduces the volume.

The culture is applied to the nursery soil for example by placing it in 10 cm deep furrows next to the seedlings at appr. 3-4 litres of inoculum per m<sup>2</sup>, or by broadcasting and mixing it with the upper 10 cm of the soil at 8-10 l per m<sup>2</sup>. The soil should be fumigated before application to avoid competition with native fungi.

A mycelium culture normally gives a fast mycorrhizal formation as compared to spore inoculation. The drawbacks are that the method is relatively expensive, labour intensive and requires special skills and facilities for growing culture under aseptic conditions, and further that some species cannot be grown or grow very slowly in pure culture. Many require specific growth substances and are sensitive to growth inhibitors (Marx 1980, Molina and Trappe 1984). Lastly, mycelium culture is relatively bulky, and the sensitivity to e.g. desiccation and temperature is higher than for spore inoculants.

For further information, reference is made to Boyle *et al.* (1987), Castellano and Molina (1989), Marx (1980), Marx *et al.* (1982), Mikola (1970), Molina and Trappe (1984).

**Rhizobium.** The production of rhizobium inoculant consists of a series of processes from extraction of rhizobia from the nodules to incorporation of the broth culture into the carrier. The production is more complicated than mycorrhizal multiplication and will only be described in brief here. In practice the production of bacterial inoculants is restricted to special set-ups, where bulk production can take place under aseptic (sterile) conditions, i.e. in a laboratory equipped for microbiology (FAO 1983, Somasegaran and Hoben 1994).

Bacteria extracted from root nodules are initially grown on agar with applied growth medium, then multiplied in broth culture. Both agar and broth cultures grow best at a temperature of 25-30°C; some species and strains have a slightly different temperature optimum. Rhizobia are aerobic and require oxygen for growth. This is relevant for broth cultures, which must be aerated during processing (Burton 1979, Somasegaran and Hoben 1994). Once the bacterial culture has multiplied well, it is incorporated into a carrier. Peat is the most common carrier material, but maize cob compost, charcoal, cow dung and other material are also used. The broth culture is mixed with the carrier and incubated at 25-30°C for approx. 2 weeks, during which the bacteria continue to multiply. The ready inoculant can be stored under dark and cool conditions. The inoculant can be applied directly to the soil by mixing it into the seed bed, by suspending it in a clay slurry and applying it to bare-root seedlings by root dipping (Dart 1988), or by applying it to the seed by coating or pelleting (see chapter 9.7).

**Frankia.** Production of pure *Frankia* inoculants basically follows the same procedure as for rhizobia, but is generally more complicated. The production procedure is described in detail in Lechevalier and Lechevalier (1990), and Diem and Dommergues (1990). *Frankia* inoculants consist of the bacteria culture only and are not imbedded in a carrier as for rhizobia. It is usually applied as the cell suspension directly, or imbedded in alginate beads.

### 13.5.7 Coating and pelleting

The basic principles of coating and pelleting are described in chapter 9.7. The method used for application of microsymbionts is described in Marx *et al.* (1982), Mauperin *et al.* (1987), Mikola (1970), Molina and Trappe (1984), and Theodorou and Benson (1983). It is applicable to some ectomycorrhizae and rhizobia. For most seed the amount of inoculant that will adhere naturally to the surface is too small, and an adhesive such as gum arabic, calcium alginate or methyl cellulose must be used. The inoculant can either be applied after the seeds have been surface treated with the adhesive, or it can be mixed with the adhesive before application.

A precondition for application is that the seed surface is not toxic to the microsymbionts. Fungi and some bacteria are sensitive to some fungicides used for seed treatment. If seeds are treated with such compounds, it is advisable to wash and rinse them carefully before inoculum application. Both coated and pelleted seeds can be stored for some time before sowing, but viability of the inoculum is generally shorter than the viability of the seeds. The methods obviously exclude most pretreatments to break dormancy unless they are applied prior to inoculation.

### 13.5.8 Inoculant granulates, tablets and capsules

Application of mycorrhizal inoculants in capsules and tablets has been described for some ectomycorrhizal species (Cruz 1983, Marx *et al.* 1984). Both methods use spore inoculants that are further processed with a carrier. One method consists of placing powdered spores mixed with powdered charcoal or another carrier in small gelatin capsules. In the other method dry spores are mixed with e.g. lime, fine particle soil or peat and compressed into tablets by the use of a medical tableting machine. One capsule or tablet is applied to each seedling.

