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Techniques for Extraction and Quantification of Arbuscular Mycorrhizal Fungi

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Abstract: Techniques to extract spores from soil and quantify external hyphae of arbuscular mycorrhizal (AM) fungi colonization in roots are essential tools in mycorrhizal research. These methods are primarily used to identify mycorrhizal associations and measure the degree of root colonization. Our review provides an overview on present techniques used to extract and quantify AM fungi in soil and roots. We hope that the present review will help the readers to choose an appropriate method to extract and quantify AM fungi in soil and roots for their specific experimental set-up.

Key words: Techniques % Extraction % Quantification % Arbuscular Mycorrhizal Fungi

INTRODUCTION

The term *mycorrhizae* or *root-fungus* encompasses several distinct types of associations. The rarest are orchid mycorrhizae and ericoid mycorrhizae, formed exclusively in the Orchidaceae and Ericaceae, respectively. More common are ectomycorrhizae, which are formed predominantly by trees in the Pinaceae, Fagaceae, Myrtaceae (e.g., *Eucalyptus*), Dipterocarpaceae and Caesalpiniaceae. The fungi involved in these mycorrhiza types come from many lineages of Ascomycetes, Basidiomycetes and a few Zygomycetes [1, 2]. The most common mycorrhizae type involved in agricultural biotechnology system are arbuscular mycorrhizal (AM) associations. The AM biotechnology is feasible for crops using a transplant stage, as is the case with horticultural systems [3]. In contrast to the above associations, arbuscular mycorrhizae (AM) are distinct in terms of their diverse range of host plants, their fungi and their anatomy. They are formed by the group of fungi that are usually present in all soils from the phylum Glomeromycota, including nine genera; *Glomus*, *Paraglomus*, *Sclerocystis*, *Acaulospora*, *Entrophospora*, *Gigaspora*, *Scutellospora*, *Diversispora*, *Geosiphon* and *Archaeospora* [4]. The phytobiont is formed by more than 90% of all vascular flowering plant families with around 170 described species [5]. The symbiosis is called “arbuscular” because all the fungi involved form specialized tree-like structures (arbuscules = tree-like) inside root cells. Other structures produced by fungi are intra and extraradical spores which are germinating structures, formed on the extraradical hyphae (some species also may form spores inside the roots), intra and extraradical hyphae, for some genera, intracellular fungal storage structures called vesicles (which are lipid containing bodies) and auxiliary cells branching from extraradical hyphae. There are three important components of the mycorrhizal root system; the root itself, the intraradical mycelium (the fungi within the root) and the extraradical mycelium (the fungi within the soil). Benefits to plants are improved water and nutrient uptake, enhanced P transport and drought and disease resistance. Benefits to fungi are the supply of photosynthates to the fungal network located in the cortical cells of the plant and the surrounding soil [2]. Because of the obligate nature of these organisms, they are often overlooked since they do not grow on standard dilution-platting media [6]. Soilborne propagules of AM fungi may include spores (chlamydospores or azygospores), colonized roots and hyphae. Extraction of spores and quantification of root colonization are the most basic procedures for working with these fungi. Although, isolation of their spores from the environment is relatively simple, multiplication of spores generally requires several months of growth under high-level light conditions [6]. Spores are required for pure, single species cultures, long-term preservation of species, propagation and species identification purposes while detection of colonization in roots is necessary to verify a functional association by visualization of arbuscules. The spores of AM fungi are

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larger than those of most other fungi, ranging from 10 to 1,000 µm in diameter. Most spores are between 100 and 200µm in diameter and can easily be observed with a dissecting microscope [6].

The present review provides descriptions and other useful information on techniques for the extraction and quantification of AM fungi soilborne propagules, in order to help the reader to choose the most appropriate method to study the fungi in soil and roots for their specific purposes.

