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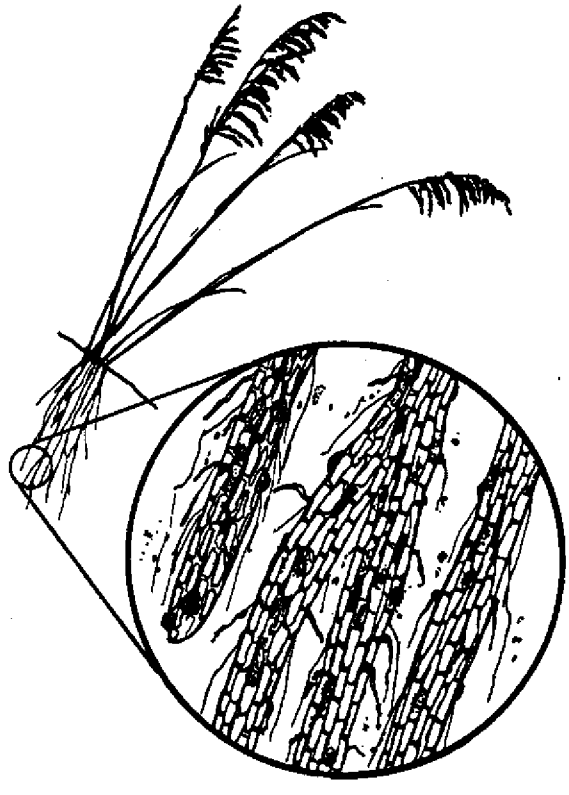

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The Production and Use of Aeroponically Grown Inocula of VAM Fungi in the Native Plant Nursery [1]

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Introduction

Selected vesicular-arbuscular mycorrhizal (VAM) fungi have been shown to enhance the growth of numerous plants of economic importance (Jeffries, 1987) including native plants used for revegetation of disturbed sites such as renourished coastal beaches (Sylvia , 1989) and strip-mined lands. Major benefits for the plants are increased uptake of phosphorus and other poorly mobile nutrients and tolerance of water stress. Nonetheless, VAM fungi are not used widely in plant production systems because inoculum sources are limited and application technologies are not well developed.

In this publication, a system for culturing VAM fungi using aeroponic culture technology is presented. The aeroponic culture system allows the roots to grow suspended in a highly aerated mist of dilute nutrient solution. The techniques for processing the colonized root material into various inocula are also detailed, as well as methods to apply the inoculum to plants and to store the inocula. Instructions are given at a technical level intended for researchers and their assistants as well as plant growers with a background in basic chemistry and microbiology.

Rationale

Roots colonized by mycorrhizal fungi are the rule rather than the exception for plants growing in undisturbed ecosystems. Vesicular-arbuscular mycorrhizal fungi are obligate symbiotic fungi present in the roots of almost all families of higher plants. Plants become colonized from other colonized plants, and from spores in soil or in the growing medium. Soilless growing mixes and pasteurized, sterilized or fumigated soils are devoid of VAM fungal propagules. Plants produced in these growing substrates will have little or no colonization when transplanted. These non-colonized plants may require more water and fertilizer inputs after transplanting. Plants should suffer less transplant shock when colonized with VAM fungi than those plants lacking VAM colonization.

At present, VAM fungi cannot be grown apart from living host roots. These fungi are often grown in soil-based pot cultures. However, soil contains many abiotic and biotic components which make it an undesirable substrate in which to grow and subsequently to distribute the VAM fungal inoculum. These undesirable attributes include a greater bulk density than roots alone, as well as the potential for contamination by insects, nematodes, and plant pathogens. Furthermore, colonized roots and spores which act as VAM propagules are diffused (approximately 10-100 per gram) in soil and large amounts (tons per year) of inocula would be needed for inoculation of the large numbers of plants grown in container nurseries.

Aeroponically-produced inocula are free of soil. The absence of the soil mineral component allows the root inocula to be finely cut, allowing for efficient distribution of inoculum by mixing with the growth media or direct application in a flowable, hydrogel suspension. The efficiency of aeroponic inoculum should make the cost of inoculation near $0.005 per plant and allow a grower to economically sell plants colonized by VAM fungi.

Production of Aeroponic VAM Inoculum

Obtaining Effective VAM Fungi

Effective VAM fungi are those which produce the plant-growth enhancement under the environmental conditions present at the planting site. Testing for effectiveness is a complicated process which we do not recommend for the grower. Fungal isolates with known effectiveness may be obtained from researchers at the University of Florida or from INVAM (International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi, 401 Brooks Hall, West Virginia University, Morgantown, WV 26506-6057). Spores of the selected VAM fungus should be obtained from a pure-isolate culture. Isolate selection is one of the most critical tasks for obtaining successful colonization under nursery conditions and plant growth enhancement at the

planting stage. Expert advice should be sought at this stage.

