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CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy of AMF: Spore-based taxonomy and identification

Morton (1990a) used all available data on selected morphological characters of somatic (hyphae, arbuscules, vesicles) and reproductive (resting spores) stages of known arbuscular species to hypothesize explicit genealogical trends. Cladistic analysis and a phylogenetic reconstruction were based on an evolutionary species definition: clonal populations which together maintained a distinct morphological identity from other clonal assemblages and which had the same evolutionary role and tendencies. A revised species concept to accommodate phenotypic variability as well as historical and contemporary evolutionary processes was elaborated by Morton (1990b). Currently, the classification of AMF has been revised by Morton and Benny (1990) to more accurately arrange groups of AMF according to patterns of common descent. It is based in part on results of Morton (1990a), with additional considerations of spore ontogeny and modes of spore germination. All the known AMF are currently classified in five families within the order Glomales(http //invam.caf.wvu.edu/Myc_info/Taxonomy).

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Order: Glomales

Suborder: Glomineae

Family: Glomaceae

Genus: *Glomus* (~89 species)

Family: Paraglomaceae

Genus: *Paraglomus* (~2 species)

Family: Acaulosporaceae

Genus: *Acaulospora* (~34 species)

Genus: *Entrophosphora* (~4 species)

Family: Archaesporaceae

Genus: *Archaespora* (~3 species)

Suborder: Gigasporineae

Family: Gigasporaceae

Genus: *Gigaspora* (~8 species)

Genus: *Scutellospora* (~28 species)

Morphological characteristics of spores are the most important for the identification of AMF. According to Morton (1988), the following are the most important:

Spore development

Spore development is one of the main criteria used to define genera of Glomalean fungi. *Scutellospora* and *Gigaspora* species have spores which develop from bulbous subtending hyphae while those of *Glomus* species form on narrow or flaring hyphae. *Acaulospora* and *Entrophosphora* have spores which become sessile after detachment from a soporiferous saccule. Spores of many *Glomus* species can form within roots as well as in the soil, but the other four genera generally do not sporulate in living roots (Morton, 1988).

Spore arrangement

Spores of Glomalean fungi can be produced singly or in aggregations, which are called sporocarps. Sporocarps differ in size, colour, shape and surface texture between and within isolates of Glomalean fungi. Sporocarps may have a peridium (outer covering of hyphae) (Morton, 1988).

Spore shape

Spores of most Glomalean fungi are globose (spherical), but some species have spores which are oval, oblong, or occasionally other shapes. Subtending hyphae which remain attached to spores can be cylindrical, flared into conical shape, or swollen, and some species have multiple or branched subtending hyphae. Spore

attachments of mature spores can be occluded by wall layers or other material (Morton, 1988).

Spore size

Spore size is considered to be less useful than many other taxonomic criteria because of its variability, but substantial differences in spore sizes can help to distinguish species. Glomalean fungi have spores which fall within a range of sizes from very small (20-50 µm) to very large (200-1000 µm) (Morton, 1988).

Spore colour

Spore colour varies between and within isolates of Glomalean fungi and can be used to help identify them (Morton, 1988).

Spore ornamentation

Examples of spore surface features include pits, reticulations, spines and papillae, and these are most often found on *Scutellospora* and *Acaulospora* spores (Morton, 1988).

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Spore wall layers and staining reactions

Walls of Glomalean fungus spores have one or more layers that may vary in their thickness, structure, appearance and staining reactions, and can be described using standardized terminology or diagrams (murographs) (Walker, 1983, Morton, 1988). *Acaulospora*, *Entrophosphora* and *Scutellospora* species typically have a complex wall structure consisting of a thicker outer wall and one or more thin inner wall layers. One or more wall layers may stain red or purple with Melzer's reagent. Melzer's staining reactions may occur in inner or outer wall layers of spores in all genera, but typical staining reactions may not occur in spores that are old, damaged or have been stored in preservatives. *Glomus* and *Gigaspora* spores are generally simpler in structure than those of other genera, but *Glomus* spores often have several wall layers. Immature *Glomus* spores may have a weak Melzer's staining reaction that is absent in older spores. Young *Glomus* spores often have a fragile outer wall layer which is lost as spores age (Morton, 1988).

