Chapter 7

Methods to Process and Identify Symbiotic Fungi in the Roots of Vascular Plants

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Introduction:

Vesicular arbuscular mycorrhizae (VAM) are present in the roots of almost all vascular plants. These common, soil-borne fungi belong to the family, the Endogonaceae (Zygomycotina) and produce fungal structures in the cortex of the roots. VAM play a crucial role in the mineral nutrition of plants by transferring phosphorus and other minerals from the soil to the plant. Techniques for obtaining VAM samples from natural sources and from inoculated pots are outlined. Methods of isolating spores, of processing, staining and clearing root samples, and of identifying the fungal structures are discussed

Isolation of Roots and VAM Spores from Natural Sources and from Inoculated Pots.

[Wetsieving and decanting methods for spore isolation, modified from Gerdemann (1955), and Gerdemann and Nicolson (1963). See Schenck (1982) for other details concerning methods for spore and root isolation.]

Methods:

1. Processing a plant plus surrounding soil or a soil core for isolation of the roots and the VAM spores present in the soil.

> Remove the plant from the pot or from its natural environment along with the soil around the roots. Cut off the top. Soak the root system in a large container of tap water and then wash the roots with a running stream of tap water to remove the soil. All of the soil particles are collected in a large container. Break-up the larger soil particles with your hands.

2. Collection of intact roots.

> Save the roots to examine for VAM and/or use as an inoculum. Place the roots in a plastic bag containing a small amount of water so they will not dry out. If the roots will not be examined or used in a couple of hours, put the bag in the refrigerator, or the roots may be fixed in formal-acetic-alcohol (FAA) to be processed and examined later for VAM structures.

3. Collection of additional roots and large pieces of organic debris (to be discarded) on a 2 mm sieve.

> Pour the soil-water mixture discussed in #1 through a 2 mm Nalgene sieve that has been placed above its bottom half, a 5 liter solid container. The soil-water mixture is collected in the bottom half. Remove the large pieces of organic matter from the sieve, and then collect the roots by picking off the larger ones and placing them in a small container of water or fixing them in FAA.

4. Collection of spores (propagules) of mycorrhizal fungi on a 38 um sieve.

> Stir the soil-water mixture and pour part of this soil suspension onto the surface of the 250 um sieve that has been stacked on top of the 38 um sieve in the sink. The top sieve will concentrate most of the soil particles; so only the fine soil particles along with the VAM spores will collect on the 38 um sieve. Use a jet of tap water

to wash the spores and finer soil particles through the top sieve. This water will quickly pass through the top sieve, but it is difficult to get the water to drain through the 38 um sieve because of the build up of fine soil particles. The two sieves are separated and a jet of water on the surface of the 38 um sieve permits the water to drain at that spot. Then the two sieves are stacked together again, and the process is repeated until the water washing through the two connecting sieves leaves the bottom sieve colorless. Generally this means the soil in the top sieve is washed 3 times. Note: The water drains slowly through the lower sieve; hence, the 38 um sieve must be continuously checked by separating the two sieves and visually looking at the height of the water. If the water does overflow the lower sieve, spores are lost.

5. Concentration of VAM spores in soil pellet.

   The screenings are collected from the 38 um sieve by washing them into a beaker using a small stream from a wash bottle. Then, the soil is placed into a series of 50 ml, round-bottom centrifuge tubes for use in a Sorvall RC-2B centrifuge (1/3 soil and 2/3 tap water up to within 2 1/2 cm of the top of the tube). The mixture is thoroughly stirred, the tubes are balanced and then centrifuged at 4000 rpm for 5 minutes. The supernatant containing the light organic matter is decanted.

6. Isolation of VAM spores in 50% sucrose.

   The pellet containing the spores is suspended in a 50% sucrose solution and is mixed well. The tubes are balanced and centrifuged again at 4000 rpm for 5 minutes. The supernatant that contains the spores are poured over the clean 38 um sieve and immediately washed with tap water to remove the sucrose. The VAM spores are washed into a beaker of water using a water bottle. This entire procedure (#6) is repeated one or two more times to obtain more spores.

7. Isolation of VAM spores free of nematode cysts.

   The VAM spores can be cleaned further by preparing a gradient sucrose solution in a 15 ml centrifuge tube (clinical centrifuge tubes). Place 3 ml of each of these solutions in this order: bottom layer, 50% sucrose solution; middle layer, 15% sucrose solution. Slowly add the spore suspension into the tube. The water in the spore suspension makes up the 3 ml top layer. Centrifuge at 2750 rpm (#7 on the speed dial of the clinical centrifuge) for 5 minutes. Collect the spores from the first (top) interface and wash off the sucrose solution. These spores will be free of nematode cysts and other contaminants found in the other interface (Tang, 1986). Make a wet mount and examine a small sample of the spores with the dissecting and compound microscopes.

8. Sterilization and storage of spores.

   Sterilization: Concentrate the spores on the 38 um sieve and then with a squeeze bottle wash the spores into the sterilization solution. Soak the spores in this solution for 20 minutes. Pour the spores onto the filter which is sticking on the funnel, and rinse with distilled water 5 times. make a hole in the filter paper and wash the spores into a 100 ml graduated cylinder. Add more water until the volume is 100 ml. Samples can be removed to determine the spore number (Tang, 1986).