Disinfection of Spores

Spores may have other organisms growing on them and should be treated with a surface disinfectant prior to inoculation of the culture plants. To disinfect spores, first prepare the disinfecting solution by placing 2 drops of a liquid detergent in 100 mL of distilled water. Then, add either 20 mL of 5.25 % sodium hypochlorite (household bleach) or 4 grams of Chloramine T. In addition, add 0.04 grams of streptomycin sulfate to the 100 mL solution. Mix the solution well and then add an additional 100 mL of distilled water. Next, place the spores on filter paper (such as Whatman # 1) in a glass or plastic funnel or on 41 μm mesh fabric filter in a magnetic filter holder (Fig. 1A). The funnel should be arranged so the waste solution drains freely from the bottom. Pour the disinfecting solution (200 mL total volume) over the spores. Follow the disinfecting solution with 1000 mL distilled water. The volume of the disinfecting solution may be increased by simply multiplying the formula, although 200 mL is sufficient for approximately 1 million spores. Wash the spores off the filter paper or mesh into a dish (e.g., Petri dishes) to use in inoculation of the culture plants. Divide the spores into groups of 500-1000 spores in separate dishes. A low-power (15 X power) dissecting microscope or a 10 X magnifying lamp may be used.

Inoculation of Culture Plants

Culture plants are those which are inoculated and colonized by VAM fungi for the purpose of growing the VAM fungus on their roots to produce inoculum. For aeroponic culture these are the plants placed in the aeroponic chamber.

Whether slips or seeds are used for starting culture plants several criteria are necessary for quality inoculum production from a given culture plant. These include: (i) that the culture plant has no pathogens in common with the plant targeted for inoculation, (ii) the potential is greater than 50% for root colonization in six weeks, (iii) the ability to grow well under the available conditions in the greenhouse, and (iv) the tolerance of low nutrient conditions which favor the fungus rather than the culture plant. Suggested plants include sweet potato, bahiagrass, sweet corn, and sorghum-sudan grass hybrids. We have used industrial sweet potato, cultivar 'White Star', with success. Slips should have the ability to root extensively within two weeks. Slips that are growing vigorously at cutting have more extensive root growth when transplanted to the aeroponic chamber.

To inoculate the culture plants, first prepare one, disinfected 2-L pot for each group of 25 plants. Plastic pots may be disinfected by washing in 10% household bleach with detergent. Clay pots should be first washed and then autoclaved for 1 hour. Plug the drain holes of plastic pots with polyester fiberfill. Fill each pot 2/3 full with new, uncontaminated vermiculite. Wash the selected, disinfected spores over the vermiculite and mix with the vermiculite over the entire exposed area of the pot to create a uniform layer of inoculum (Fig. 1B). Cover with more vermiculite to 2 cm below the top of the pot. Plant either disinfected seed or slips of the culture plant, pushing disinfected slips into the vermiculite so the terminal buds are just above the surface.

Aeroponic Chamber Construction

For ease of sanitation, an acrylic-lined chamber should be used, but white acrylic enamel paint over smooth-finished fiberglass is acceptable. Chambers should be painted white on the outside surfaces to reflect light and reduce the internal temperature. Chambers should have lids with 2.5-cm diameter holes spaced approximately 10 cm apart in a regular grid pattern. Spacing may vary with culture plant, but spacing must allow the nutrient mist to reach all roots.

Three systems of misting may be used to deliver the nutrient solution to the roots of the culture plants. The first two are represented in Figure 2. The first is after Zobel et al. (1976) and uses an atomizing disk similar to those used in cold water humidifiers (impeller stem #38994, impeller disk #38993, Northern Electric Co., P.O. Box 70 Hwy. 70 Hattiesburg,