Spore contents

Spores contain lipids and other contents which vary in colour and may be arranged in large or small droplets or granules. The size or arrangement of lipid droplets can help identify fungi, but will change when spores age (Morton, 1988).

Spore germination

Spore germination mechanisms can also be used to distinguish Glomalean fungi, especially *Scutellospora* species which have germination shields with complex infoldings on their inner walls. When *Scutellopsora* spores germinate, hyphae arise from compartments within these shields and then grow through the outer wall. There are also characteristic features of spore germination of *Acaulospora* (germination shields) and *Gigaspora* (warts inside the spore wall) spores (Morton, 1988).

Non-morphological characteristics

Careful study of biochemical, immunological and genetic properties have also provided valuable information in the classification of the Glomales (Morton, 1988; Walker, 1992).

Biochemical characteristics

Biochemical criteria to separate AMF have been limited so far to defining stained isozyme banding patterns from spores (Sen and Hepper, 1986) and mycorrhizal infection (Hepper *et al*., 1986) after polyacrylamide gel electrophoresis. The most common enzyme assayed are esterase, glutamate oxaloacetate transaminase, hexokinase, malate dehydrogenase, peptidase,and phospho-glucomutase.

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Based on results using spores of five *Glomus* species, malate dehydrogenase was most diagnostic for distinguishing between pairs of fungi (Sen and Hepper, 1986).

Immunological characteristics

Two approaches have been used thus far to identify specific AMF: the fluorescent antibody (FA) technique and the enzyme-linked immnuosorbent assay (ELISA). Immuno-localization procedures require the use of an antibody specific to either an external or internal antigen from the fungus. Though the sensitivity is excellent, immunological methods rarely overcome the problem of specificity beyond the subgeneric level (Wilson *et al*., 1983). Antisera react most strongly with their homologous antigen, but some cross reactivity to heterologous AMF occurs (Wilson *et al*., 1983). Monoclonal antibodies (MAbs), each of which is secreted by an immortal hybridoma culture derived from a single B-lymphocyte, are much more specific and therefore overcome problems associated with interspecific cross-reactions. Wright *et al*. (1987) demonstrated that MAbs against spores and hyphae of the target fungus, *G. occultum* Walker did not react against 15 other *Glomus* species or 14 AMF in other genera.

Genetic characteristics

Ribosomal genes are by far the most studied genes in glomalean fungi. In eukaryotes, the ribosomal genes code for a pre-RNA of 45S which, after processing, gives the small ribosomal subunit (18S), the 5.8S subunit and the large ribosomal subunit (28S) (Perry, 1976). The small, 5.8S and large ribosomal subunit coding regions (rDNA) are separated from each other by an internal transcribed spacer (ITS), and the ribosomal genes by an intergenic nontranscribed spacer (IGS). Sequence variations are not evenly distributed throughout the ribosomal genes and the three regions evolve at different rates. ITS and IGS are variable regions which mutate more frequently than the three conserved coding subunit regions (18S, 5.8S, 28S) (Mitchell *et al*., 1995). The coding regions of the small and the large ribosomal subunit have been used to discriminate between AMF at the species/family level (Simon *et al*., 1993; van Tuinen *et al*., 1994). The variability observed in the ITS regions has been used to distinguish between different isolates of a species of AMF (Lloyd-MacGilp *et al*., 1996).

2.2 Techniques for determining soil inoculum potentials of AMF

Practically all field studies involving AMF 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

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of the population level in soil is necessary for planning a strategy of maintenance, enhancement, or replacement with more desirable fungi (Janos, 1988; Plenchette *et al.*, 1989; Mason and Wilson, 1994). A number of techniques have been used to obtain indications of total arbuscular mycorrhizal fungal (AMF) propagules in soil. These are spore enumeration, determination of AMF mycelium biomass in soil, infection unit method, the 'Most Probable Number' technique (MPN) and the Mycorrhizal Inoculum Potential' technique (MIP) Each technique has both advantages and disadvantages. The most commonly used techniques are the MPN and MIP bioassays.

