MICROBIOLOGY COURSE MATERIAL

Semester - V

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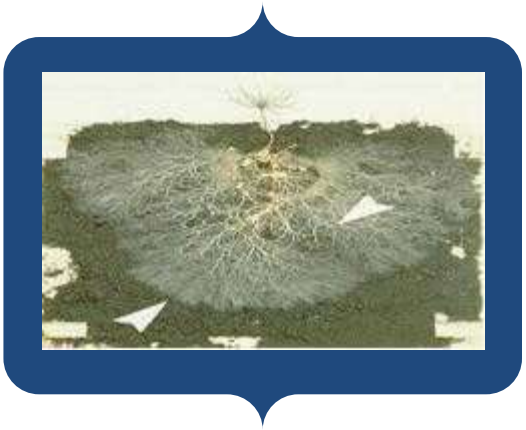
DSE-A1: UNIT-1: PART-C: MYCORRHIZAL BIOFERTILIZERS

B.Sc (HONOURS) MICROBIOLOGY (CBCS STRUCTURE)
SEMESTER – V
DSE-A1: UNIT – 1: PART- C
TOPIC: MYCORRHIZAL BIOFERTILIZERS

❖ Introduction:

Mycorrhizal fungi intimately associate with plant roots forming a symbiotic relationship with the plant providing sugars for the fungi and the fungi providing nutrients such as phosphorus, to the plants. Mycorrhizal fungi can absorb, accumulate and transport large quantities of phosphate within their hyphae and release to plant cells in root tissue.



A mycorrhiza ("fungus – root") is a type of endophitic, biotrophic, mutualistic symbiosis prevalent in many cultivated and natural ecosystems. There are three major groups of mycorrhiza: Ectomycorrhiza, Ectendomycorrhiza and Endomycorrhiza. Ectomycorrhiza and endomycorrhiza are important in agriculture and forestry. In Thailand, endomycorrhiza biofertilizer has been investigated for ten years. Initially the mycorrhizal biofertilizer production is for economic crops such as fruit trees (durian, longan, sweet tamarind, mangosteen, papaya). Now the biofertilizer can be used for vegetables and rubber.

Endomycorrhiza (vesicular arbuscular mycorrhiza; VA mycorrhiza; now known as arbuscular mycorrhiza, AM) play a very important role on enhancing the plant growth and yield due to an increase supply of phosphorus to the host plant. Mycorrhizal plants can absorb and accumulate several times more phosphate from the soil or solution than non–mycorrhizal plants. Plants inoculated with endomycorrhiza have been shown to be more resistant to some root diseases.

Arbuscular Mycorrhizal (AM) fungi (or Vesicular-Arbuscular Mycorrhizal, VAM fungi), belonging to the Phylum *Glomeromycota* are symbionts with terrestrial plant roots. It is now generally recognized that they improve not only the phosphorus nutrition of the host plant but also its growth, which may result in an increase in resistance to drought stress and some diseases. Therefore, AM fungi offer a great potential for sustainable agriculture, and the application of AM fungi to agriculture has been developed. In fact, in some countries the AM fungal inocula have been commercialized. Since it is laborious and cost-consuming for production of AM fungal inocula because of their obligate biotrophic nature, the ways to increase the function of the indigenous AM fungi in soil have also been developed.

❖ Benefits of Mycorrhizal Biofertilizer

Mycorrhiza plays a very important role on enhancing the plant growth and yield due to an increase supply of phosphorus to the host plant. Mycorrhizal plants can absorb and accumulate several times more phosphate from the soil or solution

than non–mycorrhizal plants. Plants inoculated with endomycorrhiza have been shown to be more resistant to some root diseases. Mycorrhiza increase root surface area for water and nutrients uptake. The use of mycorrhizal biofertilizer helps to improve higher branching of plant roots, and the mycorrhizal hyphae grow from the root to soil enabling the plant roots to contact with wider area of soil surface, hence, increasing the absorbing area for water and nutrients absorption of the plant root system. Therefore, plants with mycorrhizal association will have higher efficiency for nutrients absorption, such as nitrogen, phosphorus, potassium, calcium, magnesium, zinc, and copper; and also increase plant resistance to drought. Benefits of mycorrhizal biofertilizer are as follows:

1) Allow plants to take up nutrients in unavailable forms or nutrients that are fixed to the soil. Some plant nutrients, especially phosphorus, are elements that dissolve were in water in neutral soil. In the extreme acidic or basic soil, phosphorus is usually bound to iron, aluminum, calcium, or magnesium, leading to water insolubility, which is not useful for plants. Mycorrhiza plays an important role in phosphorus absorption for plant via cell wall of mycorrhiza to the cell wall of plant root. In addition, mycorrhiza help to absorb other organic substances that are not fully soluble for plants to use, and also help to absorb and dissolve other nutrients for plants by storage in the root it is associated with.