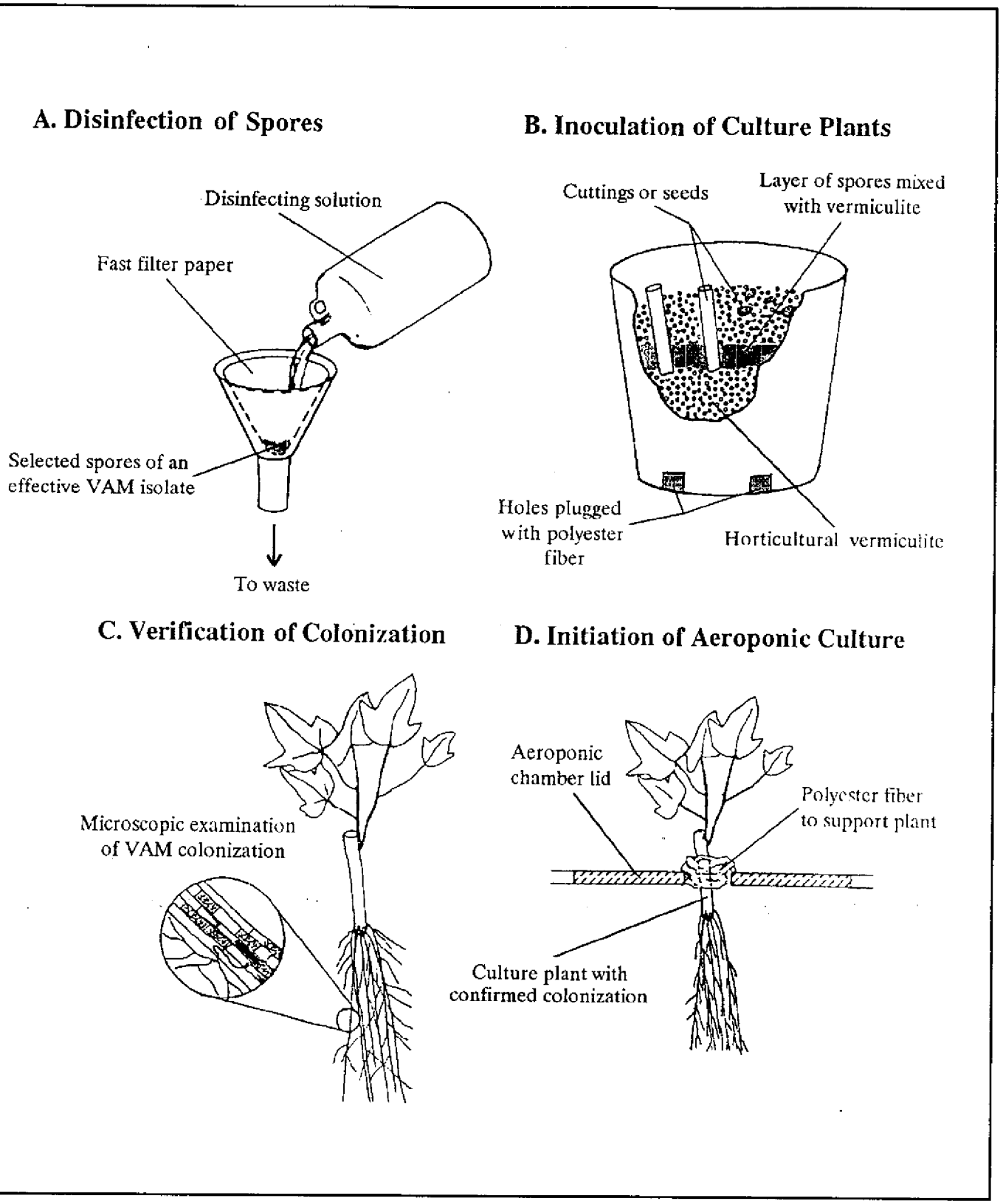

Figure 1. Procedures to initiate culture plants for aeroponic inoculum production.

MS 39401). This disk is spun at 3200 rpm on the end of a 1/4 inch threaded (1/4 inch by 20 threads; **note**: English units are used to describe some components
where metric units are not-applicable) stainless steel shaft coupled to a 1/8 hp motor (1/8 h.p. Dayton stud-mount, internal fan motor #3M292W.W. Granger, Inc., Jacksonville, FL 32205). The use of this mist delivery system is limited to a chamber approximately 61 cm W X 122 cm L X 61 cm D with between 60 and 80 L of nutrient solution. The misting must be continuous.

The second system uses a centrifugal pump (1/25 h.p. Teel magnetic drive chemical solution pump #1P677, W.W. Granger, Inc., Jacksonville, FL 32205) and micro-irrigation nozzles to deliver the nutrient solution at the upper portion of the root mass allowing the solution to flow down the roots. Multiple nozzles may be used to cover the entire root system regardless of the number of plants or depth of the chamber. A chamber with dimensions of 122 cm square and 61 cm D which contains 200 L of nutrient solution has been tested. In this configuration, the roots are sprayed every 7 seconds.