2.2.1 Spore enumeration

Direct enumeration of propagules suffer a number of problems but foremost is the difficulty of determining propagule viability. Spores of AMF 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 (Alexander *et al.*, 1992; Fischer *et al.*, 1994). Spores of some AMF are too small to be reliably extracted by wet sieving (Hall, 1977). Some AMF produce only a few infective spores, and others appear perfectly able to complete their life cycle without sporulating at all (Baylis, 1969). Spore density can only be related to inoculum potential if it is known whether the spores are alive, dead or dormant (Liu and Luo, 1994). Most often spore counts underestimate numbers of AMF since colonized roots and hyphae also serve as

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propagules. Spore counts can also overestimate numbers of AMF if AMF in soil is dead and dead spores are persistent.

2.2.2 Determination of AMF mycelium biomass in soil

There is no completely satisfactory method to quantify external hyphae of AMF in soil. Three major problems have yet to be overcome: (1) there is no reliable method to distinguish AMF 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 (Sylvia, 1992; Jarstfer and Sylvia, 1996). Colometric methods to determine chitin in cell walls of AMF have been used to estimate hyphal biomass in soil (Hepper, 1977). 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 (Jarstfer and Miller, 1985).

2.2.3 Most Probable Number

The MPN was first used to estimate the propagule density of AMF in soil by Porter (1979). 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

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aggregrates; (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 (Tommerup, 1994). The general procedure for the MPN assay is to dilute natural soil with disinfested soil. Equal portions of the dilution series are placed into small containers (5 to 10 replications of each dilution). A susceptible host plant is planted into in each container, and the plants are grown 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 propagule numbers from statistical tables (Fisher and Yates, 1963). 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 (Sieverding, 1991). Two fold dilutions are optimal but require too much space and are too laborious. Numerous factors affect the outcome of an MPN assay (de Man, 1975; Wilson and Trinick, 1982; Morton, 1985, An *et al.*, 1990) 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 advantage of providing a single number that can be compared directly with other

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tests in the same assay and is relatively easy to conduct. However, some space and time (6-8 weeks) are required for this test (Morton, 1985).

2.2.4 Mycorrhizal inoculum potential

This 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 (Moorman and Reeves, 1979). The host plant is colonized by the AMF population to an extent that corresponds to the infection potential (infectivity) of the AMF population (Sieverding, 1991). 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).

The MIP technique is less complex and time-consuming than the MPN assay (Jarstfer and Sylvia, 1996). 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 variations of measuring soil infectivity have been described. Plenchette *et al.* (1989) 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. An 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 (Janos, 1996). 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 AMF populations. Bioassays also have a limitation of estimating only those propagules which germinate, regrow, intercept a root and initiate an identifiable infection during the experiment. Estimates are affected by all the

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variables that change plant or fungal growth. Estimates derived from plant colonization tests rarely detect all the propagules present (Tommerup, 1994).

2.2.5 Infection unit technique

An infection unit technique may also be used to quantify mycorrhizal propagules (Franson and Bethlenfalvay, 1989). 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 method is applicable only in short-term experiments because infection units are discernible only during the initial stages (1 to 3 weeks) of colonization (Jastfer and Sylvia, 1996).

2.3 Visualization techniques for measuring AMF infection in roots

The primary purpose of studying root colonization in many AMF experiments is to observe and confirm mycorrhiza within the root system. The study of root colonization is also necessary to observe and describe the morphology of specific mycorrhizal structures formed within the root. Root colonization is also measured to evaluate the extent of host specificity in different host-fungus combinations Furthermore, because the anatomical features of some of the fungal structures inside the root are diagnostic for certain species, it is sometimes possible to differentiate the AMF 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 methods to meet their requirements. Most of the common assessment methods are described below.

2.3.1 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 rated positive (+) or negative (-) on a per sample or per plant basis. This is the most basic and rapid assessment method 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 (Rajapakse and Miller, 1994).

2.3.2 Calculating the percentage of root segments colonized

Several studies have calculated colonization as the number of root segments with any colonization, divided by the total number of segments examined (Daft and Nicholson, 1972, Khan, 1974; Read *et al*., 1976; Bierman and Linderman, 1981; Malibari *et al*., 1988). This is the same in principle as calculating the number of microscope fields of view with any colonization divided by the total number of fields of view examined (Baylis, 1967; Sutton, 1973; Newman *et al*.,

1981; Plenchette *et al.*, 1982, Dodd and Jeffries, 1986). This method always overestimates percentage colonization, the degree of overestimation depending on the lengths of the segments, and on the lengths of the regions of colonization.