2) Enhance plant growth, improve crop yield, and increase income for the farmers. Arising from improved water and essential nutrients absorption for plant growth by mycorrhiza, it leads to improvement in plant photosynthesis, nutrients translocation, and plant metabolism processes. Therefore, the plant has better growth and yield, reduce the use of chemical fertilizer, sometimes up to half of the suggested amount, which in turn increases income for the farmers. As in the trial involving mycorrhizal biofertilizer on asparagus it was observed that, when the farmers used suggested amount of chemical fertilizer together with mycorrhizal biofertilizer, it was found that the crop yield improved by more than

50%, and the farmers' income increased 61% higher than when chemical fertilizer alone was used.

3) Improve plant resistance to root rot and collar rot diseases. Mycorrhizal association in plant roots will help plant to resist root rot and collar rot diseases caused by other fungi.

4) It can be used together with other agricultural chemicals. Mycorrhiza are endurable to several chemical substances for example; pesticide such as endrin, chlordane, methyl parathion, methomyl carbofuran; herbicide such as glyphosate, fuazifopbutyl; chemical agents for plant disease elimination such as captan, benomyl, maneb triforine, mancozed and zineb.

❖ Isolation of Arbuscular Mycorrhizal Fungi

Taxonomy of AM fungi

AM fungi show the peculiar characteristics in morphology and physiology. Spores of AM fungi are generally formed in soil and their sizes (50-500 µm in diameter) are much larger than those of other fungi. There is no septum in their hyphae. No sexual growth-phase has been observed. Spores germinate when they are under favorable conditions, extend their hyphae and colonized plant roots. The fungi penetrate the hyphae into cortex layer of roots and form the hyphal

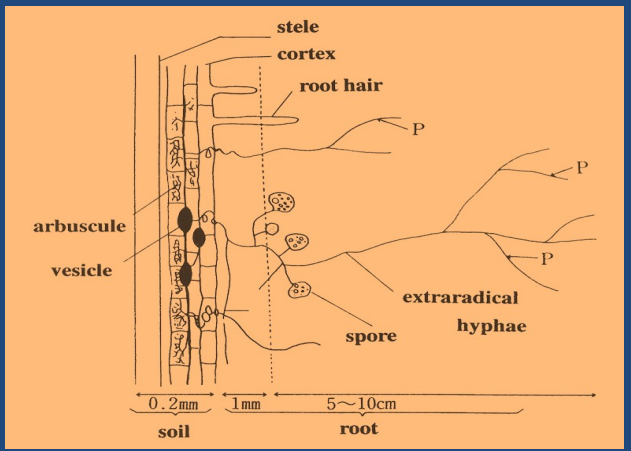
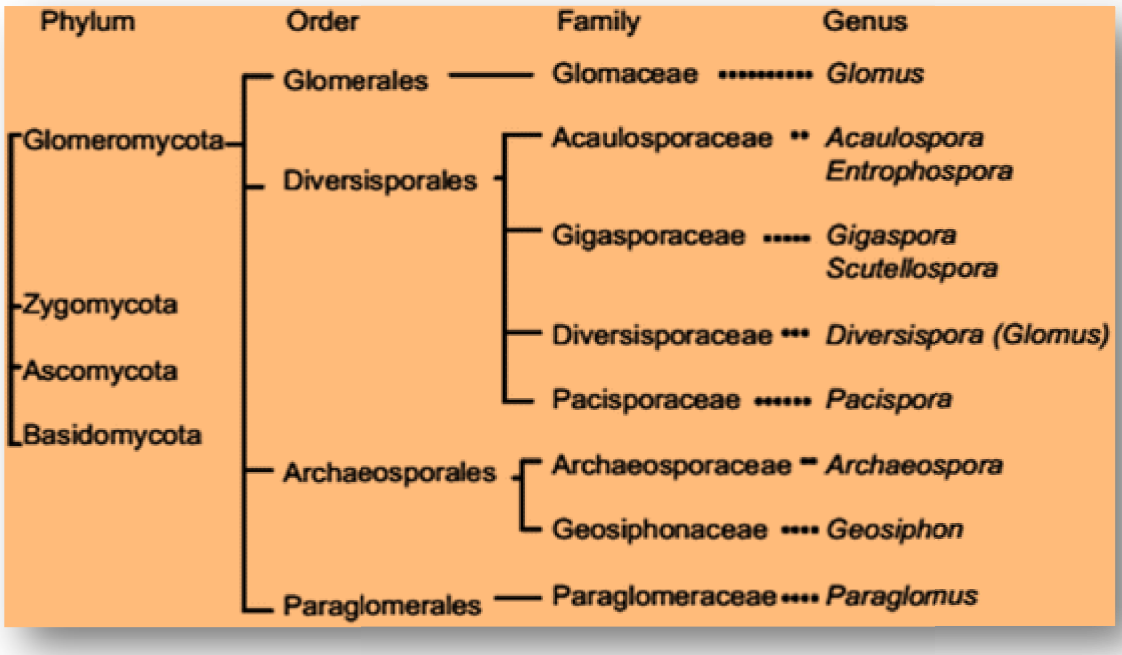


