ARBUSCULAR MYCORRHIZAL FUNGAL (AMF) INOCULUM PRODUCTION TRAINING REPORT

Conducted By:
Joyce Mnyazi Jefwa
joycejefwa@gmail.com

At
The Laboratory of Tropical Crop Improvement
Department of Agricultural Production
College of Agricultural & Environmental Sciences
Makerere University
P. O. Box 7062,
Kampala, Uganda.

Coodinated by:
John Baptist Tumuhairwe (PhD)
Makerere University

and
Sigrun Dahlin
SLU, Dept. of soil and environment,
P.O. Box 7014, SE-750 07 Uppsala, Sweden
Phone +46 18 672483;
Mobile +46 70 671 22 99; Fax +46 18 673156

1.0 Summary

The Arbuscular Mycorrhizal (AM) fungal inoculum production training was conducted from the 12th November – 17th November 2018. The aim of the training was to build capacity in methods of inoculum production.

According to the background of the participants, the training programme activities were tailored to meet this objective. The training therefore focused on methods of AMF propagule diagnosis and AMF colonization, which form the basis of inoculum production and evaluation for effectiveness. This was then followed by methods of inoculum production, assessment of inoculum vigor, and exposure to methods of application and storage.

Throughout the training, biosaftey was stressed with the following measures emphasized: laboratory handling of chemicals (dyes) and equipment (Microscope, centrifuge and autoclave).
The mode of training was through lectures, practical's and demonstration sessions. The activities undertaken are classified into six major sessions:
(1) Introductory session on mycorrhzal symbiosis (2) characteristics of Arbuscular Mycorrhizal Fungi (3) AMF Inoculants (4) practical session on Root clearing, staining and observation (5) practical training on Spore extraction, distinguishing spores under stereo-microscope, mounting and observation under compound microscope (6) AMF Culture initiation and inoculum production: trap cultures, spore and root cultures.

2.0 Introduction

2.1. Inoculum is small amount of propagules of mycorrhizal fungi which is used to initiate colonization in plants. The Mycorrhizal propagules comprises of spores, sporocarps, auxiliary cells mycelia and infected root fragments. To generate inoculum, the understanding of both the spore, sporocarps, which is the main reproductive structure and the mycelia and auxiliary cells which make up the vegetative structures is vital. This is necessary for validation of the inoculum potential on effective performance and monitoring quality of the inoculum.

The approach in the training was therefore to institute skills on how to distinguish AMF spores and colonization which form the basis of inoculum production.

2.2. Except for three, the participants had limited Knowledge on mycorrhiza and it was therefore necessary to introduce a topic on mycorrhizal symbiosis. The team trained included two (3) technicians, one (1) Professor and eight (7) MSc students (Table 1).

Table 1: List of Participants

Name	**Email address**	**Mobile No. (+256)**
Dr. John Baptist Tumuhairwe	jbtumuhairwe@gmail.com	0772463092
Mugambe Christopher	chrismugambe20@gmail.com	0702213867
Phiona Muongeza	Phionamwongeza@gmail.com	0776054744
Mutyaba Yusuf	yusmutz@yahoo.com	0777009695
Samanya Denis	dsamanya@yahoo.com	0778864032
Stephen Katende	bikoserua@gmail.com	0772628656
Ndavula Joseph	ndavula@caes.mak.ac.ug	0782774687
Asega Husseini	husseiniasega@gmail.com	0775977531
Kyeyune Gerald	gmkyeyune@caes.mak.ac.ug	0758202894
Wadri Victor	vwadri@gmail.com	0772425052
Apita Tonny	apitatony@gmail.com	0781452447

2.3. Programme Review

The training program was reviewed to accommodate the knowledge and skills of participants and the training materials available.

Table 2: Training Program

Period	Date (2018)	Activity
Day 1	12th November	Lecture: (1) Introduction to mycorrhizal symbiosis. (2) Arbuscular Mycorrhizal Fungi – Classification and Functions
Day 2	13th November	Lecture: Morphological Characteristics of AMF: Reproductive and Vegetative.
Day 3	14th November	Practical: Spore extraction and diagnosis
Day 4	15th November	Practical: (1) Spore extraction and diagnosis and mounting on slides (2) Root clearing and staining, observation
Day 5	16th November	(1) Lecture on Inoculum production (2) Practical's on Root observation (3) Initiation of cultures
Comment		Appendix 6 & 7 on

on Activity		effectiveness of Inoculum and determining abundance of infective propagules in the inoculum was not done.

2.4. Training Materials

Training materials required and where they can be procured (including indicative prices) in case we don't have them in stock.

1. Mesh Sieves (270 µm and 45 µm) = Available
2. Dissecting microscopes = two
3. Compound microscopes = one
4. Entomological Forceps fine, long point. Spring steel. Length 4.5" = Pasteur Pippete use instead.
5. Coinbill Brand New Gold Sparkle Type Tweezers Fine Tip straight Forceps = Not available
6. Pots (Yoghurt cups – small size) = Not available
7. Melzers reagent (Chloral hydrate, Potassium Iodide, Iodine) = Available
8. PVLG (Lactic Acid, Distilled water, Glycerol and Polyvinyl alcohol) = Available
9. Large pots (see picture below) for sowing leek and sorghum seeds as below = improvised with small pots
10. Leek seeds and Sorghum = Leek plants available
11. Watch glasses = Not available
12. Frosted Slides = Available
13. Cover slips = Available, good for roots but not spore mounting
14. Trypan blue and Ink (Blue and black) = Trypan blue available
15. HCL = Available
16. KOH, Hyrdogen peroxide = available
17. Oven/Autoclave = Autoclave available
18. Centrifuge = Available but not in good condition.

3.0. Summary Lecture on Mycorrhizal Symbiosis

3.1. Types of mycorrhiza and functions

Mycorrhizas are highly evolved mutualistic associations between soil fungi and plant roots. The partners in this association are members of the fungus kingdom (Glomeromycota, Ascomycota and Basidiomycota) and most vascular plants.

There are two types of mycorrhiza: (1) ecotrophic (Ectomycorrhiza, ectoendomycorrhiza) and (2) endotrophic (Arbuscular mycorrhiza, orchid mycorrhiza and ericaceous mycorrhiza) mycorrhiza of agricultural and ecological relevance. The different types of mycorrhizal associations have been recognized, involving different groups of fungi and host plants and distinct morphology patterns. The types of mycorrhiza are:

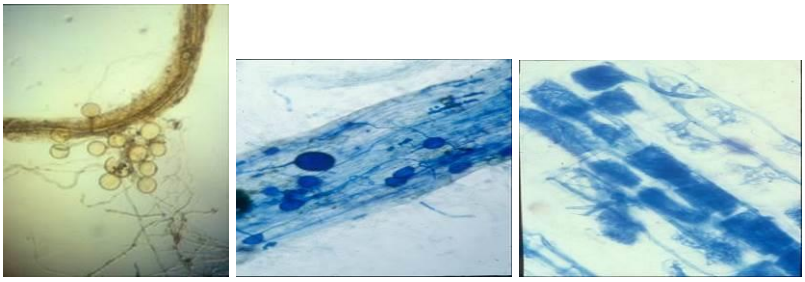
(i) arbuscular mycorrhizas (AM) in which Glomeromycota fungi produce arbuscules, hyphae and vesicles within root cortex cells,

(ii) ectomycorrhizas (ECM) where Basidiomycetes and other fungi form a mantle around roots and a Hartig net between root cells,

(iii) orchid mycorrhizas where fungi produce coils of hyphae within roots (or stems) of orchidaceous plants and (iv) ericoid mycorrhizas involving hyphal coils in outer cells of the narrow "hair roots".

