Isolation, Culture, and Detection of Arbuscular Mycorrhizal Fungi

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Mycorrhizae are mutualistic associations between beneficial soil fungi and plant roots and are common in natural soils. They have an important role in increasing plant uptake of P and other poorly mobile nutrients (74). The arbuscular mycorrhizal (AM) fungi colonize members of more than 90% of all vascular plant families. The term vesicular-arbuscular was originally applied to this symbiotic association, but since a major suborder lacks the ability to form vesicles in roots, AM is now the preferred acronym. Because of the obligate nature of these organisms, their manipulation is different from that of most other soilborne fungi, and much is still unknown about their biology in natural and managed ecosystems. Disturbance can change AM fungus distribution, abundance, and species composition within the ecosystem (63). Although isolation of their spores from the environment is relatively simple, multiplication of spores generally requires several months' growth under high-light conditions. Contemporary immunological, physiological, and molecular techniques to detect and identify soilborne organisms are now being applied to AM fungi and are leading to a better understanding of their distribution and function in the environment.

The purpose of this chapter is to summarize methods to (i) isolate and estimate numbers of soilborne propagules of AM fungi, (ii) propagate AM fungi by traditional and innovative methods, and (iii) detect and assess properties of these fungi by using recent biochemical and molecular technology. For further detail and explanation, the reader may consult various reviews of these methods (36, 47, 49, 73, 91) and a more extensive, step-by-step treatment of several of the procedures presented here (96). Methods for manipulating ectomycorrhizal fungi have been detailed elsewhere (72).

ISOLATION AND ESTIMATION OF NUMBERS OF SOILBORNE PROPAGULES

AM fungi are among the most common fungi in soil, but they are often overlooked because they do not grow on standard dilution-plating media. Soilborne propagules of AM fungi may include chlamydospores or azygospores, colonized roots, and hyphae. Isolating spores and quantifying root colonization are the most basic procedures for working with these fungi. Spores are required for pure, single-species cultures, and detection of colonization of roots is necessary to verify a functional association by visualization of arbuscules. The spores of AM fungi are larger than those of most other fungi, ranging from 10 to 1,000 μm in diameter. Most spores are between 100 and 200 μm in diameter and can easily be observed with a dissecting microscope. Hayman (36) and Schenck and Perez (91) have reviewed several methods for extracting spores of AM fungi from soil. The wet sieving and decanting density gradient centrifugation method is the most widely used method. The major variable in its application is in the use of single or multiple densities of sucrose. Multiple layers of different densities can provide cleaner spores and are useful for separating different species. Another method of separating spores from debris utilizes a series of sieves of various pore sizes. As with most of the techniques, it works best for sandy soils and less well for clay and organic soils. Soil samples with significant clay contents can be soaked in 6.3 mmol of sodium hexametaphosphate to disperse the clay fraction (64).

Method for Spore Isolation

Place a soil sample (50 to 100 g is usually sufficient) into a 2-liter container and add 1.5 liters of water. Vigorously mix the suspension to free spores from soil and roots. For fungal species that form spores in roots (e.g., *Glomus intraradices* and *G. clarum*), blend the soil-root sample for 1 min in 300 ml of water to free spores from roots. Next, let the suspension settle for 15 to 45 s (times vary depending on soil texture) and decant the supernatant through standard sieves. Sieves should be selected so as to capture the spores of interest. The spore sizes of most species can be found on the International Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) website at http://invam.caf.wvu.edu. Use a 425-μm-pore-size sieve over a 38-μm-pore-size sieve for unknown field samples. Examine the contents of the top sieve for sporocarps that may be up to 1 mm in diameter. For clay soils, it is advisable to repeat the decanting and sieving procedure with the settled soil. Roots may be collected from the larger-mesh sieve for evaluation of internal colonization. Transfer sieved material to 50-ml centrifuge tubes with a fine stream of water from a wash bottle, and balance opposing tubes. Centrifuge at 1,200 to 1,300 × g in a swinging-bucket rotor for 3 min, allowing the centrifuge to stop without braking. Remove the supernatant carefully to avoid disturbing the pellet, and then with a finger remove the organic debris that adheres to the side of the tube. Suspend

soil particles in chilled 1.17 M sucrose, mix the contents with a spatula, and centrifuge the samples immediately at 1,200 to 1,300 × *g* for 1.5 min, applying the brake to stop the centrifuge. Pour the supernatant through the small-mesh sieve, carefully rinse the spores held on the sieve with tap water, and wash the spores into a plastic petri dish inscribed with parallel lines spaced 0.5 cm apart. Spores may be counted by scanning the dish under a dissecting microscope.

