CIFOR World Agroforestry	**SOP 001**	**Date: 25.02.2022** **Author:** Lukelysia Mwangi **Authorizer:** David Lelei

STANDARD OPERATING PROCEDURE

METHOD FOR ISOLATION AND OBSERVATION OF AMF SPORES FROM SOIL

Contact Details:	**Address Details:**
Telephone: +254 (20) 7224000/4545/4433 Email: l.n.mwangi@cifor-icraf.org and D.Lelei@cgiar.org Website: https://www.cifor-icraf.org/research/theme/soil-and-land-health/	World Agroforestry Centre, **Mailing:** P.O Box 30677-00100 Nairobi, Kenya **Physical address:** United Nations Avenue Off Limuru Road, Gigiri, Nairobi, Kenya

METHOD DOCUMENT CONTROL LOG

	Name and position	Signature
Author(s)	Lukelysia Nyawira - Research Associate	[*signature for completeness and correctness of document*]
Verifiers		[*signature for completeness and correctness of document*]
		[*signature for completeness and correctness of document*]
Authorizer	David Lelei – Junior Scientist	[*signature for completeness and correctness of document*]

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Changes in this version compared to previous version:

1. Inclusion of workflow
2. Inclusion of quality control document

Contents

ABBREVIATIONS

- µm - micrometre
- cm - centimetre
- g/l - gram per litre
- GLP - Good Laboratory Practices
- M - molar
- Min - minute
- Ml - millilitre
- Rpm - rotation per minute
- Sec - second
- v/v - volume per volume
- AMF- Arbuscular Mycorrhiza Fungi

DEFINITIONS

a) ***Contaminated by microorganisms***: Every plate, tube, pipette, or other instruments (glassware, pestles, ependorff tube...) which has been in contact with microorganisms and cannot be sterilized by the flame of a Bunsen burner is considered as contaminated.

b) ***Contaminated by toxic chemicals***: Every tube, flask, pipette or other instruments (Weighing boats, glassware…) which has been in contact with toxic chemicals is considered as contaminated.

c) ***Good Laboratory Practices (GLP):*** The Principles of Good Laboratory Practices (GLP) have been developed to promote the quality and validity of results and of the analysis conducted in a laboratory. It is a managerial concept covering the organization and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported. Its principles also include the protection of man and the environment.

1. INTRODUCTION

The most successful arbuscular mycorrhizae are formed with the fungal phylum Glomeromycota. Arbuscular mycorrhizal fungi penetrate the cortical cells of the roots to form unique structures such as internal hyphae, arbuscules and coils, vesicles. The fungi exist in the soil as spores, auxiliary cells or hyphal net.

The fungal structures: spores, arbuscules, vesicles, hyphae and auxiliary cells are all used to diagnose families, genera and species and the status of the symbiosis and its functions in plants and soils. Methods for evaluating functions range from morphological, biochemical to molecular. This procedure describes the detailed protocol to isolate arbuscular mycorrhizal fungal (AMF) spores from either field soil of artificial substrate mixtures used for the pot cultures of the AMF.

2. SCOPE AND APPLICATION

Arbuscular mycorrhiza fungi (AMF) are soil microorganisms that can form mutualistic relationships with most terrestrial plants. AMF spores germinate, infect the root system, and form arbuscules structures inside the cells of the plants. Arbuscules serve as sites for nutrient exchange between the plant and the fungi. Another distinguishing feature of this symbiosis is the ability to form dense mycorrhizal network surrounding the root system aids in enhanced water and nutrient uptake by plants roots.

3. PRINCIPLE

Mycorrhizal has been in use as a biofertilizer especially in agricultural lands for maize, barley, wheat and oats. The product boosts the performance and vitality of plants. Application of mycorrhiza in agricultural fields is also sustainable compared to continuous application of fertilizers. An example of Mycorrhiza product is MycoApply