(iv) Ericales: Mycorrhizal associations are regulated by features of the host plant and rnycorrhizal fungus, as well as by soil conditions and environmental factors.

Ectomycorrhizal roots and associated ectomycorrhizal mushroom Mushroom.



Arbuscular Mycorrhiza: Aggregate of AMF spores on a root and cleared and stained roots showing colonization by Arbuscular Mycorrhizal Fungi (AMF) characterized by veicles (globose structures) and arbuscules appearing as deep blue color of trypan blue in the cortical cells of plant roots (Pictures by Joyce Jefwa).

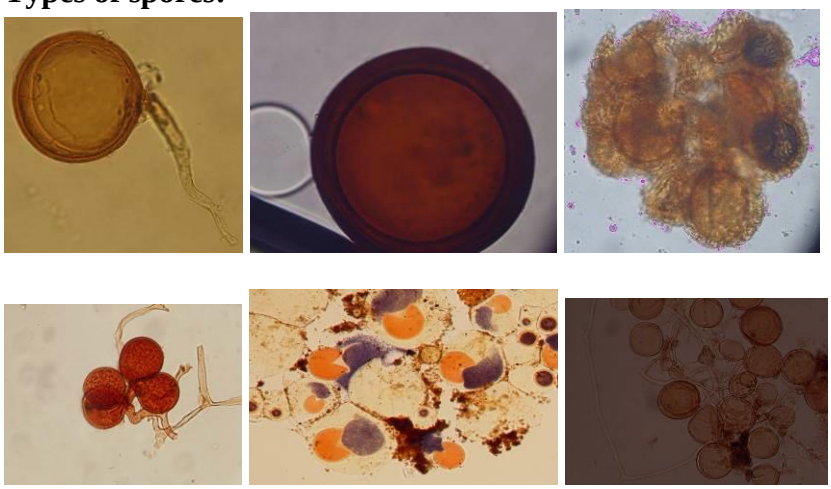
3.2 Characteristics of Arbuscular Mycorrhiza (AM) Fungi

The arbuscular mycorrhiza association is the most wide spread symbiosis, estimated in over 80% of vascular plants (Spermatophyta, Pteridophyta and Bryophyta) in all soil environments. The arbuscules and veiscles are the most dominant features characterizing this division (Glomeromycota) of fungi. The division has progressively changed names as new facts emerge and currently it is referred to as the arbuscular mycorrhizal fungi (AMF) association. It was first referred to as Phycomycetous endomycorrhiza to distinguish it from endomycorrhiza symbiosis then formed between members of the Ericaceous or Orchdaceous and higher fungi. Until recently the AMF association was referred to as the Vesicular Arbuscular Mycorrhiza (VAM) fungi based on the presence of the vesicles and arbuscules. The changes from Vesicular Arbuscular Mycorrhizal (VAM) Fungi to Arbuscular Mycorrhiza (AM) Fungi was prompted by facts that while arbuscules occurred in all, not all members of this group of fungi formed vesicles.

Arbuscular mycorrhiza have progressively changed placement in taxa based on morphological and molecular characterization. The first scientific taxa (name) assigned to AMF was Edogone in the family Endogonaceae, order mucorale and division Zgygomycotina (Molds). This has progressively changed in placement to an order Glomales and currently placement based on molecular diagnosis to its own division, the Glomeromycota.

Spores are the most important diagnostic characters of AMF and most importantly in the production of pure AMF inoculum. Spores are distinguished by size (10µm -1000µm), color, surface appearance (with or without ornamentation, hyphal attachments (subtending hyphae), sub-cellular walls), mode of spore formation. The classification of AMF is indicated in **Appendix 1.**

Types of spores:



Arbuscular Mycorrhizal spores

4.0 . Practical session: AMF Spore characterization

4.1 AMF Spore extraction (Appendix 2)

Spores are key to inoculum production process, monitoring standards and quality control measures. The objective was therefore to learn how to extract spores and how to distinguish different types of spores (spore morphotypes), pick spores and initiate clean starter cultures for inoculum production.

The spores were extracted by wet sieving and decanting method by Gerdemann and Nicolson (1963). The spores are washed into a petri dish and observed under the dissecting (stere0-) microscope. The spores are collected in water which in

most cases has soil and organic particles. To be able to distinguish spores from soil and organic particles, the water suspending the spores should not be turbid.

The initial plan was to use soils from trap cultures established with leek prior to the training. There were no trap cultures, hence this was reviewed and substituted by each participant collecting field soil from rhizosphere of plants from different locations for the isolation of spores. Each participant was given the opportunity to extract spores. The major challenge was the state of the centrifuge which was worn out and finally broke down. Another small centrifuge was used for the extraction which progressed well with all participants completing the activity.

4.2. Distinguishing spores morphotypes (Appendix 2)

The major task was to make sure all participants were able to distinguish spores from sand and organic particles. With only one microscope, each participant was to be given a chance to observe what they had extracted. This was however not possible. As I result, the first step I undertook was to scope through each extracted sample and every time I spotted spores, each participant was to view under the microscope. This took time although it was very important that all participants understood how to distinguish spores from sand particles, debris and sometimes eggs of insects. It was the only option available to make sure all participants understood the distinction between a spore and a non-spore particle.

The soils used for training were all from the field. Soils from the field unlike trap and spore cultures have many challenges. They are normally very few as was the case with all soils extracted by the participants and also considering that this was the wet season, it was difficult to get spores since this is the period of active plant growth and with few exception, most AMF is likely to be in the vegetative state colonizing the actively growing plants for enhancing the process of nutrient uptake. The spores available were heavily infested or even dead. As a result, only few spores were recovered from the soils and the search for spores was therefore long.

There were differences in spore abundance in the different soils collected by participants which could be attributed to mode of collection of soils or location of collection. Observations of isolated spores was done under the dissecting microscope when spores were still suspended in water. The participants were shown how distinguish spores from soil particles, and other debris. After observing common samples, each participant was allowed to extract again and this time observe their own extraction and identify spores morphotypes which they would then share their discovery with all team members. This activity wen on along with the clearing and staining. At the end of this activity, all participants were able to distinguish spore morhotypes from sand and organic particles.

4.3. Mounting spores on slides(Appendix 2)

This stage was the most difficult activity for the participants and the focus was to demonstrate the skill and allow each to participate and continue to practice after the training until they get it right. It involves picking spores from the petri dish

and placing them on a watch glass for mprphotyping. There was no fine forcep for picking spores and instead a Pasteur pipette was used to such up the spores. This activity required some form of steadiness and some participants were able to learn faster and were training those who had difficulty. Several spores were picked and clear distinction in morphotypes was observed.

The spores were separated into (1) spores for voucher specimens (2) Spore to initiate starter cultures for inoculum production.
To save on time and due to the limited number of microscope (one), only two senior technicians with some experience on Mycorrhiza isolation were trained in the preparation of vouchers, by mounting spores on slides for observation on the compound microscope. The spores were picked from the watch glass and mounted onto slides. Because of the size of the coverslip, only one mounting reagent was used per slide as opposed to the use of two coverslips, one with spores on (1) Polyvinyl-Lacto-Glycerol (PVLG) (2) PVLG and Melzer's reagent. In this case, the spores with either mounting reagent were mounted separately on a slide each. The mounted spores are preserved as a voucher specimen. The Voucher specimen is used for documentation and kept in a herbaria as a record for reference purposes. Every AMF species used for inoculum production must have a voucher specimen.
All participants were given the opportunity to observe the mounted spores under a compound microscope. Each participant was given a chance to describe the spores mounted on PVLG, and Melzers + PVLG (Whole and crushed) on the mounted spores.