The identification of AM fungi can be difficult because the taxonomy of this group was based almost entirely on a limited number of morphological characteristics of the spores. Unless taxonomy is the major objective, we recommend that the spores be identified initially only to the genus level. Isolates of special interest should be given a unique isolate code and then classified to the species level at a later date. For authoritative detail on the identification of spores, we direct the reader elsewhere (67–69). Maintenance of good germplasm should be an essential part of any AM fungal research program; therefore, careful notes need to be taken on the sizes, colors, surface characteristics, and wall morphologies of the spore types recovered. Initiate pot cultures with each spore type, keeping detailed records on the origin and subsequent pot culture history of the isolate.

INVAM maintains a collection of AM isolates. Samples of AM fungi may be submitted to INVAM for verification of classification and possible inclusion in their collection. For additional information, refer to the website at http://invam.caf.wvu.edu. Another culture collection, the International Bank for the Glomeromycota, exists in Europe, and its Web address is http://www.kent.ac.uk/bio/beg/.

Root Colonization

The AM fungi do not cause obvious morphological changes in roots; however, they produce arbuscules and, in many cases, vesicles in roots. To observe AM structures within the root, it is necessary to clear cortical cells of cytoplasm and phenolic compounds and then to differentially stain the fungal tissue. Phillips and Hayman (83) published the oft-cited method to visualize AM fungi in roots by using 0.05% trypan blue in lactophenol, but the use of phenol is now discouraged (54). The clearing agent for nonpigmented roots is generally 10% KOH, but treatment with H_2O_2 (83) or NaOCl (31) may be necessary for pigmented roots. Decolorizing with H_2O_2 is slower than decolorizing with hypochlorite, but there is less danger of complete destruction of fungal and cortical tissue. However, NaOCl is a very fast and effective bleaching agent, and the procedure requires no heating. Alternatives to trypan blue for staining are chlorazol black E (12) and acid fuchsin (53). For nonpigmented roots, it is also possible to observe colonization nondestructively by inducing autofluorescence (3).

Method for Clearing and Staining of Roots

Place root samples (approximately 0.5 g) in perforated plastic holders (e.g., OmniSette tissue cassettes; Fisher Scientific, Pittsburgh, Pa.) and store them in cold water until they are processed. Place enough 1.8 M KOH into a beaker (without samples) to allow samples to be covered, and heat the solution to 80°C in a fume hood. Place samples in the heated KOH for the desired time, 15 min for tender roots such as onion and 30 min for other roots. If samples are still pigmented after the initial treatment, rinse them with at least three changes of water and then place them in a beaker with either 30% (wt/wt) H_2O_2 at 50°C or 3% (wt/vol) NaOCl, acidified with several drops of 5 M HCl. Times may vary from several seconds to several minutes. Check roots frequently to avoid destruction of the cortex and fungal tissue. Rinse roots with copious amounts of water as soon as samples are bleached white or become transparent, and then rinse them with five changes of tap water. Cover the samples with tap water, add 5 ml of concentrated HCl for each 200 ml of water, stir, and drain. Repeat once. Dispense enough trypan blue stain into a beaker (without samples) to cover the samples, and heat the solution to 80°C. To prepare the stain, add the following in order to a flask while stirring: 800 ml of glycerin, 800 ml of lactic acid, 800 ml of distilled water, and finally 1.2 g of trypan blue. Place samples in the stain for at least 30 min, cool overnight, and drain the stain into the large flask for reuse. Rinse samples with two changes of tap water to destain. Additional destaining in water may be necessary for some roots.

Methods for Root Colonization Assessment

Various methods have been used to estimate root colonization by AM fungi (36, 53). The grid line intersect method has the advantage of providing an estimate of both the proportion of colonized root and the total root length (30). This is important because some treatments affect root and fungal growth differently. For example, when P is applied, the total root length may increase more rapidly than the colonized root length, and thus the proportion of colonized root will decrease even though the actual length of the colonized root is increasing.

McGonigle et al. (62) argued that the grid line intersect method is somewhat subjective because arbuscules may be difficult to distinguish with a dissecting microscope. They proposed the use of a magnified-intersect method whereby roots are observed at a magnification of ×200 and arbuscules are quantified separately from vesicles and hyphae. Another limitation of the grid line intersect method is that the intensity of colonization at each location is not estimated. To obtain an estimate of intensity, one can use a morphometric technique (100) whereby a grid of dots is placed over an image of squashed roots and colonized cortical cells are counted.

To quantify root colonization, spread a cleared and stained root sample evenly in an inscribed, 10-cm-diameter plastic petri dish. A grid of squares should be inscribed on the underside of the dish as specified by Giovannetti and Mosse (30) so that the total number of root intersections is equal to the root length in centimeters. Using a dissecting microscope, scan only the grid lines and record the total number of root intersections with the grid as well as the number of intersections with colonized roots. Verify any questionable colonization with a compound microscope. To do this, cut out a small portion of the root with a scalpel, place it in water on a microscope slide, and look for AM structures at a magnification of ×100 to ×400. Remember that the stains are not specific for AM fungi; other fungi colonizing the root will also stain, and so it is important to verify the presence of arbuscules or vesicles in the root with a compound microscope.