Spore Extraction: Several techniques for the extraction of AM fungi spores from soil have been reviewed by Hayman [7] and Schenck and Perez [8]. The wet sieving and decanting as described by Gerdeman and Nicolson [9], followed by sucrose density gradient centrifugation technique as described by Daniel and Skipper [10] is the most widely used technique. The major variable in its application is in the use of single or multiple densities of sucrose [6]. Multiple layers of different densities can provide cleaner spores and are useful for separating different species. Another method of separating spores from debris uses 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 or organic soils [6]. Soil samples with significant clay content can be soaked in 6.3 mMol of sodium hexametaphosphate to disperse the clay fraction [11].

Wet Sieving and Decanting Technique: After collection of a soil samples, suspend approximately 50 to 100 g of soil into a 2-liter container and add 1.5 liters of water. Vigorous mix the suspension to free the spores from the soil and roots. For fungal species that form spores in roots (e.g. *Glomus intraradices* and *Glomus clarum*), blend the soil-root sample for 1 min in 300 ml of water to free the spores from roots. Next, heavier particles in suspension is allowed to settle for 15 to 45 s (times vary depending on soil texture) and the supernatant decanted through standard sieves. Sieves should be selected so as to capture the spores of interest. A 425 µm pore size over a 45 µm pore size sieves is used for unknown field samples. The content of the top sieve is examined 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. The sievings retained on the other different sieves were washed into separate petri dishes for further observations or purification by sucrose centrifugation [9].

Sucrose Centrifugation Technique: Spores and minimal amount of organic particles could be further purified by re-suspending sievings in the 40% sucrose solution and centrifuge. Transfer sievings 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 x 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 x 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 scribed with parallel lines spaced 0.5 cm apart [10]

Sterilization of AM Fungi Spore: Modified techniques of Budi *et al.* [12] is used to surface sterilize the spores. Spores is immersed for 10 s in 96% ethanol and washed using a 32 µm sieve. Spores are then immersed for 10 min in a solution of 0.02% streptomycin, 2% chloramines T and a drop of Tween 20 (SCT). Subsequent washing again take place on a 32 µm sieve. A final immersion of the spores takes place in 6% bleach for 1 min and subsequent washing in sterile distilled water. Spores are stored in sterile distilled water or water agar, or on 0.1% $MgSO_4.7H_2O$ solidified with gellan gum, in Petri dishes and glass vials in the fridge at 4°C, if not used immediately [13].

Quantification of AM Fungi Colonization in Roots

Visualization of AM Fungi in Roots: The AM fungi do not cause obvious morphological changes to the roots; however, they produce arbuscules and in many cases vesicles in roots. To observe AM fungus structures within the root it is necessary to clear cortical cells of cytoplasm and phenolic compounds which usually hide them and then to differentially stain the fungus tissue. Clearing procedures, which use chemical agents to remove these cell contents and cell wall pigments, are routinely used to view internal features in plant tissues [14]. For non-pigmented roots, the clearing agent is generally 10% KOH, but treatment with H_2O_2 [15] or NaOCl [16] may be necessary for pigmented roots. Decolourization with H_2O_2 is slower than decolurization 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.

Fungal structures in cleared plant tissues are then further revealed by stains which preferentially bind to fungal hyphae without excessive background staining of plant material. These stains are usually applied in an acidic solution containing lactic acid, glycerine and water [15, 17, 18]. However, the total or almost total absence of staining is a problem for certain taxa of AM fungi such as some species of Acaulospora and Paraglomus [19]. Some Acaulospora species require careful observation at high magnification to see hyphae which usually contain distinct lipid droplets [20]. Molecular tools have been used to identify Paraglomus species in roots [21]. Phillips and Hayman [15] published the often cited method for visualization of AM fungi in roots by using 0.05% trypan blue in lactophenol as staining agent. Trypan blue is listed by the International Agency for Research on Cancer as a possible carcinogen [22]. Also, the use of phenol is now discouraged [23]. Alternatives to trypan blue for staining are chlorazol black E [18], which is possibly carcinogenic dye [22] and acid fuchsin [17] that is also a suspected carcinogen [24]. In addition, HCl although used at a low concentration, is frequently applied for the acidification of roots after clearing with KOH [25, 15].