Some rhizobia inoculants can be compressed into granules, pellets or tablets possibly with an adhesive and sowed together with the seed. Pellets can be prepared as pelleted seeds but with coarse grained sand (even size), 'alkathene' beads or other hard material as a core instead of seeds. Fine particle inoculants must be used as for seed pelleting. The granules are very easy to handle and it is easy to standardise and adjust the right amount of inoculate to be applied to each plant (Jha *et al.* 1994, Roughley 1982). Granules, beads and tablets can easily be stored under cool conditions.

Frankia cells may be entrapped in alginate beads, which also facilitates both application and storage. The cell suspension is mixed with 1-2% alginate in an appropriate nutrient solution. The mixture is added dropwise into a gently stirred 0.1 M CaCl<sub>2</sub> solution at room temperature (28°C); each drop will form an alginate bead. The beads are washed and incubated in a fresh liquid medium for 15 - 20 days. During incubation the cells will grow into colonies and form sporangia inside the beads (Diem and Dommergues 1990).

### 13.6 Inoculation Conditions

Seedlings and plantlets are generally able to form mycorrhiza or root nodules when they have formed some roots i.e. usually after one to three months. Good growth conditions of the seedlings are crucial for the inoculation success; pH and other soil conditions should be suitable for the species (Brewbaker *et al.* 1982). Although fertilizers generally reduce mycorrhizal development, and N-fertilizer specifically nodule development, it may be appropriate to apply a minor starter fertilizer in order to assure proper root development (Dart 1988, Kohls and Baker 1989, Somasegaran and Hoben 1994, Thomas and Berry 1989). In some cases it is practical to apply the inoculants at the time of sowing, for example where the inoculants are mixed with soil in the seedbed or banded at root depth, or where coated or pelleted seeds are used. If applied at the time of sowing, the fungi or bacteria must be able to survive in the soil for a certain period independently of the symbiosis, i.e. until the roots become infective. Infection and development are usually more rapid and uniform if the soil is sterilised, for instance by fumigation prior to inoculation, because competing or predating organisms have been eliminated.

The inoculant must be able to make good root contact for infection to take place. Where the inoculant is applied to the planting soil, it may be mixed with the upper approx. 10 cm by raking. All types of symbiont require moisture for infection and growth and e.g. mycelial

cultures are sensitive to desiccation. Thorough watering is generally required immediately after application if the inoculant is applied to the surface. Better root contact is achieved by banding or injecting the inoculum 5-10 cm below the soil (Crowley *et al.* 1986, Saginga *et al.* 1989). Banding can be done prior to sowing in seedbeds or containers, or in the transplanting beds or pots. For container plants the inoculant is usually applied to the transplanting soil (Mikola 1970). For bare-root seedlings, root dipping in a slurry of inoculant is occasionally applied in connection with out planting (Dart 1988). Spores can move in the soil by water percolation. Once mycorrhiza or root nodules have established on some roots, they will spread further to other roots in the soil by themselves. Rhizobia nodules usually appear 3-5 weeks after inoculation (Mary *et al.* 1994).

### 13.7 Storage of Microsymbionts

Generally, fresh inoculation material is preferred, as it is more effective in colonising roots and forming symbiosis. However, in many instances storage, at least for shorter periods, is necessary or convenient, for example in the following situations:

- a. Some inoculum sources, e.g. sporocarps, are not available (or only in insufficient quantities) when inoculation should take place. Some have erratic appearance at long intervals.
- b. The most active nursery season is during seedling production. It is often convenient to undertake other activities, e.g. collection and inoculum production outside the peak season.
- c. Some procedures of inoculum production are technically complicated and need skilled staff and special equipment and facilities. The production of such inoculants is often limited to a few central stations from where the product must be shipped to the local nurseries where the seedlings are raised.
- d. Regional or world wide distribution and exchange of exotics or superior propagation material often imply long time lapse from production until end use, both due to order and transport delay, and to import and quarantine regulations.