Propagule Assays

Spore counts often underestimate the inoculum density of AM fungi, since colonized roots and hyphae can also serve as propagules. The most commonly used methods to obtain an estimate of the total number of propagules are the most probable number (MPN) and infectivity assays. The MPN assay provides estimates of propagule numbers, but confidence

limits are usually very high. The infectivity assay is less complex and time-consuming than the MPN assay, but the actual propagule numbers are not estimated. Rather, the infectivity assay provides a relative comparison of propagule densities among various soils or treatments.

MPN Assay

The MPN assay was developed to estimate the density of organisms in a liquid culture (16). Porter (84) first used it to estimate the propagule density of AM fungi in soil. The general procedure for the MPN assay is to dilute natural soil with disinfested soil. Place equal portions of the dilution series in small containers (5 to 10 replications of each dilution), plant a susceptible host plant in each container, and grow plants long enough to obtain good root colonization. Plants are then washed free of soil, and roots are assessed for the presence or absence of colonization. Values for an MPN assay can be obtained from published tables (25); however, these tables restrict experimental design, thereby reducing the accuracy that can be obtained. A better approach is to program the equations into a computer and directly solve for the MPN value based on the optimal experimental design; increased replication and decreased dilution factor improve accuracy and reduce confidence limits. Numerous factors affect the outcome of an MPN assay (4, 19, 66, 103); therefore, caution should be exercised when values from different experiments are compared. Nonetheless, this assay has been a useful tool for estimating propagule numbers in field soil, pot cultures, and various forms of inocula.

Important considerations for evaluating AM fungi with the MPN assay are as follows.

1. Dilution factor. Preliminary studies should be conducted so that the lowest possible dilutions are used to bracket actual numbers found in the soil.
2. Sample processing. Samples should be kept cool and processed as soon as possible after collection. The sample soil needs to be relatively dry, and root pieces of >2 mm in diameter should be removed from the sample to allow thorough mixing with the diluent soil. These treatments will affect propagule numbers and viability, and all samples must be treated similarly.
3. Diluent soil. The soil preferably should be the same as the original sample and should be pasteurized rather than sterilized. Controls with no sample added should be set up with the pasteurized soil to ensure that all AM propagules have been eliminated.
4. Host plant. The host must be highly susceptible to colonization by AM fungi, produce a rapidly growing, fibrous root system, and be readily cleared for observation of colonization. *Zea mays* L. is a good choice.
5. Length of assay. Plants need to be grown long enough so that roots fully exploit the soil in each container. It is better to err on the conservative side and grow plants until they are pot bound. Roots with well-developed mycorrhizae are also more easily evaluated. A typical assay may run for 6 to 8 weeks.
6. Confirmation of negative results. The entire root system must be examined to confirm a negative reading.

Infectivity Assays

Plants are grown under standard conditions, and root colonization is estimated after 3 to 6 weeks (65). The amount of colonization is assumed to be proportional to the total number of AM propagules in the soil. The length of the assay is critical, and preliminary studies are needed to select the proper harvest time for a given plant-soil combination. If plants grow for too short a time, the full potential for colonization is not realized; however, plants grown for too long a time may become uniformly colonized despite differences in AM fungal populations.

An infection unit method may also be used to quantify mycorrhizal propagules (27). The principle is that a count of infection units is a more reliable measure of the number of viable propagules than are other methods. However, this method is applicable only in short-term experiments because infection units are discernible only during the initial stages (1 to 3 weeks) of colonization.

Quantification of Soilborne Hyphae

Even though the hyphae that grow into the soil matrix from the root are the functional organs for nutrient uptake and translocation, few researchers have obtained quantitative data on their growth and distribution. This is largely because of the technical difficulties in obtaining reliable data—there is no completely satisfactory method to quantify external hyphae of AM fungi in soil. Three major problems have yet to be overcome: (i) there is no reliable method to distinguish AM fungal hyphae from the myriad of other fungal hyphae in the soil, (ii) assessment of the viability and activity of hyphae is problematic, and (iii) meaningful quantification is very time-consuming. Nonetheless, clarification of the growth dynamics of external hyphae is essential to further understanding of their function in soil. Existing methods for quantifying soilborne hyphae have been reviewed elsewhere (95). Furthermore, the application of the molecular techniques described later in this chapter should lead to rapid advances in our knowledge of these structures.

CULTURE METHODS

Traditional Culture Methods

The culture of AM fungi on plants in disinfested soil, using spores, roots, or infested soil as inocula, has been the most frequently used technique for increasing propagule numbers (91). Many host plants have been used under a variety of conditions (94, 98). Examples of plants that have been used successfully are alfalfa, maize, onion, Sudan grass, and wheat. Generally, the host selected should become well colonized (>50% of the root length), produce root mass quickly, and be able to tolerate the high-light conditions required for the fungus to reproduce rapidly. Hosts that can be propagated from seed are preferable to cuttings since they are more easily disinfested. Most seeds may be disinfested with 10% household bleach (0.525% NaOCl) for 5 to 15 min followed with five washes of water.