The use of such chemicals should be reduced for health and safety reasons [26]. Contact with caustic chemicals may cause skin irritation [27] and their vapours may irritate the eyes, nose, throat and lungs [28, 29]. For environmental reasons it is preferable, wherever possible, to find substitutes for harmful chemicals. The "International Directory of Mycorrhizologists" lists more than 1,000 mycorrhizologists in 77 countries worldwide [30]; thus, it is estimate that tens of thousands of root samples are stained per year. In an attempt to eliminate some of the hazardous compounds, a modified procedure for staining of AM fungi in roots has been proposed [23]; however, the carcinogenic dye trypan blue is still used. Recently, a simple staining technique with an Ink and vinegar as a staining agent has been recommended by Vierheilig *et al*. [26]. This is a low-budget, supposingly non-toxic technique, which gives excellent staining results [31]. As it uses mostly non-hazardous and non-toxic chemicals which can be easily purchased, it is not only an adequate technique for research but also for teaching purposes. Due to the relatively low cost and small amount of time needed per sample, this technique is very suitable for large sample numbers. When using this technique, it has to be kept in mind that not all inks do stain AMF [31]. Quality of the staining depends on the colour and the brand of the ink. Purple, green and red inks are in general not suitable for staining, whereas nearly all black inks (Shaeffer Jet Black; Cross Black; Pelikan Black) and some blue inks (Pelikan Blue) give good staining results. Thus, before using an ink, which has not yet been reported to stain AMF, it is essential to test for staining quality. Always indicate the brand of the ink which has been used in publications. When observing the stained structures with a stereomicroscope, structures were clearest with a dark field illumination [31]. Recently, this technique has also been used to double-stain AM fungal structures after b-glucuronidase staining [32, 33]. For non-pigmented roots it is also possible to observe colonization non-destructively by inducing auto fluorescence [34].

Root Clearing: Root samples could be preserved in 70% ethanol or methanol prior to clearing [17]. Place root samples (approximately 0.5 g) in perforated plastic holders (e.g., Omnisette tissue cassettes, Fishers Scientific, Pittsburg, PA) or alternately using a screened syringe [35] before placing in a plastic or glass beaker and cover with a 10% KOH solution. Heat the specimens in a water bath at 90°C for 1 hour or in an autoclave at 15 psi for 10 min. Bevege [36] and Brundrett *et al*. [17] reported that root clearing is fastest in an autoclave, which also provides more uniform clearing of samples than other methods. Goggles gloves and vinyl apron will be worn for protection. After heating, pour off the KOH solution and rinse the root samples well with tap water. If roots are still pigmented, the cassettes (or capsules) containing it is again placed in a beaker and covered with freshly prepared alkaline H_2O_2 at room temperature for 10 to 20 min or until roots are bleached. Alkaline H_2O_2 is made by adding 3 ml of NH_4OH to 30 ml of 10% H_2O_2 and 567 ml of tap water. The alkaline H_2O_2 solution should be made up as needed; it loses its effectiveness even if stored overnight. After clearing, the cassettes in the beaker are thoroughly rinsed several times using at least three complete changes of tap water to remove the H_2O_2 and then cover with acidified dilute HCl (a x 10 dilute concentration of HCl is adequate, i.e. approximately 1 - 3.5%) and soak for 3-4 min and then pour off the solution before staining.