4.4. Methods of preparation of mounting reagents

Polyvinyl-Lacto-Glycerol (PVLG): It is a permanent mounting reagent composed of: Distilled water (100ml), lactic acid (100ml), Glycerol (10ml) and Polyvinyl alcohol (PVA) (16.6g). All ingredients are first mixed then PVA powder is added and then placed in a water bath at 70-80◦C and allow liquid to clear for 4-6 hours, then stored in a dark bottle.

Melzer's Reagent: It is an important diagnostic tool in the diagnosis of spore morphology. It is composed of: Iodine (5.0g), Potassium Iodide (1.5g), Chloral hydrate (100g), Distilled water (100ml). Reaction to Melzers reagent will vary from pale pink, red-brown, blood red to reddish purple. To get a permanent mount, Melzer's reagent is mixed with PVLG at a ratio of 1:1 and stored in the dark.

5. Practical session: Root Colonization (Appendix 3)

5.2. Root clearing and staining (Appendix 3)

The reason for root staining is to confirm the presence of AMF infective propagules in a soil and in the inoculum. Staining of roots reveals the presence and extent of AMF colonization and to some extent the degree of effectiveness of AMF. Root colonization is important in the assessment of inoculum potential of

field soils and inoculum prior to inoculation. During the training, leek roots growing in soils of anonymous origin were stained.
The leek plants were soaked in water to slowly detach the mass of soils from the roots without damaging the roots. The plants were then distributed amongst participants in groups for clearing and staining. The roots were cut into 1 cm pieces and subjected to the process of clearing and staining. The roots were autoclaved at both the clearing and staining processes. They also learnt how to prepare slides for assessment of the occurrence of colonization. After staining, the participants laid root fragments on slides as per McGonigle (1990) procedure for root examination under the compound microscope as opposed to the Gridline intercept method whose examination is under a dissecting microscope (Appendix 3). This activity was straight forward and each participant observed colonization from the stained roots. Colonization status of the leek roots ranged from high, low to none and heavily stained to pale staining. There was limited time for training in the assessment of frequency and intensity of colonization.

6. Lecture Summary: Inoculum Production

6.2. Introduction

It was important to understand the reason for inoculum production and the different types of inoculants.
The decline in AMF and effective propagules in soils due to disturbances such as intensive cultivation, application of pesticides, fungicides, monocropping, deforestation, soil erosion, extreme environments, low soil fertility, drought conditions, restoration of degraded landscapes and adaptation of introduced plant species to new habitats are some of the reasons for inoculation. AMF inoculum is comprised of AMF infective propagules such as: spores, sporocarps, mycelia, auxiliary cells and infected root fragments. All the propagules have the potential to produce inoculum. The different types of inoculum were described and their nature and challenges also elaborated in the lecture (Appendix 4).

7. Practical Session: Inoculum Production (Appendix 4)

7.1. Soil inoculum

This comprises of soils collected directly from the field and used for potting purposes or even layering landscapes because of its comparative advantage over the native soils with low AMF inoculum potential as a result of degradation. The use of soil inoculum is widely practiced especially in tree seedling nurseries and tissue culture banana nurseries. It is common for pine seedlings to be raised on soils collected from Pine forests. Pines are ectomycorrhizal and will not grow unless in association with Ectomycorrhizal fungi. Soils from Pine forest contain infective propagules of the ectomycorrhizal fungi. The introduction of pines in Africa failed until soils from plantations in Australia were used to raise the seedlings.

7.2. *Crude Inoculum*

There was no source of a starter culture from a known source, this type of inoculum was therefore not demonstrated but explained. An example of crude inoculum was given as shown on pictures below of two types of inoculants labelled mixed and *Glomus mosseae*. The mixed inoculum was derived from soils collected from the rhizosphere of banana farms in Rwanda (Kibungo and Butare). The AMF from the rhizosphere soils was trapped by use of a trap/nurse plant, Leek, to allow for buildup of AMF infective propagules for a period of four months. After a period of four months, the plant roots were chopped into approximately 1 cm pieces and mixed with the soils, this was used as an inoculum. At the same time, soils from the rhizosphere of bananas in the same region were collected and spores were isolated from the rhizosphere soils and the species *Glomus mosseae* was identified for inoculum production and spore culture initiated to start of a starter culture for inoculum production. The starter culture was then used in the production of crude inoculum (when bulked up as below in soil the spore culture becomes a crude inoculant) with leek used as the trap/nurse plant.

Crude inoculum maintained on leek and the two types of inoculum (mixed and single AMF species) after the process of trapping

7.3. Spore and root Inoculants

The participants were involved in the extraction of spores for initiation of spore starter cultures. Spores picked earlier from the spore extraction activity were inoculated onto the roots of a mature leek plants. In normal circumstances the inoculation is on roots of a seedling still with few roots raised in sterile soil. This was done by picking spores under a dissecting microscope and placing them directly on to the roots of a seedling. In some instance where there are two dissecting microscopes, the placement of the spores on the roots is also done under the microscope at specific locations of the roots. In our case, the spores were placed on the roots of a fully grown leek and replanted carefully into a pot that was filled three-quarter way with soil, the roots were then covered with soil to fill up the pot. The soils used must be sterile. The process of spore cultures may also be undertaken in-vitro by using the root organ culture method and also use of soil-less media once the seedling is colonized.

The roots are normally derived from the crude culture or spore cultures. In our case, clean roots of the mature leek were cut into 1cm pieces. The roots must be with high AMF colonization. In our case, the roots were stained and the colonization was variable but for demonstration purposes, the leek roots were used. The roots were cut into small fragments and placed as a band on a three quarter way pot filled with sterile soil. The root band was slightly covered with soil and the nurse/trap plant seedling of choice is planted and pot topped up with sterile soil (Appendix 4).


Commercial Inoculum from Dudutech generated from starter cultures initiated from spores of known AMF species. The inoculm can be bulked up prior to inoculation otherwise it is applied directly.

8.0. Appendices

Appendix 1: Classification of AMF

Classification According to Morton and Walker

Characteristics of the Glomeromycota

1. The fungus id mostly hypogeous (below the surface), sometimes epigeous (above the surface) mycorrhizal association
2. Mycelium is nonisptate and multinucleate
3. Sexual reproduction is very rare
4. A sexual reproduction is by chlamydospores and azygospores
5. Spores formed singly or in sporocarps

The order has two suborders Glomineae and Gigasporineae

Characters of suborder Glomineae

1. Arbuscules and vesicles formed in roots
2. Chlamydospores are terminal or intercarlary or lateral formed in soil
3. Auxilliary cells not found.

Characters of Gigasporineae

1. No vesicles formed, only arbuscules formed
2. Azygospores are formed
3. Auxilliary cell are produced

Characteristics of Families under suborder Glomineae:

Family Glomaceae

1. Chlamydospores formed, sometimes sporocarps are produced.

2. Different types of multiple outer wall layers
3. At least one structural wall continues with subtending hyphae.
4. Spores germinating through subtending hyphae
5. Vesicles inside the root sometimes become thick walled brown colored spores.

The family has genus *Glomus,*
Characteristics of *Glomus*:

1. Spores are globose to ellipsoid and sometimes irregular. Size ranging from 20-400µm diameter
2. Spores are thick walled, hyaline or yellow, saffron to orange, red brown, brown or black
3. Spore attached to single subtending hyphae, sometimes it may be more than one
4. Sometimes produces sporocarp.
5. Spores germinate by germ tube through subtending hyphae.