Disinfesting the fungal propagules is a critical step because other fungi, bacteria, actinomycetes, and nematodes may be propagated with or instead of the AM fungi. Hepper (39) reviewed procedures for disinfesting and germinating spores, and Williams (102) detailed a method for reducing contamination of colonized root pieces. The most effective methods use chlorine compounds, surfactants, and combinations of antibacterial agents. One effective method is to incubate pot culture-produced spores in a solution of 2% chloramine T, 200 ppm of streptomycin sulfate, and a trace of Tween 20 for 20 min followed by at least five changes of sterile water. Spores from the field usually have higher levels of contamination, and a thorough wash with water containing a surfactant should be used prior to the disinfesting treatment.

All components of the culture system should be disinfested prior to initiation of a pot culture. The method of soil disinfestation is especially important. The objective is to kill existing AM fungi, pathogenic organisms, and weed seeds while preserving a portion of the nonpathogenic microbial community (47, 91). Several methods, including fumigation with biocides and exposure to ionizing radiation, have been used to eliminate AM fungi from soil. However, the safety and convenience of heat pasteurization makes it the preferred method. Large batches (50 to 100 kg) may be treated by heating to 85°C for two 8-h periods with 48 h between treatments in a commercial soil pasteurizer. Alternatively, smaller batches (4 kg each) may be heated to the same temperature by using three 2-min exposures to 700-W, 2,475-MHz microwave radiation. Prior to either treatment method, the soil must be passed through a 2-mm-pore-size sieve and wetted to at least 10% (wt/wt) moisture.

Culturing of AM fungi in soilless media avoids the detrimental organisms in nonsterile soil and allows control over many of the physical and chemical characteristics of the growth medium. Soilless media are more uniform in composition, weigh less, and provide aeration better than soil media. Jarstfer and Sylvia (47) have reviewed the various conducive substrates. Most soilless media do not buffer P concentration, so care must be taken to avoid high levels of P in the solution in the root zone. As discussed below for soil, several strategies may be used to regulate fertility and provide conditions conducive to the culture of AM fungi in soilless media.

Conducive environmental conditions for cultures of AM fungi are a balance of high light intensity, adequate moisture, and moderate soil temperature without detrimental additions of fertilizers or pesticides (47). Good light quality (λ, 400 to 700 nm) and high photosynthetic photon flux density are necessary for colonization and sporulation. Where natural light conditions are poor (photosynthetic photon flux density, <500 μmol $m^{-2} s^{-1}$), supplemental high-intensity lamps should be used. Soil moisture affects AM fungal development directly and indirectly. Directly, excessive moisture may encourage the growth of hyperparasites on spores in the culture. Indirectly, any moisture condition that inhibits primary root growth will reduce the spread of colonization. The best strategy is to apply water regularly to well-drained substrate. Likewise, soil temperature is also important directly for the fungus and indirectly for its effect on the chosen hosts. Sporulation is positively correlated with soil temperatures from 15 to nearly 30°C for many AM fungi; however, at higher temperatures sporulation may decrease as the host is stressed. Generally, soil temperatures and moisture conditions that are optimal for the host should also prove best for the fungus.

Chemical amendments can have both beneficial and detrimental effects on the development of colonized root systems and on sporulation. Responses to P and N fertilization may be isolate dependent (22) and are affected by the relative amounts of N and P supplied. Three approaches may be used to supply the plants with nutrients: (i) apply balanced nutrients except for P, which is applied at a rate 10-fold more dilute than recommended, (ii) apply dilute but balanced nutrients frequently, or (iii) mix a time release fertilizer into the substrate. Application of pesticides can also affect AM fungal colonization and sporulation. Prior to testing any pesticide with cultures of AM fungi, we recommend that previous reviews (47, 91) and the manufacturer be consulted.

In the greenhouse, pot cultures should be isolated from contaminated soil, splashing water, and crawling insects in order to prevent contamination. In addition, specific isolates of AM fungi should be kept well separated from one another. To initiate pot cultures, place a layer of inoculum 1 to 2 cm below the seed or cutting. The inoculum may consist of spores, colonized roots, or infested soil. Infested soil is often used to obtain initial isolates from the field; however, these mixed-species cultures should rapidly progress to single-species cultures initiated from 20 to 100 healthy, disinfested, and uniform spores. For critical taxonomic studies, single-spore cultures should be produced (91). For step-by-step methods of pot culturing and more extensive discussion of these methods, consult reference 47 or 96. Cultures should be grown for 3 to 6 months under the conducive conditions stated above. At that time, soil cores of approximately 5% of the container volume should be assessed for colonization and spore production. Cultures with more than 10 spores per g may be stored at 4°C. Many isolates tolerate air drying and storage at room temperature for long periods (>5 years); however, as the shelf lives of most of these fungi are uncertain, we recommend that both methods be tested to ensure survival of isolated germplasm. Spores may be conditioned to survive at −70°C by first air drying with the host (21). New isolates should be deposited with either INVAM or the International Bank for the Glomeromycota.