Fig: Schematic picture of Arbusucular Mycorrhizal Fungi colonizing roots and their hyphal extension into soil

organs, “vesicles” and “arbuscules” which are characteristics to AM fungi (Fig). AM fungi belonging to *Gigasporaceae* are known not to form vesicles. Colonization on plant roots is essential for proliferation of AM fungi. AM fungi are thus recognized as obligate symbiotic fungi. The interaction between AM fungi and plants is generally mutualism based upon nutrient exchange.

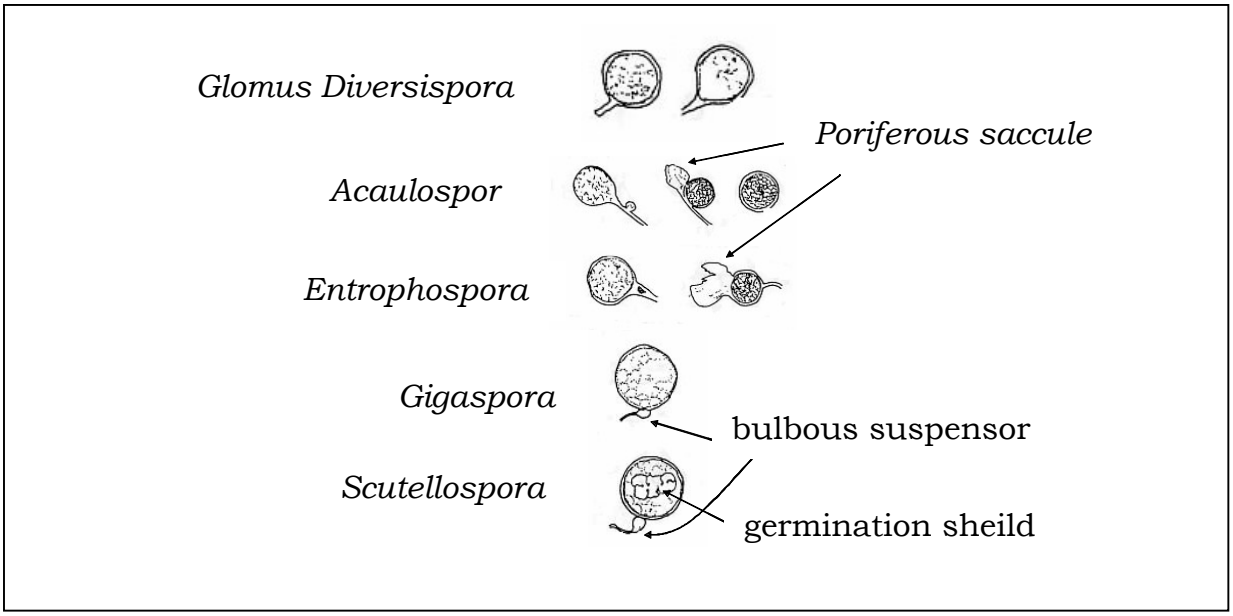
Because of morphological characteristics such as no hyphal septum, AM fungi had long been recognized as a member of *Zygomycota*. Recent molecular phylogenetic studies showed that *Zygomycota* is poly-phyletic and that AM fungi should be separated from other *Zygomycota*. A new Phylum *Glomeromycota* has been proposed for AM fungi. Current classification system is summarized in the chart below. This classification is mainly based upon the sequence data of rRNA gene. However, some new genera have been raised with relatively small numbers of isolates, so further study may revise the present classification system.