Root Staining: The cleared roots are stained using writing ink as a dye (Shaeffer Jet Black; Cross Black; Pelikan Black; Pelikan Blue) [26]. The staining solution consists of a 5% ink diluted in vinegar (5% acetic acid). After clearing, the roots are boiled (95°C) for at least 3 min in the staining solution. Following staining, the roots are rinsed several times for more than 20 min with acidified tap water (add several drops of acetic acid to the water). If water for

rinsing is not acidified and has a high pH (neutral is high), roots will destain. Total time needed for staining including clearing may take 30 min. If desired, the stained fungus can be completely destained by re-incubating the root in KOH solution. Samples will be stored in plastic bag in a refrigerator prior to measurement of colonized root length.

Estimation of Colonized Root Length: The purpose of studying AM fungi root colonization in many experiments are: (i) to observe and confirm mycorrhiza within the root system, (ii) to observe and describe the morphology of specific mycorrhizal structures formed within the root and (iii) to evaluate the extent of host specificity in different host-fungus combination. Furthermore, because the anatomical features of some of the fungal structures inside the root are diagnostic for certain species, it is sometime possible to differentiate the AM fungi responsible for root colonization. There is no standard method for quantification of root colonization in cleared and stained root samples, researchers have used various assessment techniques to meet their requirements. Most of the common assessment techniques are described below.

Detecting the Presence or Absence of Colonization: Whole stained root samples are scanned for the presence of any mycorrhizal structures, i.e. hyphae, arbuscules, vesicles or internal spores and related positive (+) or negative (-) per sample or per plant basis. This is the most basic and rapid assessment technique of those discussed here. This method is not quantitative, but adequate for some types of work such as checking host-fungus specificity and observing non-inoculated “control” plants for root colonization [37].

Calculating the Percentage of Root Segment Colonized: Several studies have calculayed colonization as the number of root segments with any colonization, didived by the total number od segments examined [38-40]. This is the same in principles as calculating the number of microscope fields of view with any colonization divided by the total number of fields of view examined [41-43]. This technique always overestimates percentage colonization, the degree of overestimation depending on the lengths of the segments and on the lengths of the regions of colonization.

Gridline-Intersect Technique: The gridline-intersect technique [44] or various modifications of it, is a procedure whereby the presence or absence of colonization at each intersection of root and gridline is noted, after dispersing the roots above a grid of square drawn on a Petri dish and observing under a dissecting microscope at X40 magnification. This technique has the advantage of providing an estimate of both the proportion of colonized root and the total root length [44]. This is important because some treatments affect root and fungal growth differently. However, even at X80 magnification it is not possible to ascertain if the roots are mycorrhizal at all intersections. This is because cortical cells or parts of the stele can become stained, the roots may be crowded with hyphae and because arbuscules can be difficult to detect when they are small. Structures formed by other fungi may also be confused with arbuscules at low magnification [43]. Different researchers are unlikely to be consistent in the way they record these difficult intersections and may arrive at different answers. The gridline-intersect technique using dissecting microscope can therefore be expected to give a relative measure of colonization.

To determine unequivocally whether arbuscules are present in all cases requires examination at X200 magnification. Ambler and Young [45] described gridline-intersect technique involving the compound microscope, but this still has the difficulty that some intersection must be classified as colonized or not when hyppae but not arbuscules are seen. Since arbuscles are the only unique feature of M fungi, decisions as to whether hyphae seen alone are mycorrhizal may vary from person to person. This technique is therefore vulnerable to bias and probably generate a relative measure of colonization.

McGonigle *et al.* [46] argued that the gridline-intersect method is somewhat subjective because arbuscules may be difficult to distinguish with a dissecting microscope. They proposed use of a magnified-intersect method whereby roots are observed at a magnification of X200 and arbuscules are quantified separately from vesicles and hyphae. Nevertheles, another limitation of the gridline-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 [47] 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 a scribed, 10 cm diameter plastic Petri dish. A grid of squares should be scribed on the underside of the dish as specified by Giovannetti and Mosse [44] so the total number of root intersections will be equal to root length (in centimeters). With a dissecting

microscope, scan only the gridlines and record the total number of root intersections with the grid as well as the number of intersects with colonized roots. Verify any questionable colonization with a compound microscope. To do this, cut out a small portion of the root with a scapel, place it in water on a microscope slide and look for AM fungus structures at a magnification of X100 to X400. 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 arbuscles or vesicles in the root with a compound microscope.