2.3.3 Grid-line intersect technique

The grid-line intersect technique (Giovannetti and Mosse, 1980) 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 squares drawn on a Petri dish, and observing under a dissecting microscope at X40 magnification. In many cases colonization by AMF can be readily determined. 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 (Dodd and Jeffries, 1986), 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. 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 the 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 (1977) described a grid-

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line intersect technique involving the compound microscope, but this still has the difficulty that some intersections must be classified as colonized or not when hyphae but no arbuscules are seen. Since arbuscules are the only unique feature of AMF, decisions as to whether hyphae seen alone are mycorrhizal may vary from person to person. This technique is therefore vulnerable to bias and probably generates a relative measure of colonization.

McGonigle *et al*. (1990b) 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. Nevertheless, another limitation of the gridline-intersect technique is that the intensity of colonization at each location is not estimated. To obtain an estimate of intensity, one can use a morphometric technique (Toth and Toth, 1982) whereby a grid of dots is placed over an image of squashed roots and colonized cortical cells are counted.

2.3.4 Visual estimation method

The subjective visual estimation technique is the most commonly used to evaluate the level of AMF infection (Mosse, 1973; Sanders *et al*., 1977; Abbott and Robson, 1978; Buwalda and Goh, 1982; Hopkins, 1987), and has been modified to estimate the intensity of colonization within the roots (Schenck, 1982). While this technique is subjective it can give quite reliable results with only a few hours training (Giovannetti and Mosse, 1980). Giovannetti and Mosse

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(1980) calculated a standard error of between 2 and 5% for subjective visual 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 AMF colonization. Gange *et al.* (1999) suggest that the extent of AMF 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 "arbuscular 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 AMF colonization. Mycorrhizal hyphae cannot be easily distinguished from those of saprotrophic fungi or root pathogens (Smith and Read, 1997), while many other fungi can produce vesicles (Jennings and Lysek, 1996). Smith and Read (1997) comment that another problem with stains is their differential tissue penetration capacities. Clapp *et al.* (1996) state that acid fuchsin (AF) preparation quality varies according to the root tissue used.

As an alternative to biological stains, Ames *et al.* (1982) described a method that involves subjecting roots to ultraviolet illumination, under which arbuscules autofluoresce. While autofluorescence might be better for arbuscule recording in some plant species, this technique also has its drawbacks. For example, the fact

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that other structures in a root, such as lignin-like compounds sometimes fluoresce (Stockwell and Hanchey, 1987), or yellow pigments might hamper the recognition of arbuscules (Klingner *et al.*, 1995). Ames *et al.* (1982) comment 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, arbuscules 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 material would not be recorded.

Merryweather and Fitter (1991) subjected roots stained with AF to epifluorescence and found that the quality of the preparation improved. By using a combination of autofluorescence and staining, an accurate arbuscule 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.* (1999) the combination of autofluorescence and staining requires less than two minutes to score the colonization.

For the quantification of AMF activity 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 (Lovato *et al.*, 1995). This has led to the development of staining procedures based on physiological activities of the fungi.

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One of these is the use of the succinate dehydrogenase reaction (SDH) to evaluate the amount of living AMF in the root cortex (Kough *et al*., 1987; Smith and Gianinazzi-Pearson 1990). 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 AMF of management procedures, in particular, whether one of these factors will lower the viability of the symbiotic fungus at an early stage of AMF development (Lovato *et al*., 1995).

Researchers working on AMF do not use one method exclusively, as no method is unequivocally superior (Rajapakse and Miller, 1994). An investigator should be able to make an informed choice of the method of assessing AMF infection that is most suitable for their needs. Not only accuracy and reproducibility of assessment are important but also the laboriousness 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 necessarily reflect the effectiveness of AMF in nutrient transfer capacity. It is not known that such functions are even related to the proportion of arbuscules present. Caution should be taken in interpreting the functional basis of differences in the level of root colonization between any two sets (Rajapakse and Miller, 1994).