4. RELATED DOCUMENTS

a) Standard Operation Procedures for evaluating COMPRO Products here

5. PROCEDURES

5.1 MATERIALS, EQUIPMENT AND REAGENTS

i. Materials

a) Centrifuge with a swinging bucket rotor
b) Waring blender
c) Microscope

ii. Equipment

a) Coverslip (preferably circular, variable diameter depending on spore size)
b) Sieves (710 µm and 45 µm or 500 and 53 µm)
c) Petri dishes, 9 cm in diameter
d) Pipette and Pro pipette 5 ml
e) Beakers (Small and big)
f) Spatula

iii. Reagents

a) Melzer's reagent (Chloral Hydrate, 484.3 g/l; Iodine, 7.3 g/l; Potassium Iodide, 24.2 g/l)
b) Polyvinyl-Lacto-glycerol (PVLG) (Polyvinyl alcohol, 73.3 g/l; Lactic acid, 44% (v/v); Glycerol, 4.4% (v/v))
c) Sugar/sucrose solution (2.5 M or 48%)

5.2 ANALYSIS DESCRIPTION

Isolation of AMF spores

- Take 50 g soil sample from the dry soil for spore extraction from field soils and 25 g from pot cultures soils.
- Soak in water (just enough to cover the soil) and thoroughly stir using a blender into a suspension for 30 sec. If soil is clay in texture, soak for at 2 min to disintegrate the aggregates before stirring.
- Wash the suspension through 710 µm sieve placed on top of 45 µm sieves with running water (other sieves may be included in this series e.g., 500, 250, 100 µm sieves, but each collection will need to be examined separately). Repeat this process several times, breaking down any lumps of soil between washes and collect suspension in a beaker.
- Examine the collection on the 710 µm sieve for sporocarps before discarding. The contents of the 45 µm sieve are backwashed into a small beaker trying to keep the volume to a minimum.

- Swirl the suspension collected and quickly pour into 50 ml centrifuge tubes up to a maximum three-quarter way up the tube.
- Balance the tubes by weight and centrifuge for 5 min at 1750 rpm.
- Decant the water carefully from tubes and discard floating debris not to disturb the sediment at the bottom of the tube.
- Add a volume of 48% commercial sugar (up to 3/4 of the tube) and mix with the sediment.
- Balance the tubes by weight and mix thoroughly before centrifuging for 15 sec at 1750 rpm.
- Pour the supernatant (sucrose and spores' suspension) immediately after centrifugation through a 45 µm mesh sieve. Retain the residue on the sieve and rinse thoroughly with water to wash out the sucrose. This is particularly important if the spores are required to initiate pot cultures.
- Backwash the spores from the 45 µm sieve into a small petri dish for examination under a compound microscope.
- On fresh soil samples, add 2-3 drops of glutaraldehyde to the petri dish to prevent spores from being attacked by nematodes and mites if not observed immediately. For long-term storage the spores are stored in vials with 2% glutaraldehyde.

5.2.1 Alternative method

- Wash the soil through 500 µm and 53 µm pore sieves with running water.
- Backwash the contents of the 53 µm sieve into a small beaker.
- Mix in a waring blender for 2-3 sec on a high speed. Pour the suspension again through the 53 µm sieve.
- Transfer the material from the 53 µm sieve to 50 ml centrifuge tube (maximum half tube).
- Gently add an equal volume of 2.5 M sucrose solution pouring it onto the wall of the vial. Gently mix the decanted soil with the sucrose by using a fine spatula.
- Balance the tubes with water.
- Centrifuge the tubes in a swing-out rotor at approx. 3000 rpm for 2-5 min.
- Remove the spores caught at the interface of the two layers with the 5 mL pipette. Start above the interface and work down into the sugar phase using a circular motion as some species produce spores which can sink in the sugar solution while others can float just above the interface.
- Pour the sucrose and the spores into a clean 53 µm sieve and wash thoroughly to remove traces of sugar solution.
- Backwash contents into a Petri dish.

5.2.2 Mounting slides and observations

- Voucher specimens of permanent slides (slides sealed with colorless nail varnish at 5-10 days after mounting) are prepared as reference material.
- Prepare three slides: On the first one, place two drops of water at opposite ends of a microscope slide. On the second, place one drop of PVLG on each end and on the third one, put PVLG + Melzers (ratio 1 :1) on both ends.
- Pick carefully spores with a fine forceps or micro-pipette and place in each drop.
- Lower a coverslip, preferably circular with diameter of 13 mm onto each drop of mounting reagent + spore.
- For each slide, observe the spore as whole for diagnosis of appearance on one end and on the other end, crush the spore gently and severely. A needle or pin may be used to crush the spores. Caution must be taken not to break the coverslip.
- In case of bubbles, gently place ethanol though the space between slide and coverslip to clear bubble, absorb ethanol with tissue and then gently place the mounting reagent again.