Subjective Visual Estimation Technique: The subjective visual estimation is the most commonly used to evaluate the level of AM fungi infection [48-50] and has been modified to estimate the intensity of colonization within the roots [51]. While this technique is subjective it can give quite reliable results with only a few hours training [44]. Giovannetti and Mosse [44] calculated a standard error of between 2 and 5% for subjective estimation. The subjective visual estimation is unquestionably the most time-efficient technique.

All of the above mentioned methods use biological stains in the preparation of plant roots for quantification of the extent of AM fungi colonization. Gange *et al*. [52] suggested that the extent of AM fungi root colonization recorded may depend on the stain used. They have reported having encountered many stained preparations in which arbuscules could not be seen. In such preparations, recording "arbusclar mycorrhiza" depends on a completely subjective decision, based on the presence of aseptate hyphae or vesicles. Neither of these characters, alone or together is a reliable indicator of AM fungi colonization. Mycorrhizal hyphae cannot be easily distinguished from those of saprotrophic fungi or root pathogens [2], while many other fungi can produce vesicles [53]. Simth and Read [2] commented that another problem with stains is their differential tissue penetration capacities. Clapp *et al*. [54] stated that acid fuchsin (AF) preparation quality varies according to the root tissue used.

As an alternative to biological stains, Ames *et al*. [34] described a method that involves subjecting roots to ultraviolet illumination, under which arbuscules autofluoresce. While autofluoresce might be better for arbuscle recording in some plant species, this technique also has it drawbacks. For examples, the fact that other structures in a root, such as lignin-like compounds sometimes fluoresce [55], or yellow pigments might hamper the recognition of arbuscles [56]. Jarstfer and Sylvia [6] commented that both live and dead arbuscules fluoresce, but conventional stains cannot differentiate them either. Furthermore, if a plant is sampled when it is in the early or late stages of mycorrhizal colonization, arbuscles might not be present and hence the recording from autofluorescence would be zero. Other useful mycorrhizal material such as vesicles, hyphae, hyphal coils and entry points present in the root materials would not be recorded.

Merryweather and Fitter [57] subjected roots stained with AF to epifluorescence and found that the qulity of the preparation improved. By using a combination of autofluorescence and staining, an accurate arbuscles count could be obtained, while still allowing determination of the presence of other mycorrhizal structures such as entry points, intercellular hyphae and vesicles. According to Gange *et al.* [52], the combination of autofluorescence and staining requires less than two minute to score the colonization.

For the quantification of AM fungi in planta, a combination of autofluorescence and non-vital staining (trypan blue, chlorazol black acid fuchsin) is not sufficient because these procedures do not indicate which part of the fungal material is active or even alive [58]. This has led to the development of staining procedures based on physiological activities of the fungi. One of these is the use of the succinate dehydrogenase reaction (SDH) to evaluate the amount of living AM fungi in the root cortex [59, 60]. This technique is useful to observe the evolution of the fungal viability in relation to plant growth and environmental factors. The evaluation of the infection using SDH staining will allow the precise determination of the effect on AM fungi of management procedure, in particular, whether one of these factors will lower the viability of the symbiotic fungus at an early stage of AM fungi development [58].

Researchers working on AM fungi do not use one method exclusively, as no method is unequivocally superior [37]. An investigator should be able to make an informed choice of the method of assessing AM fungi infection that is most suitable for their needs. Not only accuracy and reproducibility of assessment are important but also the labouriousness of the method. This is obviously increased by mounting roots on the slides but the possibility of making other, detailed observations on infection patterns, development of external mycelium and presence of other fungi may outweigh this disadvantage. Root colonization measured quantitatively by using any of the methods, does not necessary reflect the effectiveness of AM fungi in nutrient transfer capacity, it is not known that such functions are even related to the

proportion of arbuscles present. Caution should be taken in interpreting the functional basis of differences in the level of root colonization between any two sets [37].