Aeroponic and Hydroponic Cultures

A benefit of aeroponic and some hydroponic systems is that colonized roots and spores are produced free of any substrate, permitting more efficient production and distribution of inocula. At least seven species of AM fungi have been grown in various nutrient-solution systems on hosts representing at least 21 genera (47). Usually, plants are inoculated with AM fungi and grown in sand or vermiculite before they are transferred into a culture system. The plants are grown for a period of 4 to 6 weeks under conditions conducive for colonization, after which they are washed and nondestructively checked for colonization (3); however, it is also possible to inoculate plants directly into the culture system (42). The P concentrations that have been reported to support AM fungal growth in solution cultures range from <1 to 24 μmol.

A system that applies a fine nutrient mist to roots of intact plants (aeroponic culture) produces an excellent AM fungal inoculum and concentrations of spores greater than those produced in soil-based pot cultures of the same age. Because the colonized-root inoculum produced in this system is free of any substrate, it can be sheared with the sharp blade of a food processor, resulting in high propagule densities. A detailed description of these methods and applications has been published previously (48).

Monoxenic Cultures

The growth of AM fungi in pure culture in the absence of a host has not been achieved. However, selected AM fungi colonize roots of intact plants or root-organ cultures to achieve monoxenic cultures that are useful for basic research on symbiosis (26). Ri T-DNA-transformed root cultures offer the most efficient method to grow axenic roots because no plant growth regulators are required for sustained growth. Fortin et al. (26) provide procedures for initiating and maintaining transformed root cultures colonized by AM fungi.

BIOCHEMICAL DETECTION AND QUANTIFICATION METHODS

Biochemical methods have been used to improve the means of detection and quantification of AM fungi in the environment. Additionally, some biochemical methods have contributed to a better understanding of phylogenetic relationships among AM fungi. Protein analyses are providing a better understanding of the symbiosis and may lead to the discovery of unique molecules with which to tag and quantify AM fungi. Other methods allow nonspecific assessment of biomass of AM fungi in roots and hyphae extracted from the soil, but proper controls are necessary for interpretation of results. None of these techniques are yet widely used or standardized, but they are developed to the point where they can be applied to environmental analyses with reasonable success. With all of them, the purity of samples and controls is an essential consideration.

Biochemical Approaches for AM Fungus Detection

Immunoassays

Serological techniques have the potential for specific detection of AM hyphae and spores in soil as well as in planta; however, few highly specific antibodies have been proven thus far. AM fungi, like many others, have poor antigenic properties (33). It appears that many immunogenic components of both spores and hyphae are internal, cell membrane-associated components of the cell walls. In addition, associated bacteria and actinomycetes provide interfering and stronger antigenic sites on the external surfaces. These are especially difficult to remove from spores even with antibiotic agents, oxidants, or sonication because they are deeply embedded in the spore walls (11, 33). When monoclonal products are screened, isolate and microbiological purity of the AM fungi should be the foremost concern; however, very clean AM fungal material is hard to come by due to the obligate biotrophic nature of these fungi. Monoxenic AM fungal cultures may be the most suitable material for generating AM-fungus-specific antibodies. As suggested by Hahn et al. (33), immunogold labeling of thin sections will show where the antibody is attaching and thus confirm its specificity. Purified cytoplasmic proteins make good antigens (105) but may be of limited value for labeling intact spores or hyphae from soil. See reference 82 for a good introduction and specifics on the methods for use of monoclonal antibodies with mycorrhizae. Polyclonal antibodies specific for surface antigens of AM fungi generated by using soluble proteins from G. *intraradices* have also been reported to give different patterns of soluble protein antigens for different AM fungi (56). Thingstrup et al. (97) have also reported similar findings on the potential use of polyclonal antisera to differentiate between *Scutellospora* species. However, polyclonal antibodies are generally less specific and can show broad cross-reactions with other non-AM fungi. Thus, one should be very cautious when using polyclonal antibodies to identify AM fungi. Despite their possible utility for detection and species-level identification of AM fungi, serological techniques appear to have been largely neglected by AM fungus researchers in recent years.