Observation of Arbuscular Mycorrhizal Fungi in Roots

Arbuscular mycorrhizal fungal structure in roots is usually not observed without appropriate staining. Freshly collected root samples should be washed gently and be made free from soil particles. Ultrasonic treatment is effective to disperse soil particles closely adhered to roots. Roots are treated with 10% KOH solution for 30

min to 1-2 hours in a hot bath, depending on thickness of root structure. Treated roots are washed with water and treated with 2% HCl solution. Acidified root samples are stained with 0.05% trypan blue (or acid fuchsin) in lactic acid for 10-



Morphology of representative genera of Arbuscular Mycorrhizal Fungi

Table: Morphological character of spores of AM fungi

Shape:	**(i.e. globular, spherical, irregular etc)**
Size:	Globular: diameter (minimum – average – maximum) Irregular shape: length x width (minimum – average – maximum)
Colour:	(compare with Standard Colour Chart)
Hyphal attachment:	(i.e. sporiferrous saccule, bulbous suspensor etc) sporiferrous saccule = *Acaulospora, Entrophospora, Archaeospora* bulbous suspensor = *Gigaspora, Scutellospora*
Auxiliary cell:	(presence = *Gigaspora, Scutellospora*, none)
Sporocarp:	(presence, none)
Germination shield:	(presence =*Scutellospora*, absence)
Surface ornamentation:	(i.e. smooth, rough, reticulate etc)
Vesicle:	(presence or absence in mycorrhizal roots)

15 min in a hot bath or for a few hours without heating. The roots are destained with lactic acid or lacto-glycerol and are now ready for microscopic observation. The stained roots may be observed first under a dissecting microscope with

transmitted illumination and then observed under a compound microscope. Fungal structures are stained and can be easily recognized. In the soil such as grassland soil rich in organic debris, it may be hard to find the spores hidden by the debris. In such a case, sucrose density centrifugation technique is often used to separate spores from the organic debris.

Culturing AM fungi:

AM fungi need the symbiotic association with plants for proliferation. Therefore, culturing AM fungi is to inoculate AM fungi to host plant and to grow the inoculated plant. For the AM fungal inoculum, spores collected form soil can be used. However, spores in soil are not always active in colonizing plants. Therefore, trapping culture is often employed. Soil or sieving of soil is used as inoculum (Soil Trap Culture). To isolate AM fungi colonizing roots, mycorrhizal plants collected from field can be transplanted to potting medium as Plant Trap Culture.

Potting medium: Sterile soil or soil-sand mixture is usually used. We prefer to use commercially available "Akadama-tsuchi" which is collected from subsoil of volcanic ash soil and is prepared for horticulture use. Various potting materials for horticulture can be also used. However, the materials for potting medium should be low in available phosphate and preferably not rich in organic matter. In some cases the fungi isolated from some specific soils may need the specific soil properties for their growth.

Host plant: Various mycotrophic plants can be used: leguminous species (i.e. *Trifolium spp.*, *Medicago spp.*, *Lotus japonicus*) and grass species (i.e. *Lolium spp.*, *Paspalum notatum*), and other herbaceous species (i.e. *Plantago spp.*). Onion and leek (*Allium spp.*) are also good hosts. AM fungi generally do not show host specificity but some species show host preference. Therefore, the plant species from which the target AM fungus is isolated can be used as a host plant.

Growth conditions: Any conditions, which support good growth of host plants, are acceptable. To avoid contamination, a growth chamber is preferable. If greenhouse is used, it should be kept clean. It should be reminded that cross-contamination or contamination from dust is inevitable under open-air conditions, even in growth chamber.