Family Acaulosporaceae

1. Spores developing from or within neck of sporiferous saccule
2. Spores rarely have more than one structural wall
3. Vesicles usually lobed

The family includes two genera: *Acaulospora* and *Entrophospora*

Characteristics of *Acaulospora*

1. Spores are found laterally on the neck of sporiferous saccule
2. Spores are globose to ellipsoidal, ranging from 100-400µm diameter in size
3. Spores are hyaline, yellow or reddish brown
4. Spores wall ornamented

Characteristics of *Entrophospora*

1. More or less similar to *Acaulospora*
2. Spore is formed inside the subtending hyphae just below the sporiferous saccule

Family *Archaeosporaceae*

1. Arbuscules are narrow trunks (<4µm) finely branched and stain very faint, intraradical hypha also stain faintly
2. Vesicles not observed
3. Mycorrhiza distribution patchy
4. Spores formed on thallus are either monomorphic (existing in one form, showing no variation) or dimorphic (occurring in two distinct forms)
5. Some spores are indistinguishable from Glomaceae spores
6. Some spores bear resemblance with Acaulosporaceae spores

7. Distinction from other families was based on molecular characterization
8. Spore wall is maultilayered with thick inner most flexible layer with no germination orb

The family has the genus *Archeospora*

Archeospora genus has only three species *A. trappei., A. leptoticha* and *A. gerdemanii.*

Family Paraglomaceae

1. Arbuscules with narrow trunks (<4 µm), finely branched and stain very faint, intraradical hyphae also stain faintly
2. Vesicles not observed. Rarely some oil containing structures (possibly spores) detected
3. Spores produced singly or rarely in loose aggregate of 2 to 3. Spores morphology identical to *Glomus*.
4. The strongest character recognizing *Paraglomus* as a distinct genus from *Glomus* and all other AM is by signature DNA. The family includes *Paraglomus*.

Characteristics of the families under the suborder Gigasporineae

Family Gigasporaceae

1. Azygospores are formed
2. No vesicles, only auxiliary cells are formed in soil
3. Germination either directly or from germination shields or compartments
4. The families includes the genera *Gigaspora* and *Scutellospora*

Characteristics of *Gigaspora*

1. Spores are large, can be up to 600µm in diameter, with typical bulbous base
2. Bulbous base have several lateral projections
3. Germ tube arises directly from the spore

Characteristics of *Scutellospora*

1. Similar to *Gigaspora* except in germination,
2. Germtube arises from germination shield or compartments.

Table1: Comparison of families of fungi forming AM association

Structures	Archaeosporaceae/ Paraglomaceae	Acaulosporaceae	Glomaceae	Gigasporaceae
Arbuscules	Present	Present	Present	Present
Vesicles	Absent	Present	Present	Absent
Auxilliary cells	Absent	Absent	Absent	Present
Spores in roots	Present	Present	Present	Present

Intercellular hyphae	Smooth surface, some coiling, irregular branching, stain faint	Smooth surface, some coiling, irregular branching, stain moderately	Smooth surface, straight, "H" branching, stain dark	Knobby, irregular shape, irregular branching, coiling, stain very dark
Intracellular hyphae	Frequently coiling	Frequently coiling	Rare coiling	Frequently coiling
Entry points	Appressoria, frequent hyphal coils	Approssoria, frequent hyphal coils	Appressoria, infrequent hyphal coils	Lobed appressoria, frequent hyphal coils
Mycorrhiza distribution	Very patchy	Patchy	Continuous, some patchiness	Continuous, rarer patchiness

Appendix 2: Methods of Isolating and characterizing Spores

Arbuscular mycorrhizal fungi produces spores that are characteristics for each fungal species. The identity of AMF isolates can be established by means of spore characteristics such as size (10-1000µm), color, surface texture (shiny, smooth or darl), ornamentation, subtending hyphae, subcellular structures (spore walls configuration, germination structures).

Whenever possible, it is good to identify spores prior to use for the initiation of culture for inoculum production. The use of spores for starter cultures for mycorrhizal inoculum production has several advantages. For instance, spores of undesired AMF species can be removed, spores can be easily counted, spore viability and germination can be evaluated, and the presence of plant pathogens can be avoided.

Method of extraction

Soil samples from the field sites are collected from the rhizosphere of mycorrhizal native or crop plants at a depth where the most root proliferation occurs, usually 0-20cm. The samples are then passed through a 2-mm sieve. A 100-200g soil sample (dry weight) is transferred to a beaker. If the soil is dry at sampling make sure it is soaked for 30-60 minutes before attempting to extract spores.

Extraction of AM spores from the soil

1. Samples may be collected from the field or removed from pot cultures. 50 g soil samples are recommended for extraction from field soils, whereas 25 g will be enough from pot cultures.
2. If possible, pre-soak the soil in water before processing for 30-60 minutes. This is particularly important if soils are dry or high in clay content.
3. The soil is mixed with water, stirred thoroughly and decanted through 710 and 45 micron sieves (other sieves may be included in this series e.g. 500, 250, 100 micron sieves, but each collection is examined separately). The

process is repeated several times, breaking down any lumps of soil between washes.

4. The collection on the 710 micron sieve is examined for sporocarps before discarding. Roots may also be collected from this sieve for staining.
5. All sediments are washed from the 45 micron sieve into two or four 50 ml centrifuge tubes. The tubes are balanced by weight and centrifuged for 5 minutes at 1750 rpm.
6. Water is carefully decanted from tubes and floating debris are discarded.
7. 48% sucrose (227 g dissolved in 500 ml water) is added, The tubes are balanced and mix thoroughly immediately before centrifuging for 15 seconds at 1750 rpm.
8. Immediately after centrifugation, the sucrose solution is carefully decanted through a small 45 micron sieve. The spores are rinsed and retained on the sieve thoroughly with water to wash out the sucrose. This is particularly important if the spores are required to initiate pot cultures.
9. The spores are transferred from the 45 micron sieve into a small petri dish for examination under a dissecting microscope.
10. 2-3 drops of glutaraldehyde may be added to the petri dish to prevent spores being attacked by nematodes and mites. Sometimes, ethanol (70%) may be added instead. For long-term storage place in vials with 2% glutaraldehyde.

Separation into morphotypes

Spores of AMF can be transferred to a petri dish for microscopic examination and separation. A dissecting (stereo-) microscope is used at this stage. Spores are separated into distinct morphological types. A fine-tipped forceps or Pasteur pipettes can be used to transfer spores into watch glasses or micro-dishes with water for subsequent evaluation and identification. Picking of spores from water suspension is better for avoiding undesired hyphal fragments.

Identification of spores is a difficult and time consuming exercise for most researchers in the field. Purified cultures can be sent to colleagues whose focus is AMF taxonomy willing to identify species freely or at cost. Once spores are isolated and identified, they can be surface-disinfected and used as starter inoculum for production of inoculum. Spores of AMF are surface-sterilized by exposing them to a solution of liquid detergent (e.g tween 20), 0.5% sodium hypochlorite, or 2% chloramine T, and 0.02% streptomycin sulfate in a filter unit allowing contact for 15minutes and then rinsing with five changes of water.

Preparation of voucher specimen

Preparation of diagnostic slides of AM spores for identification purposes and as reference specimens/collection.

1. Permanent slides of AM spores are prepared. The spores are squashed on microscope slides to reveal details of spore wall layers and hyphal attachments.
2. All preparatory stages are carried out under a dissecting microscope.