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2.4 Procedures for mycorrhization of microplants

Three main factors are of importance in the production of mycorrhizal micropropagated plants: time and form of mycorrhizal inoculation, substrate to be used and choice of species/strain of the mycorrhizal inoculum.

2.4.1 Time and form of inoculation

Three periods during micropropagated plant production can be identified for introducing mycorrhizal fungi: during the *in vitro* phase, during the weaning phase, and after the weaning phase. Micropropagated plants can be inoculated with AMF *in vitro*, but this requires an extension of the *in vitro* phase, so the economic impact of the procedure is questionable. Inoculation during the *in vitro* phase presents other difficulties, such as: (1) differences in the conductive media for root initiation and AMF formation and (2) loss of roots produced during the *in vitro* stages after outplanting (Vidal *et al*., 1992). Plant growth increases due to the presence of mycorrhiza are obtained if microplants are inoculated at the post *vitro* phase (Ravolanirina *et al*., 1989) or at the beginning of the weaning phase when they show only two root primordia (Branzati *et al*., 1992). The trend is to couple the weaning or acclimatization period with a rooting phase, following induction of root formation during the *in vitro* phase (Lovato *et al*., 1996). Mycorrhizal inoculation at the beginning of the rooting phase has been

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successful in a number of plant species such as pear (Gianinazzi *et al.*, 1990), pineapple (Guillemin *et al.*, 1992) and apple (Branzati *et al.*, 1992).

The procedure for inoculation of micropropagated plants can be performed in a number of ways: (1) micropropagated plants can be grouped together in trays containing the mycorrhizal inoculum mixed into the weaning substratum, (2) microplants can also be dipped in water suspension that contains the mycorrhizal inoculum before transplanting into the weaning substratum, (3) or most commonly, 1-5 g of soil-based inocula is placed in the planting hole made in the soil substrate of the container. The seedling is planted into the hole with the inoculum base (Lovato *et al.*, 1996).

2.4.2 Substratum composition

Substratum composition is very important to obtain optimal mycorrhizal infection. Vidal *et al.*(1992) found that the symbiosis could be established in peat-sand mixes although soil-sand mixes were more conducive to AMF root colonization of micropropagated avocado plants. Estuan *et al.* (1999) working on micropropagated peach X almond hybrid rootstock GF677also found that soil-less growing media (peat and a commercial peat-bark compost mix) is less conducive to AMF colonization than soil-based (soil-sand mix) growing media however, compost-peat mixes result in higher AMF colonization percentages.

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2.4.3 Choice of the inoculant

Inoculants of AMF are chosen according to the target plant since, for arbuscular mycorrhizas, there are differences among fungal strains in promoting growth of diverse plant species or varieties. Most frequently a mixture of isolates, each of which is adapted to a specific set of environmental factors is used, so that a wide range of hosts and environmental management conditions can be covered. However, it is possible that in a given situation a less efficient fungal strain present in the inoculant may be more competitive for infection of host roots. Optimal plant improvement can, therefore, only be obtained through mycorrhizal fungal inoculation of micropropagated plants if soil characteristics and other environmental features are carefully considered (Lovato *et al.*, 1996).

2.4.4 Sources of AMF inocula

2.4.4.1 "Infected roots" as inoculum source

Infected roots contain internal fungal mycelium as well as external mycelium, vesicles and in some species AMF spores, i.e., *Glomus intraradices* Schenck & Smith. Before being used as inoculum, roots are often chopped into small pieces. It is well known that the infectiveness of the infected roots as an inoculum source is higher than that of spores (Sieverding, 1991).

There are several problems in using infected roots as an inoculum source in the field and nurseries. Even if the root inoculum is free of pathogenic microorganisms, the organic material introduced to the soil is probably an attractive nutrient source for omnipresent microorganisms (saprophytic as well as parasitic bacteria and fungi). The transport and commercial distribution of "infected root" is limited by the relatively short survival time of AMF in roots at ambient conditions. This applies especially to AMF which do not sporulate within the roots (Sieverding, 1991).

2.4.4.2 "Soil based" AMF inoculum sources.