Short term storage: The isolated, non-sterilized spores may be stored for 2 days in the refrigerator in a beaker of water sealed with parafilm.

Long term storage: Fill a clean petri dish 3/4 full of clean, dry sand and pour the sterilized and rinsed spore suspension over the sand. Allow the water to evaporate from the sand by leaving the cover off. This may take overnight to several days. When the sand is dry, cover the petri dish and seal it with parafilm. Store the sealed and labelled dish at 4° C. until the spores are used.

9. Retrieval of long term stored spores.

Pour the dry sand/spore mixture onto the 250 um sieve (top), which has been stacked on the 38 um sieve (bottom). Using distilled water, rinse the spores through the top sieve and collect them from the bottom one (38 um) with a squeeze bottle containing distilled water. The spores can be washed into a petri dish.

10. Isolated spores may be identified and/or used to inoculated other plants.

Variation:

A small amount of Calgon or other surfactant may be added to the 50% sucrose solution (#6) to increase the spore yield (Ianson and Allen, 1986).

Materials and Equipment:

Centrifuges-Sorvall RC-2B and a clinical centrifuge.
Centrifuge test tubes:
50 ml, round bottom tubes for Sorvall centrifuge
15 ml glass tubes for clinical centrifuge
Water bottles with bent spout, filled with distilled water.
Sieves: 2 mm Nalgene
250 um mesh sieves
38 um mesh sieves
Beakers: 250 ml and 100 ml
Petri dishes
Clean sand (small amount to fill 12 petri dishes).
Rubber tube (long enough to attach to tap water faucet). A plastic attachment at the end will provide the water jet
Compound microscopes
Dissecting microscopes

Preparation of Solutions:

15% sucrose (w/w) - 15 g. sucrose + 85 ml distilled water
50% sucrose (w/w) - 50 g. sucrose + 50 ml distilled water
Sterilization solution (Tang, 1986) -
2 g. chloramine T (2% w/v)
0.02 g. streptomycin sulfate (200 ug/ml)
a trace of Tween 80
add water until the total volume is 100 ml

Clearing and Staining of Roots to see VAM Structures: Basic Method

Methods:

1. Clearing the roots. Wash roots preserved with FAA in water. Heat at 90º C. for generally 1 hour in 20% KOH in the hood.

2. Pour off KOH solution and rinse with 3 changes of tap water or until the rinse is clear.

3. Acidify with 10% HCL and soak for about 4-5 minutes, then pour off. Do not rinse.

4. Cover roots with a 0.01% acid fuchsin-lactic acid solution, which will stain the VAM structures. Heat at 90º C. for 10 to 60 minutes.

5. Rinse off the solution with distilled water and mount the roots on a slide either in water (temporary mount) or in a permanent medium (See Powell and Bagyaraj, 1984).

6. Examine the slides with the compound microscope for VAM structures including spores, arbuscules and hyphae.

7. Allow slides to set and harden on warming tray for two days. Lead weights may be used to flatten root segments.

Variations:

1. Length of time in KOH (#1): Some roots such as cattails are destroyed if heated for more than 20 minutes (Tang, 1986); however, most grass roots need to be processed for 1 hour.

2. Type of stain: Other stains work just as well as acid fuchsin; however, one advantage of acid fuchsin is that no destaining is necessary. Trypan blue is a commonly used stain (0.01%) but destaining for 1 hour or more using the lactic acid solution without stain is required.

3. Heating or no heating: KOH (#1). The roots do not have to be heated in the base instead they can be left at room temperature. The minimum time which my lab has used is 12 hours, but shorter periods may be possible. Heavily suberized roots may need to be left for several days or even a week.

4. Phenol: Do not add to lactic acid solution. It is not necessary and is potentially carinogenic.

Materials and Equipment:

Water bath heat to 90º C.
Test tubes and holders
Tweezers
Slides
Coverslips
Compound microscopes

Preparation of Solutions:

20% KOH
10% HCL
Lactic Acid Solution -
875 ml lactic acid
63 ml glycerin
63 ml distilled water
Add 0.1 g. acid fuchsin to 1 liter lactic acid solution

Discussion:

Details of most of the procedures I have discussed are elaborated on in the excellent reference book, "Methods and Principles of Mycorrhizal Research," edited by Schenck (1982). Chapter three by Daniels and Skipper (1982) and chapter four by Kormanik and McGraw (1982) are particularly useful. Kormanik and McGraw discuss the problems with Phillips and Hayman (1970) procedure for clearing and staining roots, and make recommendations for improving the method. Photographs of VAM structures, including spores, arbuscules, vesicles and hyphae are provided at the back of this reference (Schenck, 1982). If these structures are found within the roots of the plant, then the plant if mycorrhizal.

Another useful general reference concerning VAM is the recent book by Powell and Bagyaraj (1984). Keys for spore identification are found in the books edited by Schenck (1982) and Powell and Bagyaraj (1984).

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Acknowledgement:

Funding for the Typha-VAM Project has been supported by grants from the BioEnergy Coordinating Office (BECO) to I. Charvat. The techniques covered in this workshop have been developed as part of this project.