3. Similar morphotypes are cleaned and separated in the Petri dish using a mounted needle (minimum 10 large spores: minimum 30 small spores). Spores are transfer into cavity dishes with a Pasteur pipette or fine forcep.
4. Microscope slides are prepared with very small drops of PVLG and Melzer's PVLG.
5. The spores of one type are transferred onto a watch glass using a Pasteur pipette or fine forcep.
6. The mounted needle (fine forcep/Pasteur pipette) is dipped into appropriate mountant, half of the spores from the filter paper are transferred onto a mountant on slide. The process is repeated for the other mountant using the remaining spores. Spores should not be allowed to dry up on the watch glass.
7. Ensure spores are in the centre of the mountant and carefully lower a 13 mm round cover slip onto each drop of mountant + spores.
8. Gently crush spores under the cover slip by applying pressure with a clean mounted needle.
9. Examine spores under a compound microscope for wall layers, hyphal attachments (subtending hypha), surface appearance and staining reaction in Melzer's. Then crush more firmly and re-examine for more details of wall layers and further staining.
10. Label slide. Allow mountant to dry and shrink. Add further mountant after 5-10 days and seal cover slip edges with nail varnish etc.

Appendix 3: Characterization of Root colonization

Detecting and quantifying AMF colonization of roots

Arbuscular mycorrhizal fungal colonization of roots is not generally evident to the naked eye, and diagnostic features of the fungi can be discerned only under a stereo- or compound- microscope after roots are cleared (to remove the nuclear and cytoplasm material), acidified, and then stained in specific ways. Several procedures for staining roots for detecting and quantifying AM fungi have been developed. Trypan blue and phenol are the most common used, although Trypan blue is suspected to be carcinogenic, and dyes dissolved in phenol cause headaches. Trypan blue is still the most available dye and it must be used under caution.

Collecting root samples

After the root is thoroughly washed free of soil, obtain a representative sample by removing four to five portions containing entire root length. Chop the portions into four segments and mix them together. Transfer 0.2-0.5g (moist weight) portions of mixture into glass or plastic vials. Rinse the roots with a couple changes of water if needed. Plants with slow growing roots produce small amounts of roots and the whole root system can be stained and observed.

Clearing roots

The aim of clearing is to get rid of nuclear and cytoplasm materials in order to facilitate maximal penetration of the stain. Clear roots by completely covering them with 2.5 - 10% KOH in de-ionized water (normal tap water may be used) leave at 90◦C for I hour in a water bath or autoclave at 120◦C for 15 minutes. Pour off the KOH solution and rinse the root in at least four changes of water. If the roots are dark or pigmented, they can be bleached before they are acidified and stained. The most commonly used bleaching material is alkaline H_2O_2 (mix 3ml of 30% NH_4OH with 10ml of 30% H_2O_2 and 567 ml tap water). The NH_4OH may be replaced by the same volume of household Jik (ammonia). The duration of bleaching is 10-20 minutes but incase of deep pigmentation, the roots are allowed until they appear clear.

Acidifying roots

Roots must be acidified to facilitate retention of the stain by the target specimen. Cover the roots with 1% HCL for 1hr. Remove the acid but do not rinse the root after this step. The roots at this stage may be left for as long until one is able to stain.

Staining roots

Cover roots with 0.05% Trypan blue in acid glycerol (Staining solution: Trypan blue = 0.25g; Glycerol = 250ml; 1% HCl =25ml; H_2O = 225ml) and autoclave for 3 minutes. Decant the stain and add acid glycerol. The staining solution is prepared by dissolving 1,5 g of acid fuchsin in a solvent consisting of 63 ml of glycerine, 63 ml of water, and 875 ml of lactic acid.

De-staining roots

To de-stain roots, decant the stain from the vials containing the roots and rinse the roots with de-staining solution (Glycerol = 250ml; 1% HCl =25ml; H_2O = 225ml) to get rid of the excess stain. Cover roots with de-staining solution which consists of the solvent mixture used for dissolving the dye. Incubate the vials at ambient temperature for 24-48 hours. At the end of this period, decant the de- staining solution and add unused de-staining solution. The root is now ready for observation.

In each step in which incubation is involved, the 24-48 h incubation period can be replaced by heating in a water bath at 90◦C for 1 h or autoclaving at 121◦C for 15 min.

Observing stained roots

Stained root fragments can be spread in petri plates or mounted on microscope slides and examined for the occurrence of typical AMF structures.

Assessing AM infection using compound microscope

1. After staining in trypan blue, a minimum of 10 x 1 cm fragments are chosen at random from the sample. Normally, 5 - 30 fragments, arranged in parallel, can be mounted in PVLG under a 22 mm square cover slip and gently squashed.
2. AM structures (appressoria, intercellular hyphae, intracellular coiled hyphae, arbuscules and vesicles) can be observed in the root cells using a compound microscope.

3. a linear eyepiece graticule, move along each root fragment in approximately 1 mm intervals (with a x10 eyepiece this probably means working with the x10 objective lens). At each 1 mm interval, assess the root intersect for AM structures. For 10 cm of root sample this should give about 100 assessment points.
4. This method enables different AM structures within the root to be quantified and related to function. However, with such a small sample size (compared to the minimum 100 cm for the gridline intersect method) assessments of overall levels of infection may be inaccurate.
5. The method may be necessary for the roots of many tree species when staining or pigmentation of the epidermal cells means that roots need to be squashed before AM infection is visible.

Safety measures

Use rubber gloves during the preparation and use of the clearing, staining, and acidifying solutions. Collect used staining and de-staining solutions in separate and labeled screw-capped bottles for recycling or disposal. Used KOH and HCL can be mixed together, further neutralized, and discarded in the sink.

Appendix 4: Inoculum Production

Types of Inoculants

Soil Inoculum

Soil from the rhizosphere of plant hosting AMF can be used as inoculum. The soil inoculum is composed of soil, dried root fragments, and AMF spores, sporocarps, and fragments of hyphae. Soils can only be a reliable inoculum once the spore abundance, diversity and activity of the indigenous community is established. Challenges in the use of soil inoculum is the possible transfer of weed seed and soil borne pathogens; determining the amount of inoculum to be added in growth media or the field is a challenge, because the abundance and viability of AMF propagules from soil inoculum is uncertain.

Spore Inoculum

Spores can be extracted from the soil inoculum and used as inoculum though such spores are low in viability. Viable spores may be recovered from the rhizosphere of an actively growing plant, which is highly infected with AMF. If the spores are not viable, soil or root tissue from the site can be taken to start a trap cultures to boost the number of viable spore propagules for isolation and further multiplication. The roots and soils are either mixed into the growth medium or applied as a band below the soil surface. Germinated seeds of the trap/nurse plant are then planted and grown long enough for spores, which are then extracted, separated into morphological types, identified, and used as starter cultures. Identification can be done concurrently with the production of inoculum.

Crude Inoculum

Crude inoculum is obtained after a known isolate of AMF and suitable host are grown together in a medium to optimized for AMF development and spore formation. This inoculum is the most commonly available for large scale crop inoculation. Crude inoculum consists of spores, fragments of infected roots, pieces of AMF hyphae, and the medium in which the inoculum was produced. Spore inoculum initiates AMF colonization less rapidly than crude inoculum.

Root inoculum

Infected roots of known AMF host separated from the medium in which crude inoculum was produced can also serve as a source of inoculum. Root inoculum has certain advantages over spore and crude inoculum. Root inocula are generally superior to spore inocula in the speed with which they colonize plant roots. They are also much lighter than crude inocula and, most importantly, they require much less time to produce than crude inocula. Root inoculum is on production of large quantities of roots heavily colonized by AMF, rather than on the production of mature spores. Root inoculum can therefore be produced in about half the time required to produce crude inoculum.

Production of root mass can be influenced by factors including the type of trap/nurse plant and solid media, the number of plants per unit volume of growth medium, and the quality of the starter culture. Sand or crushed basalt are suitable media for root inoculum production from the standpoint of ease of root removal and rapidity of drying at the end of the production period, but they generally yield less root mass under nutrient regimes commonly used for inoculum production compared to media consisting of pure soil or soil-sand mixtures. Root inoculum can also be produced in non-soil media.