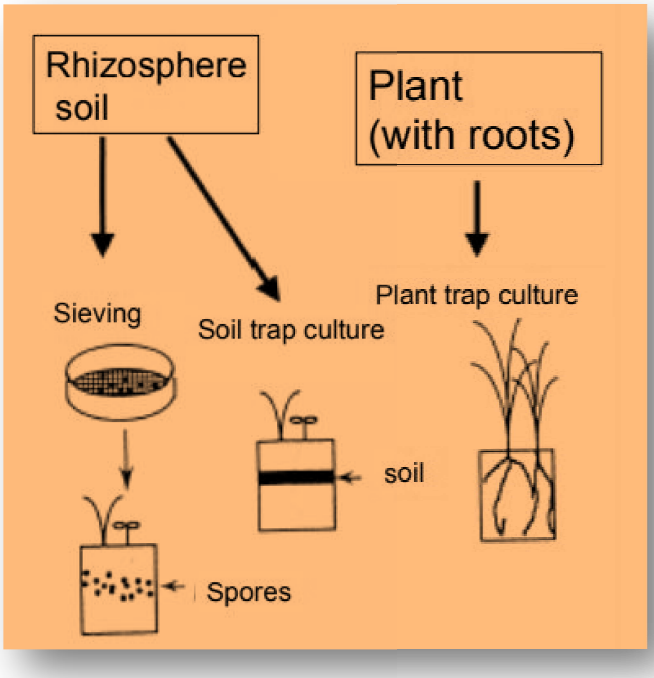


Fig. 5: Methods for trapping AM fungi

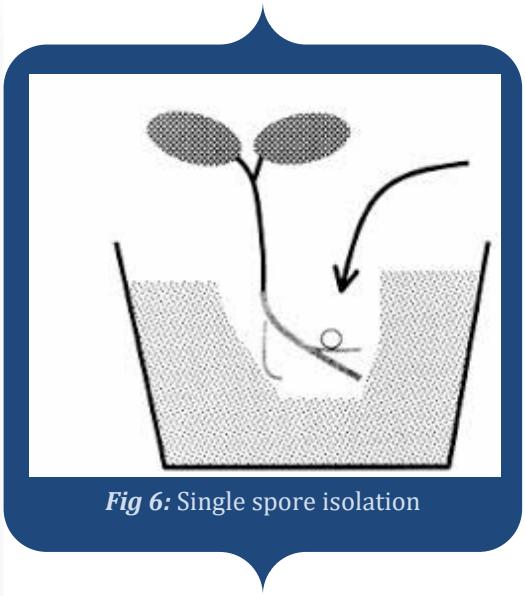


Fig 6: Single spore isolation

Single spore isolation: To purify an isolated fungus, single spore isolation is needed. Even if the spores are morphologically identical, it often contains contaminants whose morphology is very similar. Successive pot culture of such multispore isolates would cause unexpected outbreak of the contaminant. Furthermore, even if the culture contains only one species, it may be composed of genetically diverse populations. For such genetic studies or population genetics, the purification through single spore isolation is essential. For single spore isolation, no specific equipment is needed. For efficient handing, two sets of dissecting microscopes are placed side by side. One microscope is for picking up single spore from spores in a dish. Another is for inoculation of a spore on roots. Seedling placed in the pot is placed under another microscope. Under the first

microscope, single spore is picked up and transferred under the second microscope. Under the second microscope, the spore is placed on fine roots or root tip of the seedling (Fig.6). If culture is successful, the detailed morphological observation is required. Potting medium can be dried by stopping watering to the pot. After the host plant wilt, the dried soil containing spores can be stored for a year at 4-5°C. It is advisable that the isolated fungi are recultured every year. Flow of isolation and culture of AM fungi is summarized in Fig. 7.

Throughout this procedure, the followings should be reminded:

1) Origin information of the isolated fungi should be recorded in details as much as possible. (i.e. site description (latitude, elevation, vegetation, soil type, cropping history etc.), soil properties etc.)

2) At each culture step, voucher specimen of spores should be prepared and stored.