Soil inoculum contains all AMF structures. Soil inoculum is most frequently used in greenhouse and field experiments. However, Mosse and Thompson (1984) have indicated that soil inoculum is too bulky to handle particularly for use on a larger scale. It is also difficult to keep this inoculum from contamination of unwanted microorganisms (Lombaard, 1993).

2.4.4.3 Spores of AMF as inoculum source

Spores are important sources of inoculum for the establishment of clean cultures of AMF on host plants in previously sterilized substrates. This is because (1) small numbers of spores can be isolated relatively easily from soil substrates, (2) spores can be morphologically distinguished for the identification of the endophytes, and (3) spores can be satisfactorily disinfected on their surfaces

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with certain chemicals (with the object of producing inocula free of other microorganisms)(Budi *et al*., 1999). Spores are suitable sources of inoculum for experimentation and for special cases, i.e., in nurseries or in conditions where aseptic inoculation is required (Sieverding, 1991).

The germination of AMF spores depends on certain environmental conditions such as temperature, soil moisture and photoperiod. Koske (1987) showed that AMF could be directly influenced by temperature or indirectly by the influence of the temperature on the host. Reports indicate that in general spore germination is best at temperatures between 20 to 25°C (Hetrick, 1986; Bagyaraj, 1991). Bagyaraj (1991) indicated that spores of the same AMF species would germinate at different temperatures, depending on other environmental conditions. Land and Schonbeck (1991) showed that the germination of spores cannot occur under 5°C.

The germination of AMF spores also depends on the species of AMF itself. There is evidence that the germination of spores of some species of AMF is blocked by a dormancy factor for a certain period of time depending on the species of AMF (Tommerup, 1983; Bowen, 1987; Bagyaraj, 1991). Bowen (1987) found that this dormancy period is longer in dry soils than in wet soils. Tommerup (1983) reported a dormancy period of approximately 6 weeks for *Glomus caledonium* (Nicolson & Gerdemann) Trappe & Gerdemann and *Glomus monosporum* Gerdemann & Trappe in wet soil while, *Gigaspora calospora* (Nicolson &

Gerdemann) Gerdemann & Trappe was found to have a dormancy period of 12 weeks. This period was significantly reduced to 1 week for the *Glomus spp* and 6 weeks for *G. calospora* in dry soil. *Acaulospora laevis* Gerdemann & Trappe was found to have a dormancy period of 6 months under all conditions. Several studies showed that AMF spores will not germinate in sterile soil and that this dormancy factor should be broken before the spores will germinate. The presence of certain soil microflora has been shown to break this dormancy and subsequently stimulates the germination of spores (Hetrick, 1986; Bowen, 1987). According to Bagyaraj (1991) cold treatments or desiccation in the laboratory can also break this dormancy. Linderman (1991) indicated that the germination of AMF spores is influenced by several factors including self-inhibitors within the spores. Chemical and physical conditions of the soil may affect AMF spore germination (Lombaard, 1993).

Infected root segments, soil-based inoculum and spores isolated from open pot cultures of AMF-inoculated plants have been the usual source of AMF inoculum for research purposes (Ferguson and Woodhead, 1982). However, this type of inoculum is prone to contamination even with good phytosanitary care (Ames and Linderman, 1978). As opposed to the above-mentioned inoculum sources, axenically produced spores have lower levels of fungal contamination. Axenically-produced spores as AMF inoculum are more suitable for large-scale production as well as for biochemical and molecular investigations of the AMF symbiosis (Vimard *et al.*, 1999).

2.5 AMF and micropropagated banana

Available data on banana trials derive mostly from controlled conditions, however, efficient associations between AMF and banana plants are formed under greenhouse conditions (Declerck *et al.*, 1994). Inoculation experiments have resulted in increased banana plant growth due, in the great majority of cases, to their ability to deliver essential nutrients like phosphorus to the plant (Knight, 1988; Rizzardi, 1990; Jaizme-Vega *et al.*, 1991; Lin and Fox, 1992; Declerck *et al.*, 1994; Yano-Melo *et al.*, 1999), especially in soils of low fertility. Shashikala *et al.* (1999) have demonstrated that micropropagated banana plantlets raised in soil inoculated with *Glomus mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe and amended with 50% of recommended P fertilizer exhibit a significant increase in plant height, number of leaves, stem girth, root volume, total biomass and total P content over those grown with 100% recommended P fertilizer.