Production of high quality inoculum depends on the following factors:

1. State of the starter culture
2. Type of nurse plant
3. Support medium
4. Growth environment.

The plant and AMF should be brought together in a physical and chemical environment that is most conducive for the activity of the fungi and the formation of abundant hyphae and spores.

The physical environment

The solid media most commonly used for the production of crude inoculum are soil and sand. Coral sand which is rich in calcium carbonate is not suitable for inoculum production. Silica sand, and sand-soil mixtures have distinct advantages of drying more rapidly than soil alone once the inoculum production cycle is completed. This is important to minimize the growth of other microorganisms in the inoculum during the drying process. The sand particles preferably used is <2mm. Soils with poor drainage may not be used for the production of inoculum because removing roots from soil at the end of inoculum production is more difficult than from sand or sand-soil mixture. For a

host-fungus combination of interest tolerant to soil acidity, the AMF colonization will not be hampered by Al or Mn toxicity if soils of pH 5 or lower are used without liming.

The initiation and development of AMF activity depends on the host's supply of photosynthate and on root exudations. If these are reduced by conditions such as shading or defoliation, AMF colonization can be reduced. The host must have sufficient photosynthate to support the formation and development of AMF on its roots without adverse effects on itself. Consequently, environmental variables such as light intensity, soil and air temperature, and soil water status should be favorable for normal plant function.
AM fungi development is favored when the moisture content of the medium is slightly less than optimal for plant growth. A moisture content of approximately 0.1-0,2 bars appears to be adequate for inoculum production. Soil temperature is generally considered to be more important than air temperature, and temperatures that are slightly higher than the optimum for host plant development appear to favor AMF development.

Container types

Various containers can be used to hold solid matrixes during inoculum production, including plastic bags and pots made of concrete, clay, and plastic. They should have holes in the bottom to ensure adequate drainage. To minimize the amount of light reaching the medium, the containers should be translucent. If clear material must be used, it should be painted or enclosed by wrapping in an opaque material. Up to 2-10 kg of medium per container may be used to give satisfactory results.

Starter Culture

The inoculum from which a crude inoculum is started can be a pure isolate obtained from another researcher, a culture collecting and a culturing organization, or a reliable commercial culture producing firm or, an isolate can be made from specific soil by a person producing inoculum.
The amount of starter inoculum to use will depend on its quality. The culture must be highly infective, contain at least four infective propagules per gram, and be free of pathogenic microorganisms. The aim is to inoculate the inoculum-production medium at a rate of 500 infective AMF propagules per kilogram of medium.

Nurse or Trap plant species

The nurse plant grown to host AM fungi in the inoculum production medium should be carefully selected. It should grow fast, be readily colonized by AMF, and produce a large quantity of roots within a relatively short time (45-60 days). It should be resistant to any pests and diseases common in the inoculum-production environment.

Nutrient requirements

Managing the chemical composition of the medium in which the AM fungi interact with their host can be more problematic than managing the physical environment for inoculum production. Because AMF directly influence the uptake of only those nutrients whose

movement towards root surface is limited by diffusion, nutrients not limited by diffusion must be supplied in the medium in sufficient amounts for normal host growth. Moreover, the supply of immobile nutrients, particularly phosphorus (P), and the supply of nitrogen (N) must be carefully monitored, because these nutrients appear to regulate the formation of arbuscular mycorrhizal association. Also, P in high concentrations is known to suppress AMF colonization of roots. Because of this suppression and because different species of plants can have different P uptake efficiencies, it is important to make sure that the concentration of P in the growth medium is appropriate for the particular nurse plant. Species that are very highly to highly dependent on AMF for nutrient uptake and growth are generally known to have higher external P requirements than those with a lower degree of mycorrhizal dependency. The highly dependent species can grow in soils with solution P concentrations of 0.02-0.2mg/L or higher and still sustain high levels of mycorrhizal colonization on their roots. However, such P concentrations will significantly limit AMF colonization in species that are only moderately to marginally dependent on AMF, and these species must therefore be grown at a soil P concentration lower than 0.02mg/L.

If inoculum is produced using media with extremely low P buffer capacity, such as silica sand or crushed basalt, the best approach is to feed the nurse plant through periodic additions of nutrient solution such as Hoagland's solution with the P concentration adjusted to 8 mg/L (Appendix 4). This solution can be added to support media at the rate of 200mL/kg of medium once a week. Phosphorus-free Hoagland's solution can also be used in combination with rock phosphate, which can be mixed with the media the rate of 5 mg P/kg.

The effect of N on AMF is still not well understood. At high concentrations, the effect of inorganic N is believed to inhibit root colonization, and the ammonium form is reported to be particularly toxic. This form of N is particularly problematic if its concentration exceeds 200mg/kg. Between 80-120mg/L are adequate for inoculum production purposes. If the nurse plant is a legume and the seed or growth medium is inoculated with appropriate rhizobium, most or all of the N demand of the plant can be met by biological N_2 fixation. However, in many instances a starter N level not exceeding 25-50 mg/kg will be required during the initial phase of the establishment of the legume-rhizobium symbiosis.

All other essential nutrients must be supplied in quantities sufficient for normal plant growth. The levels of these nutrients generally used involve a 1:1 sand:soil mixture (pH = 6.2) in mg/kg of medium, K = 250, Mg = 212 (as MgSo4), Zn = 10, Cu = 5, B = 0.1, Mo = 0.5. Contamination of the pots culture by undesired organisms can be minimized by covering the surface of the medium with sterilized sand or gravel.

Duration of Growth

To ensure that most of the spores in the inoculum are mature, it is essential to grow the nurse plant in the inoculum-production medium for 12-14 weeks. The medium is then allowed to dry slowly by reducing the frequency of watering over a week and then withdrawing water completely for another week, If at the end of the last week the plant is dry, it is removed from the growth medium. The roots of the plant can be chopped into fragments 1 cm long and mixed with the medium, or they can be used separately as root inoculum. The moisture content of the medium in this time should be 5% or lower. If not,

the crude inoculum must be spread on a clean surface in an environment with low humidity (RH≤65%) and allowed to air-dry until the desired moisture content is reached.

Nurse plant species

Plant species vary in the amount of root mass they produce in a given amount of time and in the extent to which their roots can be colonized by AMF fungi. Nurse plants for crude inoculum production and for root inoculum must be carefully selected on the basis of criteria such as adaptability to the prevailing conditions, rapid infectability by numerous AMF, ability to produce abundant root mass within a short time, and inherent resistance to disease and insects, particularly those that attack plant species for which the inoculum is targeted. Example of nurse plants meeting these criteria and tested are *Sorghum bicolor* or Sorghum Sudanese, Leek (*Allum cepa*), *Zea mays* and others. The more species of appropriate nurse plants one has to choose from the better, because the nurse plant used should be as dissimilar as possible from the plant species for which the inoculum is produced so that the possibility of spread of disease and parasites through the inoculum to the target plant is minimized.

Common Hygienic Procedures

Another precautionary measure against disease spread via inoculum is to surface-disinfect nurse plant seeds before germination and then transplant only clean, healthy seedlings into the inoculum production medium. Standard hygienic practices for greenhouse or growth chambers designated for inoculum production include using clean disinfected greenhouse ware, maintaining clean bench spaces, and avoiding sloppiness in transferring materials and maintaining the plants.

Nurse plant density

The number of nurse plants per unit weight of medium may influence the quality and quantity of root inoculum produced through its effect on root mass and AMF colonization level. The number of nurse plants per unit weight of sand-soil medium has very little impact on the level of AMF colonization, but it has significant impact on root mass of *Zea mays* grown in the medium. Maximum amount of AMF-colonized root mass is obtained at a density of one corn plant per 2 kg of medium.