A third system offers promise, but it has not been thoroughly tested for VAM inoculum production. A fog of droplets about 5 μm in diameter is produced by an ultrasonic head and is distributed by a small fan in the fogging unit. Nutrients are injected into the water supply eliminating the bi-weekly nutrient solution maintenance required by the two previous systems.

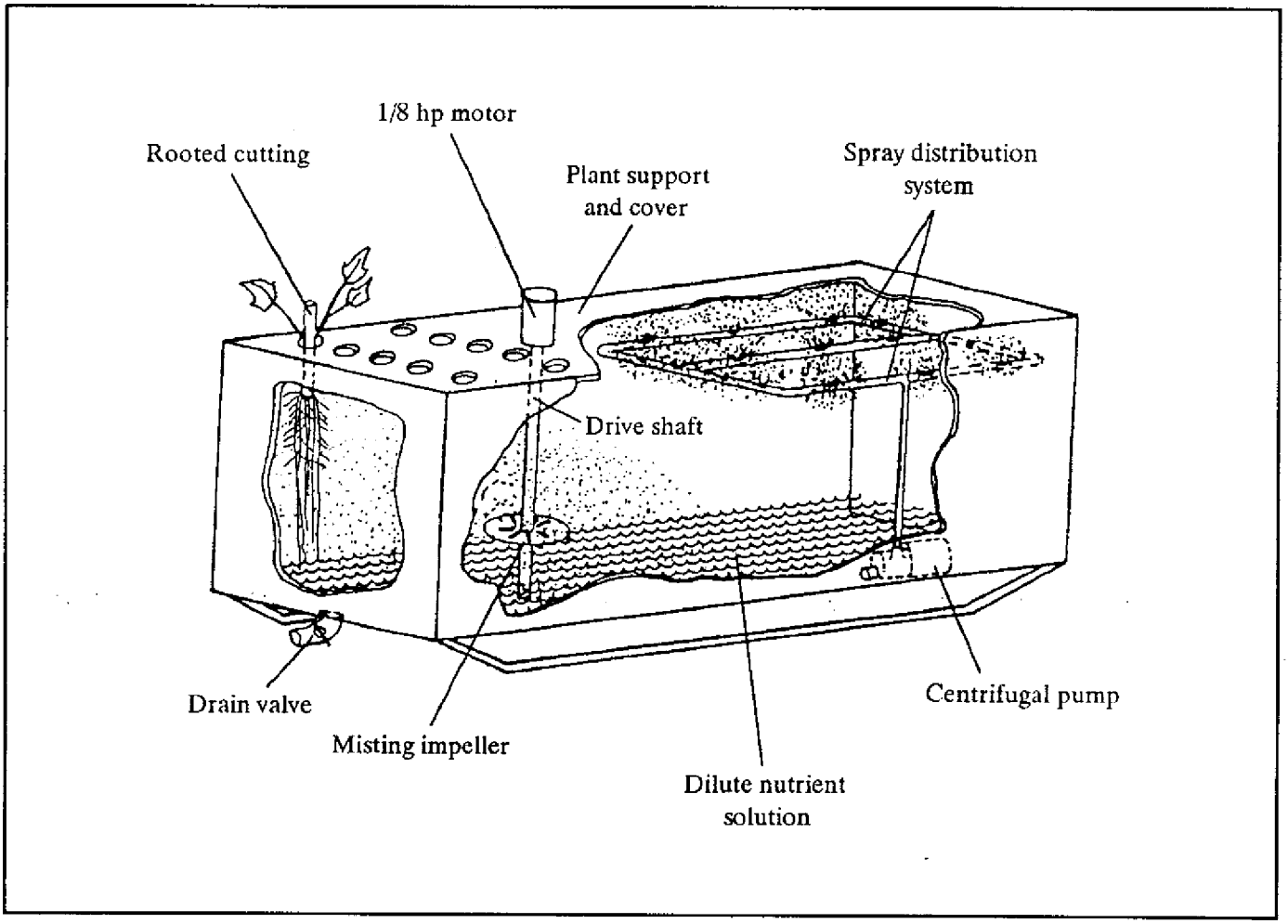

Figure 2. Aeroponic chamber construction.

Nutrient Solution

Quality water must be used to make the dilute nutrient solution which constantly bathes the roots of culture plants. Deionized or distilled are superior to untreated ground water for several reasons, including pH, buffering capacity, and potentially toxic micro-nutrient concentrations. We recommend the exclusive use of deionized or distilled water for preparation of the nutrient solution for aeroponic cultures.