Protein and Isozyme Analyses

Qualities of proteins, in either spores or the colonized root, also may be used to detect and identify AM fungi. Protein assays can be based on total or enzyme-related protein molecules. For colonized root, identification with isozymes depends on the resolution of differences between host enzyme and diagnostic fungal enzyme banding patterns after electrophoresis (40, 99). The interactions between the particular hosts and fungi are extremely important, and some combinations yield protein bands that are indistinguishable. Careful preparation of root and hyphal materials by using protocols that prevent phenolic oxidation is necessary. With these methods, as with the other biochemical methods, isolate purity and positive controls are of utmost importance. It should also be noted that specific detection from the mycorrhizae requires an identical noncolonized root system as the control. Rosendahl and Sen (86) provide the specific methods for sample preparation and analysis, as well as a review of previous applications. Researchers have also exploited spore protein patterns of AM fungi by using either denaturing or native polyacrylamide gel electrophoreses for reasonably accurate discrimination and identification of AM fungal species and isolates (5, 89). Their results consistently indicate that neither the host species used for spore production nor different generation and storage times affect these spore protein profiles; however, the physiological states (germinating versus dormant) of spores may affect the results (5). Nevertheless, some isozyme analyses appear to be promising as complementary approaches to PCR-based methods for AM fungal species identification (6).

Fatty Acid Analyses

Fatty acid methyl ester (FAME) profiles of AM fungi have proven to be reliable measures of similarity below the family level (32). These profiles are stable across hosts and with storage and subculturing (8). Graham et al. (32) found that the fatty acid 16:1ω5 content of roots provides a reproducible index of colonization by *Glomaceae*, *Acaulospo-raceae*, and *Scutellospora* and the fatty acid 16:1ω5 is not a good indicator of infection by *Gigaspora* species, *Paraglo-maceae*, or *Archaeosporaceae*. *Gigaspora* can be detected by using 20:1ω9 (88). The FAME techniques for detecting and identifying AM fungi may be used with as little as 10 to 20 mg of spores (130 to 500 spores) or 15 to 30 mg of oven-dried root material (8, 32). After collecting and washing spores or root material and air or oven drying, the methods detailed by Sasser (90) are used to extract and quantify the FAME contents of AM fungi. Individual AM fungal species do not produce unique fatty acids, but some AM fungal taxa can be distinguished by distinct fatty acid profiles (18). At present, no comprehensive database of FAME profiles for AM fungi exists, and thus researchers wishing to investigate and identify AM fungal species from environmental samples based on fatty acid analysis must generate their own reference FAME profiles for each AM fungal species. Additionally, AM fungal species from a mixed sample have not been differentiated. Furthermore, it has been shown that rates of production of these lipids not only can vary between AM fungal species but also can be influenced by the physiological status of mycorrhizal symbioses (76). Although this analysis clearly can be used to detect the presence of AM fungi both in colonized roots and in soil, its suitability for AM fungal species identification may be limited only to well-controlled experiments.

Biochemical Approaches for AM Fungal Quantification

Determination of Chitin Content

Chitin content has been used to estimate fungal biomasses in roots under controlled experimental conditions (9, 38).

However, the utility of this method for natural soils is limited because chitin is ubiquitous in nature. It is found in the cell walls of most fungi and the exoskeletons of arthropods, and certain soils exhibit physical and chemical properties which interfere with the analysis (46). However, the content of chitin in roots may be used effectively to determine the fungal biomass when comparable noncolonized control plants are available. Chitin content determination has also been used to estimate the hyphal biomass in soil; however, the limitations discussed above for this method also apply here. For this method, dried soil samples are mixed with concentrated KOH, and the mixture is autoclaved for 1 h to degrade chitin into chitosan. Subsamples of the soil-KOH suspension are transferred into centrifuge tubes and assayed for chitin content as described above for roots. By subtracting the chitin content of control soil without AM fungi from that of soil containing AM fungi, the biomass of AM fungi can be estimated.

Ergosterol Detection

Ergosterol, a fungus-specific sterol, has been widely used for biomass estimation for other fungi, including ectomycorrhizal fungi. Although it has been reported to occur in AM fungi (28) and has been used to estimate the biomass of AM fungi (34), its suitability as a biomass indicator for AM fungi is questionable due to its low content in AM fungi compared to that in other fungi in the environment (75, 79), as well as variation in production of ergosterol among AM fungal species (35).

Fatty Acid Analyses

In addition to their potential use in AM fungal detection and identification, fatty acid analyses also have great potential for AM fungal biomass estimation both for colonized roots and for soil (76, 78). The signature fatty acid 16:1ω5 is rather unique because it is not usually produced by other fungi but is found in relatively large proportion in total fatty acids of many AM fungal species (32). Although some bacteria are capable of producing this particular fatty acid, as long as proper controls and additional measures are included, it should be possible to use this analysis for relatively accurate biomass estimation for AM fungi (7, 75, 77). However, since some AM fungi do not produce 16:1ω5, total biomass estimation for mixed communities of AM fungi may be problematic.

