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Ajit Varma (Ed.)

Mycorrhiza Manual

With 114 Figures

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Foreword

The survival of mankind requires that we modify deeply our relationship to natural resources, and especially those with living organisms. In particular, new management roles have to be defined to exploit what for us is an unavoidable resource: the plant. We can postulate from present-day knowledge that plant survival and fitness in a given ecosystem are not conceivable without the establishment of beneficial relationships between plants and microorganisms, in particular those living in the soil. However, we are still a long way from understanding the contribution of each type of microorganism to such processes, although those developing preferentially or exclusively in roots, like mycorrhizal fungi, must play a key role.

The intimate symbiotic relationships developed between mycorrhizal fungi and plants, since land colonization by the latter, have led to an interdependence between these organisms for many basic processes. The fungi require plants to accomplish their life cycle; this is the case, for example, in fructification of many edible fungi, such as truffles or bolets. Plants depend heavily on mycorrhizal fungi for many important functions, such as mineral nutrition and abiotic or biotic stress resistance. Substantial evidence has accumulated in recent years about how rational use of these microsymbionts could significantly contribute to decreasing fertilizer and pesticide use in agriculture, especially if combined with other beneficial soil microorganisms (N_2 fixers, pathogen antagonists, PGPR, etc.).

The commercial importance of mycorrhizal fungi lies in their potential to maintain a reasonable level and quality of production, whilst minimising the negative impacts on the environment resulting from agricultural practices developed during this century, which entirely ignore the existence of beneficial soil microorganisms. Mycorrhizal fungi act as a major link between plants and soil, and should therefore be considered a central pivot for new strategies in the development of biologically orientated agricultural practices.

However, although the science of mycorrhizology is expanding rapidly, it is still generally considered of secondary importance (see, for example,

research carried out on root development and physiology without considering mycorrhizal fungi!).

The great merit of this book is to bring together worldwide specialists in the science of mycorrhizology, in order to present up-to-date techniques for research aimed at understanding and exploiting mycorrhizal systems, and so meet future challenges of using them in sustainable agricultural practices.

Dijon, September 1997 Dr. S. Gianinazzi

Preface

Nearly all terrestrial plants in this universe have or could have mycorrhizae of one type or another. The major types of mycorrhizae of importance in agriculture, forestry, florihorticulture, viticulture and arboriculture are arbuscular mycorrhizae (AM) and ectomycorrhizae. The evidence gathered so far is very strong that plants evolved with mycorrhizae, and, in natural ecosystems are highly dependent on them for their contributions to growth, health and sustainability. Mycorrhizae are involved in many fundamental plant processes because they link plants and soil and induce changes in the host plant physiology. If our crop plants, other than those that are nonmycotrophic, lack mycorrhizae, it is probably because of the detrimental effects of agricultural and forestry practices on these fungi. Plant growth and "health" are supported in many ways by the rhizosphere microbes, and the key among these microbes is mycorrhizal fungi.

Rhizosphere or, more appropriately, "mycorrhizosphere" microorganisms influence many chemical reactions by means of their metabolites, and mycorrhizae play a crucial role in facilitating both microbial and plant functions as mediators of exchanges between them. Mycorrhizae improve the "health" and development of plants by enhancing nutrition, modifying physiological functions of plants, reducing plant response to environmental stresses, and modifying the chemistry and biology of the rhizosphere in ways that alter nutrient cycling and suppress activity of root pathogens. The extraradical phase of mycorrhizae extends into the soil, and generates significant changes in soil aggregation, organic matter accumulation, and microbial activity in the "hyphasphere" soil; all these changes improve the structure and "health" of the soil (sustainability of soil). If any type of microorganism could induce and orchestrate interactions and functions of the soil in relation to the growth and health of plants, it is the friendly mycorrhizal fungi.

The benefits of mycorrhiza in suppressing plant diseases are: aiding in the reclamation of mine spoils; maintenance of nutrient recycling systems, structure and stability of soils; reducing the need for excessive fertilizers and pesticides; stabilizing ecosystems exposed to air pollutants;

and maintaining the biodiversity of all ecosystems, making them a key element in maintaining healthy plants on the planet Earth.

The science of mycorrhizology is expanding rapidly and will soon make mycorrhiza connections with other sciences of plant systematics, ecology, and physiology; horticulture; agronomy; soil science; climatology; molecular biology, biochemistry, genetics, and certainly plant pathology.