The concentration of nutrients in the solution for aeroponic culture needs to be dilute since the roots are in constant contact with them. The phosphorus concentration is critical and must be kept low (about 0.1 ppm) to promote the growth of the VAM fungus. A set of concentrated stock solutions may be kept in refrigeration and diluted as necessary to make the dilute aeroponic solution. When measuring the concentrated nutrients, the magnesium sulfate stock solution should be separate from the others or insoluble precipitates will form making the solution unusable. Add the magnesium sulfate only when adding deionized water to the chamber to make the final dilute culture solution. Table 1 lists the necessary stock solutions and the amount of each chemical compound to be added to deionized or distilled water to make the stock solutions. Table 2 gives the elemental concentrations in the final dilute culture solution which constantly bathes the roots. Table 3 is a suggested dilution and maintenance record for the periodic changes of the culture solution. The monitoring of pH and temperature help to assess the culture conditions. A low pH (below 6.0) will inhibit root elongation and will also lead to necrotic leaves. A high pH may make nutrients unavailable to the culture plant. If the pH changes dramatically, readjust as directed below. Temperatures over 35° C slow plant and fungal growth and indicate inadequate cooling in the greenhouse or growth chamber. Action should be taken to change the conditions so the solution will not overheat. Reducing light intensity will decrease the VAM fungal growth (see below) and should not be used as a solution to overheating.

To initiate a culture or routinely change the nutrient solution, (i) fill the chamber with the desired amount of water, (ii) add the mixture of concentrated nutrients measured from the stock solutions, (iii) add the magnesium sulfate, (iv) mix very well, (v) check the pH after at least 15 minutes, and (vi) adjust the pH to 6.5 for most culture plants. Use drops of a 20% sulfuric acid solution to decrease the pH or drops of a 10% potassium hydroxide solution to increase the pH. Add a few drops only between pH measurements, mix well and recheck the pH. Repeat as often as necessary to achieve a pH of 6.5 $\pm$ 0.1.

Supplemental Light

Vesicular-arbuscular mycorrhizal fungi use carbohydrates from the host roots. Increased day-length and intensity of light increases the available carbohydrates to the fungus and thus its growth and reproduction. The light measure which is important for plants is called the photosynthetic photon flux density (PPFD) and is measured as micro-moles per meter squared per second ($\mu mol \cdot m^{-2} \cdot s^{-1}$).

Locate the aeroponic chamber in a nonshaded greenhouse to provide maximum light. Alternatively, a high-intensity-discharge (HID) growth chamber may be used. If light conditions are poor (a PPFD < 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in the greenhouse, as during the winter months, provide supplemental light by using metal-halide lamps. Hang a single 1000 watt (HID) fixture no closer than 1.3 m above the culture plants with the reflector set for the broadest spread of light. This arrangement provides excellent light conditions (PPFD $\geq$ 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for a single aeroponic chamber of dimensions up to 1.5 m square.

Aeroponic Culture Initiation

After the culture plants have been grown at least 4 weeks with the inoculum, carefully remove the plants from the vermiculite. Work on a surface such as new kraft paper or plastic laminate which has been disinfected with 10% bleach. Separate individual plants while giving care not to injure the roots. Some roots will be lost in this process. Wash the roots by dipping and swirling in several changes of water to remove as much vermiculite as possible. Keep the plants in water while checking for colonization.

For the most efficient use of aeroponic chamber

Table 1. Nutrient stock solutions for aeroponic culture of VAM fungi.

Stock Solution	Pure Chemical Compound $g \cdot L^{-1}$
0.01 M KH_2PO_4	1.36
1 M KNO_3	101.11
1 M $Ca(NO_3)_2$	236.15
0.1 M NaFeEDTA	36.70
0.1 M NaCl	5.84
1 M $MgSO_4$	246.48
Micronutrients (Combined in one solution)	
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.22
$CuSO_4 \cdot 5H_2O$	0.80
$Na_2MoO_4 \cdot 2H_2O$	0.016

Table 2. Final concentration of elements in aeroponic culture solution.