Glomalin Detection

A nonspecific soil glycoprotein called glomalin is produced by hyphae of AM fungi and has been implicated in soil aggregate development. It can be detected in situ by immunofluorescence techniques or by various specific or general protein assay methods (104, 106). Many questions remain regarding its role in the ecophysiology of AM fungi and soil genesis, yet the techniques for extraction and quantification are now available. Briefly, a soil sample of 1 g is autoclaved for 30 to 90 min with 20 or 50 mM sodium citrate. The extraction time and sodium citrate concentration determine whether recent or total glomalin is removed from the soil sample. This extract may then be further analyzed by such techniques as enzyme-linked immunosorbent assay, Bradford total protein assay, dot blot analysis, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Because glomalin is highly recalcitrant, its utility for field application for AM fungal quantification may be limited and even for a greenhouse experiment, a proper control sample is needed. The amount of glomalin in the field may not be a good indicator of AM fungal biomass, but rates of glomalin production may reflect total AM fungal activity and possibly total biomass (61, 107).

DNA- AND PCR-BASED DETECTION AND QUANTIFICATION METHODS

Molecular Approaches for AM Fungal Detection

Molecular genetic techniques have revolutionized AM fungal systematics (69, 92) and allow identification of the species of fungi present in roots or soil that may not be detected from examination of spores. Although the application of molecular genetic tools for the detection of AM fungi has become commonplace, the approaches have not been standardized and are not trouble free. A persistent problem with all molecular approaches is the possibility of contaminant sequences. Other species of fungi as well as bacteria often inhabit spores of AM fungi (11, 41), and there are countless organisms that may be associated with field-sampled roots. As a result, molecular approaches rely on phylogenetic analysis of a targeted gene sequence to identify the PCR amplicons as belonging to AM fungi. Contamination can be reduced through the use of primers that selectively amplify sequences from AM fungi. Although AM fungi appear to be monophyletic (with the exception of *Geosiphon*), families within the group are highly divergent (93), and at present, there is not a single set of primers that selectively amplifies sequences from all AM fungal species. The widely used primer AM1 selectively amplifies a region of the small subunit of the rRNA genes of *Gigasporaceae*, *Acaulosporaceae*, and *Glomeraceae*; however, it does not amplify species in *Archaeosporaceae* and *Paraglomeraceae* (13), and even this primer also amplifies non-AM-fungal contaminants (13, 58).

A second constraint in the application of molecular markers for AM fungal species identification is the quality of publicly available databases. While the number of AM fungal sequences in GenBank is large (http://www.ncbi.nlm.nih.gov/), many AM fungal species are not represented even by the best-studied genes. Moreover, annotation of GenBank data is difficult, so that non-AM-fungal contaminant sequences remain labeled as AM fungal species and there is poor correspondence of sequences to AM fungal species for some taxa, perhaps due to inconsistent identification of spores in different studies.

The best gene to target depends on the taxonomic level being studied (i.e., resolution at the family or genus level). Most studies thus far have focused on the nuclear ribosomal sequences of AM fungi, which occur in multiple repeats within the genome and therefore require less initial DNA. To date, the small subunit of the rRNA gene has been the most popular targeting sequence among AM fungus researchers and has the most complete publicly available database and several published selective primers, including the AM1 primer mentioned above. The small subunit of the rRNA gene appears to provide relatively good resolution for differentiating between AM fungal morphospecies. The large subunit of the rRNA gene also appears to be promising for species identification. The intergenic spacer region of the rRNA gene is highly variable—even within individual AM fungal spores (81, 85)—and, therefore, is suitable for distinguishing among isolates of the same AM fungal species.

The approaches to DNA extraction and PCR amplification are now routine, with several commercial products

being used successfully. Once amplicons have been obtained, they can be prescreened based on product sizes, restriction sites, or strand conformations (see below). If the targeted sequence is highly conserved or the template's purity is high, amplicons may be directly sequenced. On the other hand, if the targeted sequences are highly polymorphic or the template is of questionable purity (i.e., from a mixed community as expected from environmental samples), it may be necessary to clone these PCR products into bacteria prior to sequencing. Once amplicons have been cloned, several molecular techniques can be employed for screening of these clones (see, for example, chapters 49 and 55 in this manual).

Methods of Screening and Separation of Variants

Clones may be separated by size or variation at restriction enzyme sites (restriction fragment length polymorphism [RFLP]) (43, 80). Single-strand conformational polymorphism analysis has also been used to detect genetic variation among and within AM fungi (14, 45, 51, 52). This analysis is based on the principle that differences in sequence will result in different single-strand DNA conformations that can be detected by electrophoresis. Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) techniques have also been used routinely in microbial ecology studies (71). The main principle for both methods is that differences in sequence composition affect the melting behavior, leading to different motilities. DGGE has been used to describe the diversity in AM fungal communities (20, 55). Similarly, TGGE has also been used to characterize AM fungi (17). Although we have introduced these methods in the context of screening cloned amplicons, DGGE and TGGE could be applied directly to PCR products or to high-quality genomic DNA to assess the level of variation. Bands could then be excised from the gel, purified, cloned, and then sequenced to confirm their glomeromycotan origins.