DNA Extraction from spores

Molecular phylogenetic information is essential for taxonomy of AM fungi. Sequence data for conserved genes such as rRNA is obtained by PCR amplification followed by sequencing with DNA extracted from spores. Many protocols for DNA extraction are reported, one of them is:

- **Cleaning of spores:**

Clean spores are collected with tweezers or fine Pasture pipette. Spores should be washed several times with sterile water with sonication for 10-20 seconds. If spores show water repellency, surfactant such as Tween 80 can be used. Microplate with 6 or 12 wells is convenient for successive washing.

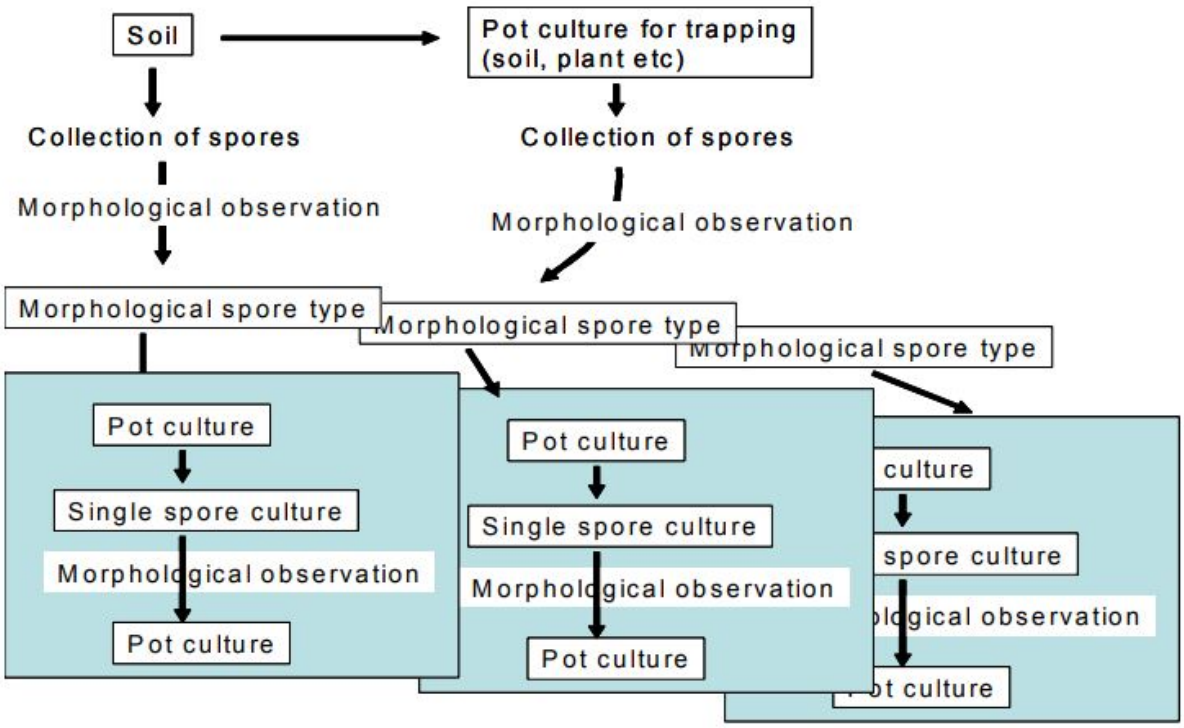


Fig. 7: Flow of isolation and culture of AM fungi

- **Crushing spores:**

We use a cap of Eppendorf tube. The cap is cut from the tube and placed upside down on the stage of a dissecting microscope. Twenty µl of Instagene (Biorad) is added to the cap. A spore or spores are put into the cap and crushed thoroughly with a micro-pestle or any fine rod. The tube itself is put on the cap with crushed spores. Then the tube is centrifuged for a while for spinning down the reagent with crushed spores from the cap into the tube.

- **Extraction of DNA:**

Further purification of DNA with ethanol/chloroform precipitation is sometimes needed to remove inhibitor for PCR amplification.

- **PCR amplification and DNA sequencing:**

Conditions of PCR amplification depend on the primers you will use. AM fungi are multi-nuclear organisms and often show polymorphisms in their sequence. It is

advisable to sequence several clones from the target fungus and to check the phylogenetic position of the sequence by constructing phylogentic tree if the target genes are located within a reasonable clade of the tree.