Element	Concentration (ppm)
Nitrogen	62.8
Phosphorus	0.0927
Potassium	58.7
Calcium	60.1
Magnesium	14.5
Sulfur	19.2
Chlorine	1.79
Sodium	1.72
Iron	1.6
Boron	0.15
Manganese	0.15
Zinc	0.015
Copper	0.006
Molybdenum	0.0018

Table 3. Sample Sheet

Aeroponic Chamber Maintenance Data Sheet

Current Date ___/___/___ Next Maintenance Date ___/___/___

Original Set-up Date ___/___/___

Chamber # ____ Host _____ VAM Isolate _____

Stock Solution	mL/L	Total mL Used
0.01 M KH_2PO_4	0.3	_____
1 M KNO_3	1.5	_____
1 M $Ca(NO_3)_2$	1.5	_____
Micronutrient Stock	0.3	_____
NaFeEDTA Stock	0.3	_____
0.1 M NaCl	0.45	_____
1 M $MgSO_4$	0.6	_____ *

* Keep the magnesium sulfate solution in a separate container!

Culture Maintenance Steps

1) Measure stock solutions.
2) Record pH : _____; temp.: _____ ° C before draining old solution.
3) Trim roots above full water line.
4) Drain old solution and rinse chamber thoroughly.
5) Add above concentrates to ____ liters of purified water.
6) Record pH of the solution after 15 minutes: _____.
7) Adjust the pH to 6.5 0.1 (Use 20% H_2SO_4 to decrease; 10% KOH to increase).
8) Record final pH :_____; temp. : _____ ° C.

Performed By: _____

space, place only colonized culture plants in the aeroponic chamber. Ideally, living roots of each plant are examined with epifluorescent microscopy for granular fluorescence (indicating arbuscular formation) (Ames et al. 1982) (see Figure 1C). It will be necessary to consult an expert at this stage to verify VAM colonization of the culture plants. If you lack the facilities or expertise to verify colonization nondestructively using this method, place the most vigorous plants in the chamber as detailed below. Then, send a randomly selected subsample of roots from all the plants placed in the chamber to the Bureau of Plant Pathology, Division of Plant Industry, Florida Department of Agriculture and Consumer Services, P.O. Box 147-100, Gainesville, FL 32614-7100. This agency has agreed to provide this service in Florida. If the assessment for colonization is negative, the procedure for starting culture plants must be repeated. Carefully consider each step to determine the reason for previous failure.

After the dilute nutrient solution is in the chamber and the pH is adjusted to 6.5 ± 0.1, the chamber is ready for plants. Wrap the original stem piece or crown area with polyester fiberfill and place each plant in the chamber. Thread the roots carefully through the 2.5 cm holes and assure that the crown is securely held in the lid (see Figure 1D).

Periodic Maintenance

Remove plant debris and control insects as necessary. Use less toxic, nonsystemic insecticides to control insect pests so as not to injure the VAM fungi. Insecticidal soap may be used to control some area pests. Locate the aeroponic chamber to minimize any chance of insect-borne contamination. Use a vacuum to remove plant debris around the chamber.

On a bi-weekly schedule, cut the roots to 2 cm above the highest nutrient solution level. Remove the waste portion of roots and scrub the walls of the chamber to remove any algal growth. Drain the nutrient solution quickly from the chamber and wash the walls and base with a stream of deionized water. Remove any root pieces that will not flush out of the drain. Immediately refill the chamber with water to the correct level and mix in the concentrated nutrients. Allow at least 15 minutes for the solution to mix and then adjust the pH as outlined above.

For aeroponic chambers which use the atomizing disc, replace the impeller stem and disc on the same bi-weekly schedule as above. Clean them in household bleach and soap. Shut the motor off for this exchange. Keep an extra impeller stem and disc for each chamber to exchange when changing the nutrient solution. Changing the stem and disc every two weeks will maximize mist output. Lubricate the drive motor periodically according to the manufacturer's directions.

Harvest and Processing of VAM Inoculum

Timing of Harvest

Aeroponic culture of VAM fungi produces colonized roots which will provide three basic forms of inoculum (see Figure 3). Using the procedures and expert resources detailed above, check a small quantity of roots for colonization and spore production before preparing large quantities of inoculum. Remove roots after 12 weeks for preparing root or sheared-root inoculum. Harvest roots after at least 18 weeks to collect mature spores for inoculum or other research purposes. When harvesting roots to be used as inoculum, remove entire plants.

When preparing sheared-root inoculum from the roots, harvest only the number of plants needed at one time and hold the remainder in the chamber to maintain high quality inoculum. Root inoculum may be stored as detailed below, but the quality will decrease with time.

Root Inoculum

To prepare root inoculum remove the desired number of plants from the aeroponic chamber and plug the holes with new polyester fiberfill. Allow excess water to drain from the root mass and then blot on clean paper towels. Cut the entire root mass into 0.5- to 1.0-cm lengths with scissors. Separate into individual root pieces. Add slightly moist vermiculite (10 mL per 100 mL of vermiculite) to the roots to aid in separation and maintenance of viability. Transport and maintain in sealed plastic containers or bags until use. Keep the inoculum refrigerated at 4° C. Use the root inoculum within 3 days.