**Mycorrhizal inoculum** can be stored as spores or mycelium culture. Spores must be dry and mycelial cultures moist during storage. Cold (2-6°C), dark and aseptic conditions extend viability of either. Since light is generally detrimental to fungi, direct sun drying should be avoided, and use of fungicides should be excluded or minimised during preparation and storage. This is especially pertinent when inoculum is handled together with seeds (e.g. coated or pelleted seeds).

Spores generally store better than mycelial culture and are less bulky. It is therefore practical to store spores and, if mycelial culture is preferred, postpone germination and multiplication in culture until shortly before inoculation. However, germination and culture are slow processes, and the receiving nursery may not have the facilities to undertake that; hence, temporary storage of mycelial culture cannot be avoided. Low temperature generally slows down or stops the growth of the mycelium.

Potential storability depends on inoculant type:

- Soil can sometimes be stored from one year to the next but the amount of viable fungi will decrease; prolonged storage is not recommended in the tropics (Cruz 1983). If soil is to be stored for a period, it must be kept moist (not wet) and shaded (Menge 1984).
- Dried sporocarps of ectomycorrhizal fungi can be stored for up to several years. It is advisable to surface sterilise them (e.g. 2% chloramine T + 200 ug streptomycin/ml (Hepper 1984) to kill possible pathogens.
- Dried powder of spore inoculum made of surface sterilised, chopped, dried and crushed sporocarps can be stored cold and dark in sealed polythene bags. Temperatures above 45°C should be avoided during drying; freeze drying is possible but often kills some of the spores. Spore inocula of some temperate species can be stored up to 4-5 years without significant loss of viability (Marx 1980). This refers to ectomycorrhiza as there is little experience with long time storage of VAM fungal spores.
- Spore suspensions of various ectomycorrhiza species have been stored in the dark at 5°C for one year, without significant reduction of inoculum effectiveness.
- Mycelium culture can reportedly be stored for up to 9 months at 4-6°C (Hung and Molina 1986). If the mycelium is to be stored, the cultivation process is preferably stopped before transferring the culture to larger tanks, i.e. still in small flasks (section 13.5.6). This also facilitates transport. The final stage of multiplication can be carried out at the local nursery. The viability (= infectivity) of mycelial culture generally decreases rapidly during the initial period but the deterioration rate decreases later in storage. Most species will lose about 50% of their infectivity during the first two to three months.
- Roots of host plants (e.g. *Ipomea*, *Paspalum*, *Sorghum*) colonised by VAM fungi (pot culture) can be stored in sterilised water or vermiculite for 2-4 months at 4°C. Predrying the VAM crude soil inoculum to 1-10% moisture content (no free water) and stored in sealed plastic bags at 5°C is reported to extend the viability up to 4 years (Menge 1984).
- Pelleted seeds, inoculum tablets and capsules are likely to store equivalently to dried spores provided storage conditions are appropriate.
- Living seedlings with mycorrhiza can be used to 'store' mycorrhiza in the nursery, where they form a resource base from where mycorrhiza (both ecto and VAM) can spread to new seedlings or from where material can be collected for inoculation, possibly via pure culture technology. The seedling resource base must obviously be renewed or pruned to keep them from overgrowing.

**Rhizobia** are generally sensitive to heat (> ca. 45-50°C), desiccation, and UV light. Many chemicals are also dangerous to the bacteria. Bacteria excrete metabolic waste products during their growth and multiplica-

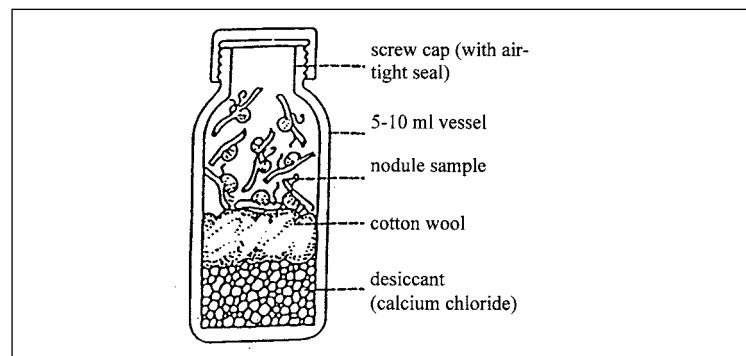
tion which, in large quantities, are toxic to the bacteria. Metabolism and multiplication rate should therefore be kept low during storage by storing cool (refrigerator at 4°C), dark (away from UV sunlight), moderately moist and, for broth cultures, aerated (Roughley 1982). In addition, it is very important to keep the cultures absolutely free from contaminants. Cleanliness during the operations cannot be overemphasised.