Starter Culture

The quality of AMF culture with which one starts inoculum production will make a big difference in the quality of the final product and the length of time required to produce the inoculum. If a starter inoculum containing few propagules is used, the time allowed for the production of the inoculum must be extended, or roots will not be colonized with AMF to the degree desired. Best results both in terms of root mass and AMF colonization levels were observed if the starter inoculum contained 520 infective propagules per kilogram of medium. Increases in the density of infective propagules in excess of this value did not improve AMF levels. The starter culture also must be free from pathogenic and parasitic organisms.

Hydroponic and Aeroponic inoculum

The most common means of producing inoculum employ media like sand, soil or a mixture of the two. inoculum can however be produced in non-solid media. This includes (1) the flowing solution culture technique, (2) the flowing nutrient film technique both hydroponic, and (3) the aeroponic technique.
Hydoponic and aeroponic systems require constant monitoring and adjustment of the nutrient solutions involved. The two hydoponic techniques are useful for producing limited quantities of clean root inoculum, but their usefulness in spore production is low.
Flowing solution culture technique*:** plants are supported in a structure that allows their roots to be bathed by a continuously flowing solution of dilute nutrients, Plants are colonized by AMF either prior to their introduction into the apparatus, or they become mycorrhizal after they are introduced into the apparatus. ***Flowing nutrient film technique: roots of plant a bathed with a thin film of flowing nutrient solution. The stationary solution culture technique is similar to the flowing solution culture technique except that there is no flow and the solution is continuously aerated.
Aeroponic Technique: In the aeroponic technique of inoculum production, plant roots are continuously exposed to a nutrient solution mist in a closed chamber. This technique has proven useful in producing clean root inocula and spores.

The In-Vitro Technique: Root organ culture
A transformed carrot root is used. Root-organs provide valuable tools for producing large quantities of high quality AMF propagules. However only a few AMF species are able to grow and sporulate under in vitro conditions.

Inoculum storage

Both root and crude inoculum must be dried to moisture content of less than 5% before they are stored. It is recommended that inoculum be stored in closed plastic containers in a low humidity room at 22◦C. The inoculum should be dried as rapidly as possible to minimize growth of other microorganisms. Crude inoculum can be dried at room or greenhouse temperature by spreading it thinly on a clean surface in a clean, non-humid environment (RH 65% or lower). High quality Crude inoculum can be stored at 22◦C at a minimum of two years with minimal loss of viability. Air dried cultures of this kind are packaged in plastic bags and stored at 5◦C for at least four years. Root inoculum is best dried in a forced-air oven at 60◦C. Root inoculum dried under greenhouse conditions has a very short shelf life compared to oven dried matter, and even when dried in the oven has a shelf life of less than 100 days at 22◦C. After only 14 days of storage the effectiveness of root inoculum is similar to the reference crude inoculum.

As the duration of storage increases, the effectiveness of the root inoculum progressively decreases, the decrease being more pronounced if roots were dried in the greenhouse or in an oven at 40◦C than if they were dried in the oven at 60◦C. it is possible to extend the shelf life of root inoculum through cold storage.

Inoculum application

Methods of applying AMF inoculum include mixing inoculum with soil, placing inoculum as a layer at various soil depths, applying it as a core below the seed, banding it in much the same way as fertilizers are applied in bands, dipping roots of seedlings in viscous suspension containing AMF propagules, and placing AMF propagules adjacent to roots at the time of transplanting.

Mixing inoculum thoroughly with soil is the most straight forward method of applying inoculum in the field as well as in the greenhouse, but it is effective only when large amounts of inoculum are applied. This approach is better with crude inoculum than it is with root inoculum, because root fragments do not easily disperse in soil. Inoculum can be placed at various depths (up to 5cm) from the surface of the soil as a layer or applied in bands near the seed row (generally 5 cm below and 5 cm to the side of it).
Any type of inoculum can be ***placed close to seedling roots at the time of transplanting***. For example, ***spores can be pipetted directly onto roots either at the transplanting*** or to roots of an established plant after making a hole adjacent to the roots. Crude inoculum and root inoculum can also be applied to established plants ***by placing inoculum in holes bored into soil*** where roots are likely to be contacted. Before planting, seedling roots can be inoculated by ***dipping them in viscous medium*** (1% methyl cellulose or 10-20% gum Arabica) containing AMF propagules, usually spores. Seed application of AMF inoculum is rare.

Amount of inoculum applied

The amount of inoculum to apply directly to soil is dependent on the quality of inoculum. If a crude inoculum contains four to eight infective propagules per gram, application of 50g/kg soil usually produces rapid initiation of AMF colonization of target plants with minimal lag period. The number of infective propagules in any material containing AMF is determined by the Most Probable Number (MPN) method (Appendix 5). Spore counts and AMF root colonization may also be used. Root Inocula are generally more effective in stimulating plant growth in quantities substantially lower than are normal for crude inocula. Root inoculum contains 4000 cm of infected root per gram, application of 0.5-1g/kg of medium produces good results.

Abundance of AMF Propagules

Effectiveness of mycorrhizal fungi may not be rapidly expressed if the number of infective propagules contained in an inoculum is low. Many instances of poor inoculum performances may in fact be a result of a low level of infective propagules. All other things being equal, if high-quality inoculum is introduced into a soil containing a very low density of indigenous AMF, the probability of obtaining a positive response to inoculation is high. However, if soil contains high levels of infective propagules to begin with, it is unlikely that plants will respond to additional inoculation. It is, therefore, important to know about the quality of the inoculum as well as the abundance of native AM fungi in the target soil before one attempts AMF inoculation.

Soil P status

There are critical ranges of soil-solution P concentration at which the host-fungus association is truly mutualistic, i.e., where the benefit each partner derives from the association outweighs the costs. The primary cost of the association to the host is the photosynthate that provides for the maintenance and production of the fungus. Under normal conditions, this expenditure is more than compensated by enhanced rate of photosynthesis resulting from an increased leaf index area and perhaps also enhanced chlorophyll levels induced by the mycorrhizal association.

As the soil P concentration approaches a level nearly adequate for mycorrhiza-free growth of the plant, the contribution of the AM fungi to the plant productivity becomes negligible and may even be detrimental.
Mycorrhizal inoculation will have its maximum effect on plant growth at soil P concentration near optimal for mycorrhizal activity or at soil P concentration that are barely accessible to the unaided root. This P concentration is host-dependent. The optimal soil-solution P concentration at which a balance between the fungus and host is maintained for fast growing coarse rooted plant species is 0,02mg/L. At this concentration of soil P, the mycorrhizal association more than compensates the host for the cost associated with supporting the fungus. If the phosphorus concentration in the soil solution is sub-optimal for mycorrhizal function, AMF symbiotic effectiveness is curtailed, and the fungus and the host may compete for scarce P. When solution P concentration is much above the optimum for a given host-fungus combination, mycorrhizal colonization will be suppressed. If the host fails to suppress the development of the fungus at soil P concentration near-optimal or above-optimal for mycorrhiza-free growth, the fungus will act as a parasite rather than a mutualist, and host growth may be depressed as a result. The best approach to optimizing the soil solution P concentration is first to determine the P-sorption isotherm of the soil. The many benefits associated with inoculation with AMF will not be realized unless the soil-solution P concentration is optimal or near optimal for AMF colonization and function. Consequently, AMF play crucial roles in certain conditions:

1. Native ecosystems (e.g forests) where application of large quantities of fertilizer P to extensive land areas is not usually done or is not practical.
2. Agriculture system on soils with strong P fixing capacity, or where P fertilizer is unavailable or prohibitively expensive.
3. Situations where it is essential to reduce soil fertilizer applications because of environmental concerns such as nutrient pollution of surface waters
4. Situation in which rock phosphate is readily available and used instead of more soluble P sources.