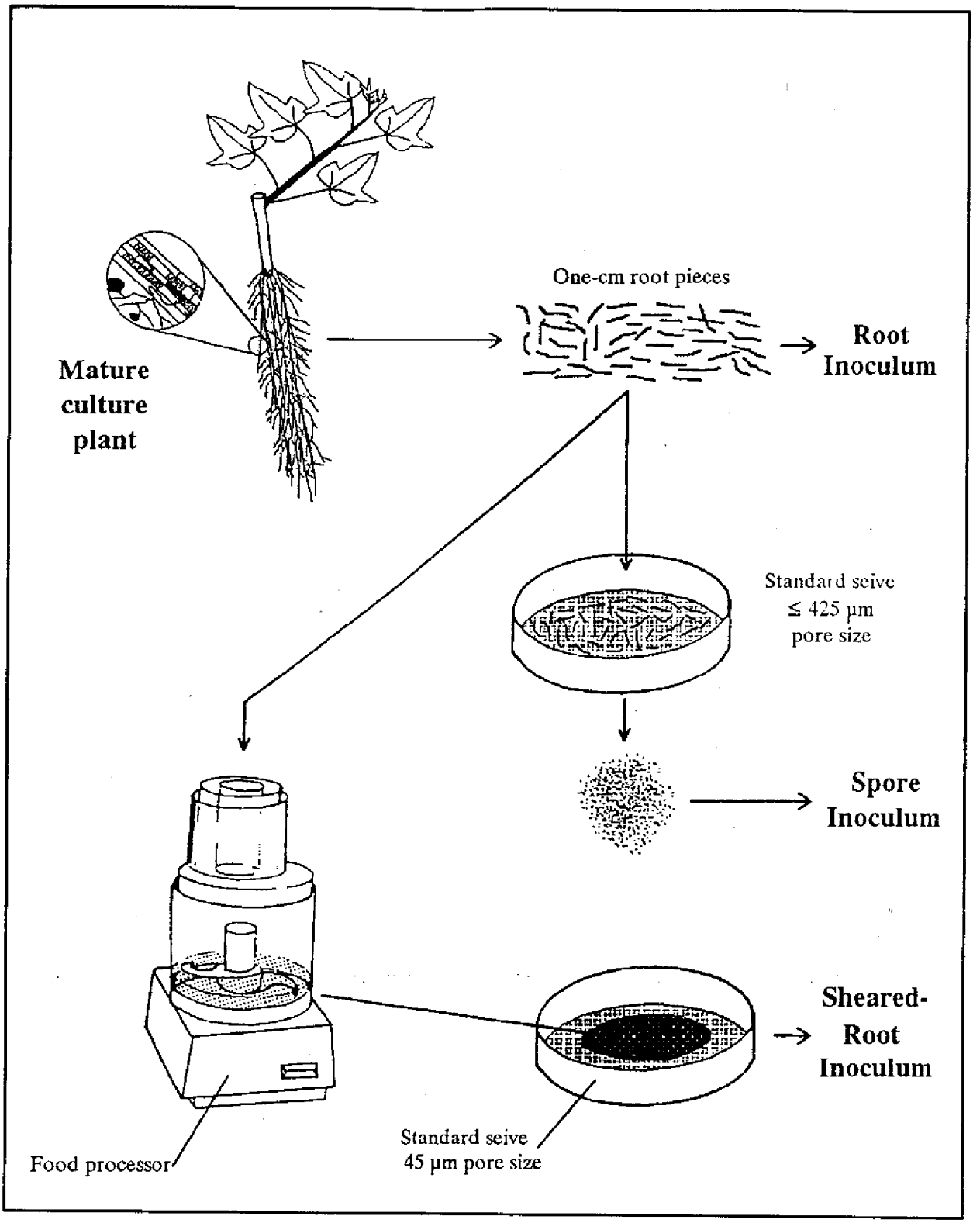

Figure 3. Harvest procedures for various inocula.

Spore Inoculum

Spores may be collected from fresh roots or from roots stored air dry by washing over standard sieves. Use sieves of 425 μm to 45 μm pore sizes (see Figure 3). To prepare spore inoculum, remove the number of plants necessary from the aeroponic chamber and place half of the root system on the 425 μm pore-size sieve over a 45 μm pore-size sieve. Spray with a strong stream of water for 5 minutes taking care to separate and wash the entire root mass. Collect the spores from the bottom sieve.