6. MAINTENANCE

Servicing of the Microscope, Luminar flow, Centrifuge and Blender to be done twice annually.

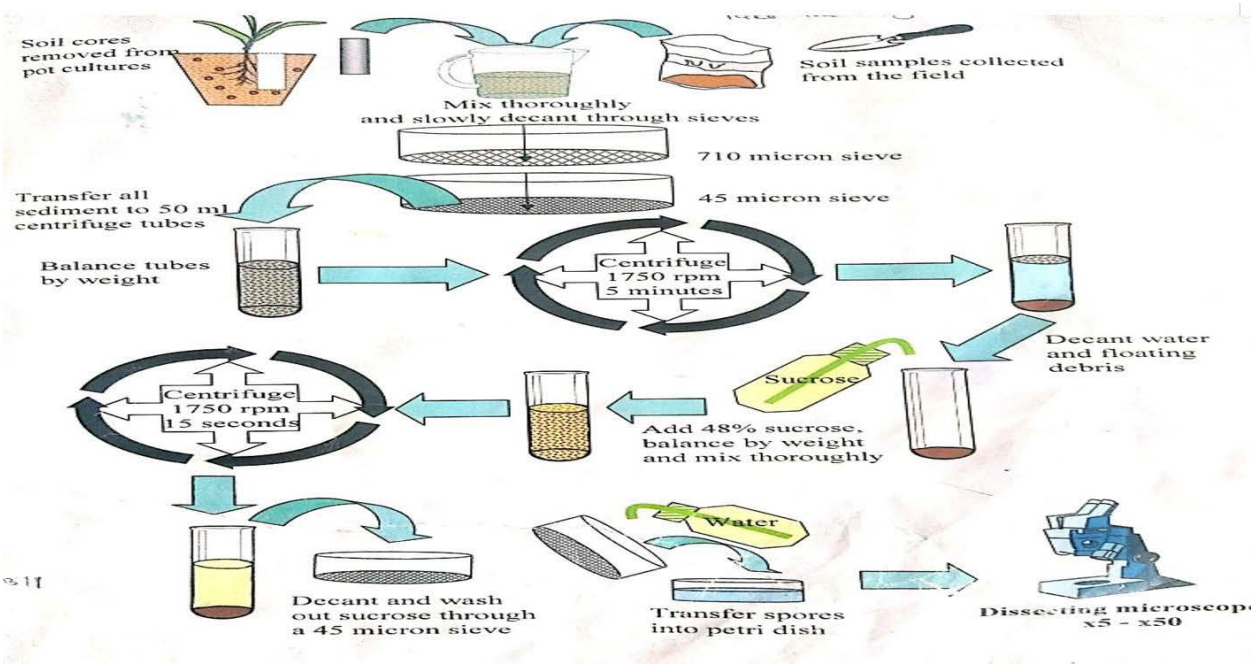
7. OCCUPATIONAL HEALTH AND SAFETY

- All activities performed under this SOP comply with the recommendations of ICRAF Health and Safety policy
- Laboratory dress code: clean laboratory coats and low-heeled closed shoes worn at all times while performing the procedures.
- Hand gloves are worn when handling chemicals and the microorganisms
- Laboratory working areas are cleaned daily and benches regularly disinfected and sterilized using 70% alcohol during various procedures.

8. REFERENCES

- Brundrett, M., Abbott, L.K. and Jasper, D.A. (1999). Glomalean mycorrhizal fungi from tropical Australia. I. Comparison of the effectiveness and specificity of different isolation procedures. Mycorrhiza 8, 305-314.
- Talukdar, N.C. and Germida, J.J. (1993). Propagation and Storage of Vesicular-Arbuscular Mycorrhizal Fungi Isolated from Saskatchewan Agricultural Soils. Canadian Journal of Botany-Revue Canadienne De Botanique 71, 1328-1335.
- Wagner, S.C., Skipper, H.D., Walley, F. and Bridges, W.B. (2001). Long-term survival of Glomus claroideum propagules from soil pot cultures under simulated conditions. Mycologia 93, 815-820.

6. ANNEX



- For description of spores refer to INVAM WEBSITE and Schenck and Perez, 1990.