Quantification of AM Fungi Propagules in Soil: AM fungi involving all field studies practically require an assessment of infection in the roots of plants studied/used in the investigation. This may be a simple check that infection has occurred or quantitative record of soil infectivity. Measurement of the population level in soil is necessary for planning a strategy of maintenance, enhancement or replacement with more desirable fungi [61-63]. A number of techniques have been used to obtain indications of total AM fungi propagules in soil. These are spore enumeration, AM fungi mycelium biomass in soil, the most probable number (MPN) technique, the mycorrhizal inoculum potential (MIP) technique and infection unit technique. Each technique has both merits and demerits. The most commonly used techniques to obtain an estimate of total propagules are the MPN and MIP bioassays. The MPN assay provides estimates of propagules numbers, but confidence limits are usually very large. The MIP assay is less complex and time-consuming than the MPN assay, but the actual propagules number are not estimated. Rather, the MIP assay provides a relative comparison of propagles density among various soils or treatments [6].

Spore Enumeration: Direct enumeration of propagules suffers a number of problems but foremost is the difficulty of determining propagule viability. Spores of AM fungi can be extracted from soil, identified and counted relatively readily but because thick walls of dead spores are persistent, total spore counts can be poorly correlated with mycorrhiza formation/ activity [64, 65]. Spores of some AM fungi are too small to be reliably extracted by wet sieving [66]. Some AM fungi produce only a few infective spores and others appear perfectly able to complete their life cycle without sporulating at all [67]. Spore density can only be related to inoculum potential, if it is known whether the spores are alive, dead or dormant [68]. Most often spore counts underestimate numbers of AM fungi since colonized roots and hyphae also serve as propagules. Spore counts can also overestimate numbers of AM fungi if AM fungi in soil are dead and dead spores are persistent.

Determination of AM Fungi Hyphal Biomass in Soil: This technique allows non-specific assessment of vitality and biomass of AM fungi in roots and hyphae extracted from the soil, but proper control is necessary for interpretation of results. 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 technique to quantify external hyphae of AM fungi in soil. Three major problems have yet to be overcome: (1) there is no reliable method to distinguish AM fungi hyphae from the myriad of other fungal hyphae in soil, (2) assessment of the viability and activity of hyphae is problematic and (3) meaningful quantification is very time-consuming [69, 6]. Colometric methods to determine chitin in cell walls of AM fungi have been used to estimate hyphal biomass in soil [70]. The utility of these methods for natural soils is limited because chitin is ubiquitous in nature. It is found in the cell walls of many fungi and the exoskeletons of insects. Certain soils exhibit physical and chemical properties that interfere with the chitin analysis [71].

Most Probable Number (MPN) Assay: The MPN assay was developed to estimate the density of organisms in a liquid culture [72]. Porter [73] first used it to estimate the propagule density of AM fungi in soil. It provides a relative measure of the density of propagules capable of colonizing roots. Four main assumptions of the method are: (1) that the propagules are randomly distributed in the soil; (2) that propagules are single and aggregates; (3) that dilution is proportional to the number of propagules; (4) and that if one organism is present it will be detected by the assay method [74]. The general procedure for the MPN assay is to dilute natural soil with disinfested soil. Place equal portions of the diluted series in small containers (5 to 10 replications of each dilution), plant a susceptible host plant in each container and grow the plants long enough (6-8 weeks) to obtain good root colonization. Plants are then washed free of soil and roots are assessed for the presence or absence of colonization. Results are interpreted as a probability estimate of propagules numbers from statistical tables [75]. 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 on the basis of optimal experimental design, i.e., increased replication and decreased dilution factor improve accuracy and reduce confidence limits. Five or ten fold dilutions are often used when inoculum is perceived to be highly infective but are not recommended because precision in quantification is lost [76]. Two fold dilutions are optimal but require too much space and are

too laborious. Numerous factors affect the outcome of an MPN assay [19, 77, 78]; 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. The MPN assay has the merit of providing a single number that can be compared directly with other tests in the same assay and is relatively easy to conduct. However, some space and time (6-8 weeks) are required for the test [19]. Important considerations for evaluations of AM fungi with the MPN assay are as follows [6]:

Dilution Factor: Preliminary studies should be conducted so that the lowest possible dilutions are used to bracket actual numbers found in the soil.

Samples 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 >2 mm in diameter should be removed from the sample to allow thorough mixing with the diluent soil. These treatments will affect propagules numbers and viability and all samples must be treated similarly.

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.

Host Plant: The host must be highly susceptible to AM fungi colonization, produce rapidly growing fibrous root systems and be readily cleared for observation of colonization. Sorghum (*Sorghum bicolor* L. Moenche), Maize (*Zea mays* L.) and cowpea (*Vigna unguiculata* L.) are good choice.

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.

Confirming Negative Colonization: The entire root system must be examined to confirm a negative reading.

Mycorrhizal Inoculum Potential (MIP) Assay: The MIP assay measures the percentage mycorrhizal colonization in a host plant over time, after the host plant has been grown in a series of inoculum dilutions and root colonization is estimated after 3 to 6 weeks [79]. The host plant is colonized by the AM fungi population to an extent that corresponds to the infection potential (infectivity) of the AM fungi population [76]. 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 populations [6]. The MIP is an indirect bioassay because there is not a 1:1 correspondence between number of infectious propagules and the assay result. The amount of mycorrhizal colonization includes a measure of both primary ingress (from propagules) and secondary spread (new infection units from those already established) [80].

The MIP technique is less complex and time-consuming than the MPN assay [6]. The technique is simple and the test can be conducted with basic laboratory equipment. However, this technique is sensitive to the environment. Actual propagule numbers are not estimated; rather the assay provides a relative comparison of propagule density among various soils or treatments.

Other variables of measuring soil infectivity have been described. Plenchette *et al*. [62] described a technique whereby soil infectivity can be estimated by a standard bioassay from a dose-response relationship. The technique described for measuring soil infectivity involved cultivation of a population of susceptible plantlets in controlled conditions on a range of concentrations of natural soil diluted with the same disinfected soil. Soil infectivity was expressed as arbuscular mycorrhizal soil infectivity (MSI) units/100g of soil. As MSI unit is the minimum dry weight (g) of soil required to infect 50% of a plant's population under the bioassay conditions (MSI_{50}). For each soil the percentage of mycorrhizal plants is plotted against the logarithm of unsterilized soil concentrations. Regression equations are calculated for each soil and the soil infectivity is determined by calculating the value at which 50% of the plants are mycorrhizal.

Typically, bioassays (MPN and MIP) are performed in a greenhouse upon soil samples removed from the field. The inevitable problem of extractive bioassays is that removal from the field may alter inoculum potential [81]. The length of the bioassay is critical. If plants are grown for a short time, the full potential for colonization is not going to be realized. On the other hand, if plants are grown for too long a time, they may become uniformly

colonized despite differences in AM fungi populations. Bioassays also have a limitation of estimating only those propagules which germinate, re-grow, intercept a root and initiate an identifiable infection during the experiment. Estimates are affected by all the variables that change plant or fungal growth. Estimates derived from plant colonization tests rarely detect all the propagules present [74].

Infection Unit Technique: This technique may also be used to quantify mycorrhizal propagules [82]. The principle is that a count of discrete points of infection is a more reliable measure of the number of viable propagules than are other methods. However, this technique is applicable only during the initial stages (1 to 3 weeks) of colonization [6].

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