Rhizobia can be stored in various ways:

1. Excised nodules. Nodules can be stored in screw-cap vials (fig. 13.5) with desiccant (anhydrous  $\text{CaCl}_2$ ). The nodules can be stored up to one year under cool (4°C) conditions (Somasegaran and Hoben 1994).
2. Rhizobia culture. Cultures may be stored for a shorter or longer period provided the temperature is low and the bacteria thus inactive. The viability is, however, rarely more than a few weeks (Somasegaran and Hoben 1994).
3. Inoculant with carrier. This is the final product of rhizobium culture and the commercial product to be used for inoculation of seeds or seedlings. The carrier (e.g. peat, charcoal or compost) protects the bacteria to some extent, but the viability differs. Most inoculants can be stored at least up to one year (Burton 1979) and inoculants of some species kept in cold storage have remained viable up to 7-10 years (Biederbeck and Geissler 1992). Sterilised carriers generally prolong the viability of rhizobia, especially at high moisture content (Roughley 1982).

The viability of rhizobia in storage is relative in the sense that the number of viable bacteria in the inoculant goes down with time. The inoculant must have a certain number of bacteria per unit weight or volume in order to be effective.

Figure 13.5.  
Screw cap vial for  
storing root nodules.



*Frankia* may be stored as nodules or culture similar to rhizobia. Nodule lobes can be stored temporarily over  $\text{CaCl}_2$  or silica gel desiccant at room temperature or in the refrigerator <1 year. Fresh or (freeze) dried nodules, or finely ground nodules can be stored in a freezer (-20°C) for up to 3 years (Lechevalier and Lechevalier 1990, Benoit and Berry 1990, Perinet *et al.* 1985). Pure culture inoculants can be stored by freeze drying in the medium in which they are grown. Finally, pure cultures can be stored entrapped in alginate beads and remain viable for years at room temperature (Diem and Dommergues 1990, Sougoufara *et al.* 1987).

### 13.8 Fumigation, Pesticides and Pretreatment

Soil sterilization by fumigation with e.g. methyl bromide is a widespread practice in nurseries. Alternatively, specific fungicides or other pesticides are used to eliminate special pests and pathogens. Whatever method used, such remedies will kill beneficial soil organisms along with the target organisms. Fumigation has no long-term effect and inoculation can be carried out shortly after; two days may be sufficient (Donald 1975).

Some fungicides, e.g. thiazoles, have no or only limited negative effect and may even promote mycorrhizal formation possibly by eliminating competitive organisms (Trappe *et al.* 1984). Certain fungicides are effective over a longer period and inoculation must be postponed until the harmful effect has disappeared. Although certain fungicides like thiram, benomyl and captan are sometimes reported to be harmless to spores when the inoculant is applied by pelleting (Marx *et al.* 1984), contact between fungicides and inoculants in general should be avoided, e.g. by rinsing seeds with fungicides before seed inoculation. Certain herbicides are beneficial, and some soil insecticides and nemacides (against nematodes) promote mycorrhizal development in the way that they eliminate predation on spores and mycelium (Trappe *et al.* 1984).

Fumigation normally kills rhizobia together with other soil microbes. In rhizobial management it can, as with mycorrhiza, be used to promote inoculation with a selective strain by eliminating possibly competitive native strains (Alexander 1982).

Sensitivity to herbicides, fungicides and insecticides sometimes varies with species and stage of infection. Bacteria already enclosed in nodules are often relatively well protected while free-living bacteria are sensitive. For rhizobia relatively fungicide-resistant strains exist. If such resistance can be obtained, the fungicide may even be beneficial to the inoculated rhizobium by eliminating competitive native rhizobia or predating protozoa (Alexander 1982).

Pretreatment of legume seeds to break physical dormancy, e.g. by immersion in hot water or sulphuric acid (chapter 9), obviously does not go well together with inoculants. Since pretreatment is usually undertaken just before sowing, seeds that require such pretreatment cannot be pre-inoculated e.g. by pelleting. Seeds must be pretreated before inoculation and, if toxic compounds like sulphuric acids are used, they must be carefully cleaned off before inoculation.

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