Mycorrhizal research is entering a challenging and exciting period when molecular and genetic tools can be used synergistically. The development of techniques permitting studies of the mycorrhizal fungi, which are at best difficult to culture, will expand our understanding of the value and functioning of below-ground root-fungal symbiosis. In this manual, every possible effort has been made to describe the protocols in a simple and illustrative version for a wide audience, including specialists, students, and beginners, to pick up the thread and conduct comprehensive research on these complex but eco-friendly symbiotic fungi.

In planning this treatise I extended invitations to prepare chapter(s) to distinguished scientists throughout the world. I wish to sincerely thank these eminent authors for their scholarly contributions, their enthusiasm for this joint venture, and their patient cooperation in responding generously and submitting the manuscripts promptly. I have learned much during the editorial process and hope that this volume will provide a similar stimulus to its users. I offer my sincere appreciation to Dr. Dieter Czeschlik, Executive Editor, Biology, Springer and his coworkers for timely publication.

This book is dedicated to my friends and well-wishers, especially Dr. Hannes Schuepp (Wädenswil, Switzerland), Professor David J Read (Sheffield, UK), Professor Bertold Hock (München, Germany), Professor Paola Bonfante (Torino, Italy), Drs. Vivienne Gianinazzi-Pearson, Silvio Gianinazzi (Dijon, France), my students especially Sudha and my wife Gita, our children Amit with Neeti, Anshul with Sravana and Ruchita with Avijit.

TUM, Freising-Weihenstephan, Germany Ajit Varma
September 1997

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Chapter 1

Mycorrhizae – the Friendly Fungi: What We Know, What Should We Know, and How Do We Know?

Ajit Varma*

INTRODUCTION

Roots, the "hidden half" of plants, serve a multitude of functions. They are responsible for anchorage, supply the plants with water and with nutrients, and exchange various growth substances with the shoots. The root-soil interface is the site where most interactions between the plants and their environment occur. Roots constitute a major source of organic material for the soil and thus affect its structure, aeration, and biological activities. While organic chemicals move out of the roots into the soil, inorganic ones move in. Insufficient or excessive accumulation of most elements would damage plants, and therefore their uptake is controlled at the root surface (Wilcox 1991).

I propose in this introductory chapter to take stock of the position at which we have arrived in certain aspects of the study of mycorrhiza, and to try to see in what direction research might go to enter important new fields of biodiversity, molecular biology as well as the important practical aspects in the field of biotechnology. Mycorrhizal symbioses are part of a great array of symbioses between heterotrophic and autotrophic organisms – from corals to neem trees, from lichens to legumes. In this symbiotic system one of the partners is carbon autotrophic and provides fixed carbon compounds for the system. That is, the heterotrophic partners are adapted to receiving a supply of carbon direct from photosynthetic products of the autotrophic partners rather than primarily or solely from humus or dead tissues indirectly derived from photosynthesis after the death of the autotroph. In autotrophic mycorrhizal systems carbon compounds synthesised in the green tissues not only nourish the host itself but also pass ino the fungus and so into the external mycelium as its source of carbon. Soil-derived nutrients absorbed by the mycelium in the soil pass into the fungal phase and so into the host tissue.

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Under natural conditions there is a harmonious combination of fungal microflora with the roots they occupy. Mycorrhizae occur in about 83% of dicotyledonous and 79% of monocotyledonous plants thus far investigated (Trappe 1987). All gymonsperms are reported as mycorrhizal (Newman and Reddell 1987). Arbuscular mycorrhizal fungi (AMF) are one of the few plant-fungus associations with fossil record and may even have facilitated the origin of land flora. The estimated origin of arbuscular-like fungi is 353–462 Myr ago, which is consistent with the hypothesis that these fungi were instrumental in the colonization of land by ancient plants (Simon et al. 1993). Only *Glomus*-like fossil fungus has been reported (late Palaeozoic 250 Myr ago).

Although the family Brassicaceae is typically considered to be non-mycotrophic (Gerdemann 1968), reports of arbuscular mycorrhizal development in crucifers are common (Harley and Harley 1987; DeMars and Boerner 1994). Despite such reports, the functionability of the mycorrhizae remains unknown, especially since few authors have observed arbuscules in the root segments examined. The lack of arbuscules suggests that any mycorrhizal development in the family is nonfunctional, since these structures serve as the interface for symbiotic nutrient transfer (DeMars and Boerner 1995). Future studies must incorporate analysis of root turnover and inoculation potential to fully understand the dynamics of AM development in this family.