Community Fingerprinting with T-RFLP

Terminal RFLP (T-RFLP) analysis is similar to other techniques in that it is based on PCR amplification of targeted glomeromycotan sequences. However, this technique has a different goal in that it provides a "shotgun view of community" without the need for cloning and sequencing (50, 70). Briefly, targeted sequences are amplified and end labeled with fluorescence dyes previously attached to the primer. The end-labeled PCR products are purified and then digested with a restriction enzyme, and end-labeled fragments are analyzed (15, 59). The number of peaks (different fragment sizes) in a given profile approximates the number of species in the sample. Resolution can be improved by using more than one digestion enzyme or by labeling both primers so that both ends of the PCR products can be analyzed (50). The success of this technique is dependent on the primer choice and consistent stringency of PCR to enable data from different studies to be directly compared. Given the problem of amplification of non-AM-fungal contaminants, the glomeromycotan origin of the fragments needs to be confirmed. This could be done by generating a reference T-RFLP profile database by using a high-quality DNA template derived from known AM fungal species. This approach is not definitive for environmental samples as unknown isolates may share restriction sites in similar locations. A more direct approach would be to isolate and sequence the actual fragments from the T-RFLP profile, but to our knowledge this has not been done with AM fungi.

Genome-Wide Approaches

A few investigations of AM fungi have employed a genome-wide approach that uses either arbitrary primers (1, 87, 108) or primers based on repeated motifs (29, 57, 60, 101, 109) to generate unique banding patterns for different AM fungal species or isolates. These approaches are relatively easy to employ and are economical; however, there is not a way to confirm the glomeromycotan origin of the amplified DNA, which limits utility in working with environmental samples. One of these approaches, random amplification of polymorphic DNA analysis (1, 29, 57, 60, 101, 108, 109), has been used as an initial step in the generation of specific primers for individual AM fungal species (1, 57). The AM fungal origin of these species tags would then need to be confirmed.

Molecular Approaches for AM Fungus Quantification

Quantitative studies of AM-fungal molecular ecology have commonly characterized and screened multiple cloned amplicons from individual samples. With this approach, high confidence in the AM fungal origin of the amplicons comes with great effort invested in each individual sample, thereby limiting the sample size and the statistical power of the studies. A few molecular approaches have potential for direct estimates of relative AM fungal densities that may allow more efficient measurement of large numbers of samples. Attempts have been made to use conventional PCR methods in a quantitative fashion (23); however, with this method there may be nonlinear relationships between the abundance of the PCR product and the initial sequence abundance that may prevent reliable scoring of relative density. There have also been a few attempts to quantify AM fungal root colonization by using real-time (RT) PCR (2, 24, 44). The RT-PCR has an advantage over competitive PCR in that it does not require a development of external competitor standards for quantification and it is possible to determine the amount of genomic DNA or the starting copy number of the targeted sequence (37). Isayenkov et al. (44) found that RT-PCR provided a good estimate of the density of arbuscules within roots in a pot experiment with a single species of AM fungi, but the utility of RT-PCR for field samples remains to be demonstrated.

T-RFLP

In addition to its application for molecular investigation of AM fungal communities, T-RFLP has potential to provide a quantitative analysis of the relative composition of these fungi. Instead of simply counting how many peaks there are for each T-RFLP profile, it may be possible to analyze the peak height (level of fluorescence intensity) of each end-labeled fragment to estimate the relative abundance of AM fungal species in the samples (70). However, there are potential problems with nonlinear relationships between the amplicon abundance and the initial concentrations of DNA in the sample generated by the competitive PCR amplifications. These problems may be reduced by standardization of the amount of templates used for PCR, but the potential for T-RFLP peak heights to estimate AM fungal relative abundance remains to be demonstrated.

Current Limitations and Future Directions for Molecular Approaches

Although current molecular methods improve our ability to detect AM fungi in the field, monitoring of the abundance and distribution of individual fungal species remains

laborious and expensive. Moreover, the molecular approaches are currently limited by reliance on rRNA gene and molecular data sets that include only a small subset of the total AM fungal diversity (10). These limitations can be alleviated only through more extensive collection and taxonomic description of AM fungal species, along with cloning and sequencing of rRNA and single-copy genes. Over time, the molecular data sets will continue to improve, creating the potential for additional applications. In particular, more complete molecular data sets would allow the creation of microarrays for one-step characterization of AM fungal composition. This DNA bar-coding approach would improve the detection of AM fungal species and may ultimately facilitate the quantification of AM fungal species, perhaps by coupling this approach with fatty acid methods.

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