Sheared-Root Inoculum

Sheared-root inoculum has the advantage of greatly increasing propagule density and efficiency of handling when compared to root or spore inoculum. A detailed paper has been published by the authors on this sheared-root process (Sylvia and Jarstfer, 1992). In this process, roots are reduced to very small pieces (< 0.5 mm). Sheared-root inoculum must be prepared from fresh roots. To prepare sheared-root inoculum, harvest fresh roots from the aeroponic chamber and cut to 1-cm lengths. It is not necessary to separate root pieces by hand as with root inoculum. Mix roots with water in a 1 to 10 ratio (fresh weight/volume) with water and shear with a food processor (e.g. Little Pro food processor, Cuisinart®, P.O. Box 546, Norwich, CN 06360) for 40 seconds to produce a slurry of root fragments, vesicles, and free spores. Collect these propagules by pouring the slurry over a 45 μm pore-size sieve. Alternatively, collect sheared-root pieces over nylon or polyester mesh (pore size 35-41μm, Spectrum, 1100 Rankin Road, Houston, TX 77073) or fast filter paper (i.e. Whatman #1, Fisher Scientific Inc., 7445 Exchange Dr., Orlando, FL 32809).

Handling of VAM Inoculum

Inoculum Application

All three types of inocula may be mixed directly and thoroughly with growing media if plants are to be immediately planted or transplanted. Sheared-root or spore inocula may be added to hydrogels such as Natrosol® (Aqualon Co., Wilmington, DE 19894) to make a flowable inoculum. Use only hydrogels which are manufactured for agricultural purposes and are certified as nonphytotoxic. The pH of the hydrogel at the desired concentration should be between 6 and 8. Poorly purified gels may contain toxic concentrations of metals or organic by-products of the manufacturing process which may be harmful to the germination of the VAM fungi in the inoculum.

To prepare a hydrogel-type formulation of inocula, make the gel according to the manufacturers directions to achieve a pH between 6 and 8. When using Natrosol®, heat 1000 mL of deionized water to 90° C. Stir in 25 grams of the dry powder. Allow the gel to cool to room temperature and verify that the pH falls between 6 and 8. Add enough sheared-root material or spores, as collected above, to achieve a spore density of at least 10 spores per mL of gel suspension.

To inoculate plants use a plastic syringe or squeeze bottle to deliver approximately 1 mL of gel inoculum to each plant or planting-flat cell. Stick cuttings or plant seeds after inoculating. Transplants may also be inoculated by adding the gel inoculum at transplanting time.

Inoculum Storage

Inoculum viability declines with storage time. Root inoculum air-dried prior to storage at 4° C retains a significantly greater density of VAM fungal propagules than roots stored moist; however dried roots cannot be sheared. For short-term storage of living roots continue to maintain the aeroponic culture and remove entire plants to meet inoculum needs. When plants are harvested, excess moisture should be removed from the roots to prevent anaerobic conditions from developing. Roots may be stored for short periods (less than one month) in the moist state, but should be air dried for long-term storage.

For long-term storage, remove roots from culture plants and cut into 1-cm-long pieces. Separate by hand to facilitate drying. Air dry root pieces at room temperature (21-25° C) for 72 hours on clean kraft paper. Cover with a sheet of paper also. After drying, store roots in air tight containers at 4° C.

Sheared-root inoculum in a hydrogel may be refrigerated at 4° C for one year but the viable propagule density is greatly reduced.

Summary

The rationale and procedures for producing inocula of VAM fungi have been presented. Although these methods have been specifically tested using a VAM fungal isolate from coastal dunes in North Florida, the methods have broad application to many plant growing systems around the world. The aeroponic culture system has been used in a commercial nursery which produces sea oats for revegetation of beaches in the southeastern United States. Inoculations were successfully performed in a commercial nursery using both root inoculum and sheared-root inoculum.

Several significant benefits may be obtained by using aeroponically-produced VAM fungi to inoculate coastal revegetation plants in the nursery. This soilless culture technique avoids soil-associated plant pests. Absence of soil mineral matter allows roots to be sheared to higher propagule densities and thus provide more cost effective inoculations. On-site inoculum production using aeroponic chambers allows optimal timing of inoculations and higher propagule densities from fresh inocula. Small batches of selected isolates of VAM fungi should result in fewer losses and better growth when colonized plants are transplanted into nutrient poor and water stressed environments.

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