Pre-colonization of roots by AMF has been shown to reduce root damage in micropropagated banana plants due to soil-borne nematodes. Studies conducted by Umesh *et al.* (1988) resulted in a reduction in the nematode *Radopholus similis* (Cobb, 1893) Thorne 1949 by *Glomus fasciculatum* (Thaxter) Gerdemann & Trappe emend. Walker & Koske inoculated in banana plantlets. Furthermore, inoculated banana plants had a lower root lesion index as compared to plants

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with nematodes alone. Jaizme-Vega *et al.* (1997) showed that *G. mosseae* suppressed root galling and nematode buildup in micropropagated banana roots Pinochet and Fernandez (1997) reported that the association between micropropagated banana plantlets and *G. intraradices* increased host tolerance to the nematode *Meloidogyne javanica* (Treub) Chitwood.

A number of mechanisms of interaction between AMF and nematode pathogens have been considered, and the evidence supporting each is reasonable. All the proposed mechanisms depend on AMF-mediated changes in host physiology. Changes in root exudation by AMF plants may change the attractiveness of roots to nematode pathogens. AMF may improve host plant vigor, and thus reduce yield losses caused by nematode infection, especially in low P soils and if AMF are established early in the growth cycle, before nematode infection (Linderman, 1994). Increased resistance to nematodes may also involve competition for food or space between AMF and the nematodes present together on the same roots. Physiological changes in AMF roots could also change resistance to nematodes by increased production of inhibitory substances or by changes in root exudation, which could alter mycorrhizosphere population and affect nematode populations and survival. It is important to note that the mechanisms of interaction between AMF and nematode pathogens depend on conditions of the test, the host plant, edaphic conditions, and the species of AMF involved (Linderman, 1994).

Declerck *et al.* (1995) reported that for a given banana cultivar, the extent of root colonization was influenced by the species of fungus used as inoculum: *Glomus macrocarpum* Tulasne & Tulasne colonized roots less than *G. mosseae*. Jaizme-Vega and Azcon (1995) also found differences in colonization of banana by AMF species. Declerck *et al.* (1995) points out that although *G. macrocarpum* colonized roots less than some other species, the fungus produced higher relative mycorrhizal dependency for each banana cultivar tested. Differences in levels of root colonization by AMF is probably attributable to differences in mycorrhizal dependency among varieties of banana and to abiotic factors (Yano-Melo *et al.*, 1999).

Mycorrhizal dependency has been defined by Gerdemann (1975) as "the degree to which a plant is dependent on the mycorrhizal condition to produce its maximum growth or yield at a given level of soil fertility." The magnitude of response is known to vary both between and within species and is mainly attributed to the ability of these species to absorb P from soils that are low in available P (Mosse *et al.*, 1973). Morphological root properties (i.e. root geometry, rate of root growth, density and length of root hair) as well as physiological root properties may influence P uptake of plants from soil and therefore influence mycorrhizal dependency. Baylis (1975) suggested that the length and density of the root hairs could be indicative of the degree of Relative Mycorhizal Dependency (RMD) of a plant species or cultivar. Long and abundant

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root hairs allow for better uptake of P and other nutrients, thereby reducing the need for a strong mycorrhizal relationship (Declerck *et al.*, 1995).

AMF species vary in effectivity at promoting growth of micropropagated banana plantlets. Declerck *et al.* (1994) reported that *G. mosseae* was more effective than *Glomus geosporum* (Nicolson & Gerdemann) Walker at improving growth of micropropagated banana plantlets. Later, Declerck *et al.* (1995) observed that promotion of growth varied among banana cultivars and that plants inoculated with *G. macrocarpum* grew more than those inoculated with *G. mosseae*. Studies conducted by Jaizme-Vega and Azcon (1995) indicated that *G. fasciculatum* was more effective than *G. mosseae* at improving growth and nutrient uptake of micropropagated banana plants. Difference in growth promoting effects could arise from different rates of spread of infection, different amounts of external mycelium or different efficiency of the latter in uptake or translocation of phosphorus (Sanders *et al.*, 1977).