Variation in the dependence of plants on AM fungi

Mycorrhizal dependency is a measure of the degree to which a plant species relies on the mycorrhizal conditions for nutrient uptake and growth as the concentration of P in the soil solution is increased. It is well established that plant species and cultivars within a given species vary in their response to AMF colonization. Most of the variation may have to do with the ability of plant species to take up P at very low soil-P concentrations in the absence of mycorrhizal fungi. The property of P uptake, efficiency, as discussed earlier,

is related to a great extent to root mass and root morphology. Species that produce large quantities of fine roots and many long root hairs generally tend to be less responsive to AMF inoculation than those with sparse and coarse root systems and few root hairs.

Other properties that allow some plants to have a low external P requirement and hence a low response to AMF colonization are the ability to acidify the rhizosphere or excrete chelating agents that bind to P-fixing cations like aluminium. The degree to which these morphological and biochemical root mechanisms meet the host plant's demand for P will determine the degree to which the plant responds to AMF inoculation at a given soil-solution P concentration.
The dependency of plant species on the mycorrhizal condition is a function soil solution P concentration. All other things being equal, AMF inoculation will have its maximum effect on host plant growth when the level of P in the soil solution is barely accessible to nonmycorrhizal plant. Because the effect of mycorrhizal colonization on host plants, by and large, could be duplicated by amendment of the soil with fertilizer P, one could establish categories of mycorrhizal dependency of host plants by assessing plant host responses to AMF colonization at different soil solution P concentration.
When soil solution P concentration is appreciably lower than 0.02mg/L, most plant species will respond dramatically to mycorrhizal colonization. As P concentration is increased from this level to 0.1-o.2mg/L, the dependency of plants on AMF for P uptake diminishes progressively, so that at 0.2mg/L only very highly mycorrhizal-dependent species respond significantly to mycorrhizal inoculation.

Soil disturbance

The activities of AM fungi can be severely curtailed by soil disturbance in both native and agricultural ecosystems. In native ecosystems, soil disturbances caused by land clearing and mining operations can be so severe that mere inoculation of the affected areas with AMF may not be able to restore the symbiotic function of the fungi. In soils severely disturbed by tillage, native AMF populations are not likely to initiate AMF formation on the target crop rapidly, and the process can be enhanced by inoculating the soil with high-quality AMF inoculum.

Appendix 5: a modified hoagland's solution for use in AMF inoculum production

Stock Solution	Working solution (ml/L
$_{M}NH_4NO_3$	1
$_{M}KNO_3$	6
$_{M}Ca(NO_3)_2$	4
$_{M}MgSO_4$	2

Micronutrient solution
Dissolve the indicated amounts in 1 liter of deionized water, 1ml of this solution is added to each litter of final solution.

A separate iron solution

Prepare a 5% iron tartrate solution and add it at the rate of 1.0ml of final solution just before the solution is added to the plant.

Element	Carrier	Amount (g)
B	H_3BO_3	2.86
Mn	$MnCl_2.4H_2O$	1.81
Zn	$ZnSO_4.H_2O$	0.22
Cu	$CuSO_4$	0.08
Mo	$H_2MoO_4.H_2O$	0.02

Appendix 6: Evaluating effectiveness of AMF inoculum

One way to assess the quality of an inoculum is to determine the density of viable spores it contains (Methods of spore extraction); and another way is to determine the total number of infective propagules in the inoculum. This is done by employing the most-probable-number technique.

The quality of inoculum can also be assessed in terms of the degree and the speed with which the inoculum colonizes roots of an indicator species or stimulates the P uptake and growth of a highly mycorrhizal dependent indicator plant species grown on a medium optimized for AMF activity. The rate of development of AMF colonization can be determined by growing the indicator plant in a medium optimized for mycorrhizal activity in the presence of the test inoculum and then monitoring AMF colonization of roots as a function of time through destructive sampling roots. Growth of the indicator plant can be monitored over time nondestructively by measuring leaf number, plant height, stem diameter, and leaf-area index, or by destructively determining biomass accumulation. The P status of the indicator plant can be used to assess inoculum quality by growing the plant in the presence and absence of the test inoculum in a medium optimized for mycorrhizal formation and activity. P status can be determined nondestructively over time by monitoring the P content of pinnules, leaf disks or leaf tips, depending on the species of the indicator plant used.

Appendix 7: Determining the abundance of infective propagules in crude inoculum and in soil

Determining the number of infective propagules in soil and crude inoculum can be complex for various reasons. First, fungal structures such as spores, vesicles, arbuscules, mycelium, and even colonized roots act as infective propagules. Secondly, AMF cannot be cultured in vitro conditions apart from their host plants. Although spores can be isolated and counted, not all of them are ready to germinate, and hence spore numbers are often not strongly correlated with AMF infectivity. The most reliable method of assessing the number of infective AMF propagules contained in a crude inoculum, soil, or sheared mycorrhizal roots is most-probable number (MPN) technique, which permits a statistical estimation of microbial population density without direct count of single cells or colonies. The MPN technique is the most precise method to estimate mycorrhizal

propagule numbers because It considers the infectivity of viable spores, mycelial fragments and fragments of colonized roots.

The Most Probable Number (MPN) Methods

The technique is based on determining the presence or absence of microorganisms in several individual aliquots of each of several consecutive dilutions of a sample of soil or other materials containing microbial propagules. A serial dilution, usually 10-fold (or four fold), of soil or crude inoculum sample is prepared using sterile sand, soil, or sand-soil mixture as the diluent. From each dilution, a predetermined amount of material, say 20 g, is used to inoculate each of the five cups containing 270-350 g of sterile soil or sand-soil mixture optimized for mycorrhiza activity with soil-solution P concentration of 0.02mg/L. Germinated seeds or seedlings of a suitable mycorrhizal plant are sown in these cups, which are placed in a reservoir containing water of P-free nutrient solution.

The plants are allowed to grow in the greenhouse or growth chamber for four weeks. At the end of the growth period, the roots are excised, washed, cleared, and stained. The stained roots are spread in a petri dish and scored for the presence or absence of AMF colonization. Do not count detached hyphae or germinated spores.
To calculate the most probable number of infective propagules in a sample, the statistical table developed by is essential. In the table, p_1 stands for the number of positive replicates in the least concentrated dilution, and , p_2 and , p_3 represent the numbers of positive replicates in the next two higher dilutions. The most propagule number of infective propagules in the quantity of the original sample is obtained by multiplying the reciprocal of the middle dilution by the number in the table located at the point of intersection of the experimentally observed values corresponding to p_1 , p_2 and p_3 . The value represents the most probable number of infective propagules for the quantity of soil used to inoculate test plants (e.g 20g). The number of infective propagules per gram of soil can be obtained by dividing the number of infective infective propagules observed by the quantity of soil.

Example:
Suppose the following number of positive replicates are obtained for the following dilutions:
$10^{-1} = 5$
$10^{-2} = 4$
$10^{-3} = 1$
$10^{-4} = 0$
$10^{-5} = 0$
In this series, $p_1 = 5$, $p_2 = 4$ and $p_3 = 1$
For this combination of p_1 , p_2 and p_3 , Cochran's table gives 1.7 as the most probable nunber of infective propagules applied in the 10^{-2} dilution. Multiplying this value by the dilution factor 10^{-2} gives 107 as the number as the number of infective propagules in the original sample. The number of infective propagules per gram of soil is calculated (107/20 = 5.35 to be approximately five.