❖ Inoculants Production

Preparation of trap culture inoculum of Arbuscular Mycorrhizal Fungal spores

For the establishment of inoculum from monospecific cultures trapping of healthy arbuscular mycorrhizal fungi (AMF) spores is often a necessity. Spore trap cultures can also aid in AMF identification. AMF spores sampled directly from a field plot may appear healthy but are not viable. The spores may appear differently, due to weathering and intrinsic soil environment effect, physical, chemical or biological. Trap cultures are important in the following situations:

- ✓ When AMF colonization is high in roots of a plant community, but with little or no spores produced, especially in arid and hydric soil conditions.
- ✓ Where the soil has high microbial activity, especially in tropical environments, with relatively high temperature and humidity moisture. Organic matter content at these sites can be high. Under these environments, AMF spores may physically transform resulting in difficulty in species identification.
- ✓ To gather abundant healthy spores of different species and establish monospecific cultures for specific purposes.

Procedures

I. Rhizosphere soil is collected, with shoots of trap plant cut at the crown, and roots are finely chopped and mixed with the soils using a sharp chopper.

II. The chopped roots and soil are mixed 1:1 (v/v) with autoclaved coarse sand in a mechanical mixer, or massaged well in a durable plastic bag.
III. The soil mix is then transferred to a 15 cm plastic pot.
IV. Plant seeds of suitable trap plant such as tropical signal grass, *Brachiaria decumbens*, into the pot.
V. The pot cultures are maintained in a greenhouse for at least 3 months, and check sporulation from time to time. By the fourth month AMF sporulation may be at the peak. Sanitary tests may also be carried out to ensure no contamination from parasitic fungi occurs.
VI. Keep fertilizer application to a minimum, to encourage AMF proliferation.
VII. Trap culture pots are later left to dry under shade for up to 2 weeks.
VIII. Harvest the spores using the sieving and decanting techniques or the density-gradient centrifugation technique.
IX. The monospecific spores are ready for inoculation onto seedlings of the desired crops.

Inoculation of AMF

Two weeks before spore inoculation, the desired seedlings (e.g. oil palm, vegetable, pasture grass) are prepared in suitable containers filled with sandy loam soil.

1) The seedlings are gently uprooted singly on in a small bunch, and have a gentle stream of water sprayed onto the roots so that they stick together.
2) Spores collected from 3.3.1 are suspended in water and about 200 µl of the spore suspension are pipetted onto the moist roots.
3) The inoculated seedlings are immediately transplanted into containers of suitable size, containing sterilized soil.
4) The soil is topped with a sterile growth medium, watered gently under shade, before transferring into the greenhouse.
5) To encourage colonization of AMF onto seedling, fertilizers are not given during the early growth stage of the seedlings.

Problems and potential for AMF inoculum production and utilization:

- ✓ Situations where effective indigenous AMF population is low.
- ✓ Inoculation is best for transplanted crops, where soil disturbances has reduces AMF inoculums potential.

Inoculant Application

1) Application rate of VA mycorrhiza biofertilizer is 10 g or 1 spoonful per plant.
2) VA mycorrhiza biofertilizer can be used at any stage of plant growth.
3) However, for maximum benefits it should be applied during seedling stage or placed at the base of plant hole before planting. After two weeks of application, other suitable fertilizers can be applied.
4) For planting by stem cutting, the growing media are mixed with VA mycorrhiza biofertilizer prior to planting. The cutting stocks can be transferred to field one month after roots have developed.
5) For transplanting, simply sprinkle VA mycorrhiza biofertilizer adjacent to the plant roots and cover with soil.
6) For grown trees, soil under the plant canopy is trenched or the leaf litter under the tree is removed. About 10 g (1 spoonful) per plant of VA mycorrhizal biofertilizer is applied to the root hair system and then covered with soil.
7) VA mycorrhizal biofertilizer can be used in combination with several types of biofertilizers (e.g. *Rhizobium* biofertilizer, or PGPR).

Preservation and Precautions

1) Mycorrhizal biofertilizer can be kept under shade at room temperature. Normally AM fungi can live for 1-5 years, depending on the species.

2) Avoid using VA mycorrhizal biofertilizer on plants with root rot or stem rot. Mycorrhizal biofertilizer is more useful when applied prior the infection.

3) Avoid using VA mycorrhiza biofertilizer with some chemical products such as fosetyl, metalazyl and metalaxyl mancozeb since these substances can inhibit growth of VA mycorrhizal fungi.