The philosophy that air pollutants (ozone, acid deposition, carbon mono- and dioxide and sulphur compounds) may alter relationships between plants and mycorrhizal fungi is not as arcane as one might suppose. This concept appeared in the popular press article entitled *Fewer Fungi Bode Poorly for Forests* (Cwyndar 1992). The salient observations of this article were: (1) fewer fungal fruiting bodies are being found in European forests than in the past, and air pollution is a primary suspect in their decline, (2) a decrease in fungal populations spells trouble for the trees because these are dependent upon the fungi for nutrients and water, (3) without mycorrhizal fungi, trees may have increased susceptibility to other stresses, (4) it is not clear whether the decline of the fungi is a cause or a consequence of forest decline, (5) when fungi begin disappearing, it is certain that forest trees are in trouble. For inferences and conclusions, readers are advised to see the article by Shafer and Schoenberger (1994).

The role of fungi in the functioning and biodiversity of terrestrial ecosystems has received little attention. Amongst those interacting with land flora, AMF represent an important component by their ubiquity in the soil microbial biomass and their direct involvement in essential processes at the plant-soil interface. Mycorrhizal associations are potential factors determining diversity in ecosystems; they can probably modify the structure and functioning of a plant community, in a complete and unpredictable way (Read 1990; Giovannetti and Gianinazzi-Pearson 1994). Any

shift in the mycorrhizal fungal population could have consequences for the composition of plant communities, causing changes in the biology of natural ecosystems (Harnikumar and Bagyaraj 1996). The same is true for the change and alteration of host communities and the abiotic factors. Therefore, it is important to have a good knowledge of the different factors influencing the population biology of AM fungi in any attempt to use them in environmental conservation (Allen 1991), biotechnology (Mulongoy et al. 1992; Gianinazzi et al. 1995; Varma and Schuepp 1995) or in sustainable agriculture (Bethlenfalvay and Linderman 1992; Bagyaraj and Varma 1995). Knowledge of how soil management and cultural practices affect the dynamics and diversity of arbuscular mycorrhizal fungal communities is a prerequisite to managing these symbiotic microorganisms in agriculture, viticulture, arboriculture and florihorticultural systems.

Nomenclature of Mycorrhizas

The most widely used classification recognizes five broad mycorrhizal groups. They are based solely on the position of fungal mycelium in relation to root structure; the categories are purely descriptive and imply no functional significance. Although these subdivisions may serve useful purposes in promoting mycorrhizal research, their significance is not completely understood. They are:

ECTO – ectotrophic; ectocellular; sheathing; hartigian
ENDO – endotrophic; phycomycetous; vesicular-arbuscular; arbuscular
ENDO – endotrophic; ericaceous; ericoid
ECTENDO – ect-endotrophic; ericaceous; arbutoid
ENDO – endotrophic, orchidaceous

Ecto- and orchidaceous groups may also sometimes have common fungi since some orchids are connected by rhizomorphs and hyphae to ectomycorrhizae.

Taxonomic decisions based on the morphology of spores of arbuscular mycorrhizal fungi are most problematic in the genus *Glomus* for a number of reasons (Morton 1996). First, the number of species described is almost triple that in any other genus, but the sub-cellular morphological characters defining species are less discrete and, therefore, more difficult to separate and interpret. Many diagnostic characters such as type of "walls" of the spore, surface "walls" that slough with age or degrade by hyperparasitism; abiotic factors; and the fact that specimens tend to change their appearance from the time they are collected: a combination of all these factors has hindered unequivocal identification. Ontogenic interpretations of spore wall subcellular characters appear to provide a definite basis to reconcile differences between fresh and type specimens (Morton et al. 1995).

Morphological characters among endomycorrhizae have been a continual source of frustration for mycorrhizasts concerned with identification of experimental materials. Problem areas have been methodological and conceptual rather than the low information content. The sterotype view that morphological characters of glomalean fungi are too simple or too variable does not hold true once comparisons are made from cultured organisms within a developmental context. The positive point is that the subcellular characters in spores are discrete, conserved and highly ordered within rigidly constrained differentiation sequences. These characters are hierarchically organized, and those at higher levels of the hierarchy are so conserved phylogenetically as to be predictive in classification. However, no correlation appears to exist between morphological patterns and ecological gradients, suggesting autonomous processes evolving new diversity at each level. Because of rigid developmental constraints on morphological diversity, a similar autonomy is predicted for diversity originating at the molecular level.

Taxonomy and identification of arbuscular mycorrhizal fungi is almost exclusively based on the distinct morphology of their spores and it is very difficult to distinguish between genera or species when fungi are within root tissues. Molecular characterization offers an alternative approach for more reliable and reproducible identification. The chief features are protein profiles and isozyme polymorphism, DNA analysis, and immunological characterizations.

Protein Profiles and Isozyme Polymorphism

Attempts have been made to characterize arbuscular mycorhizal fungi by analysis of total or enzymically active proteins extracted from spores and separated by electrophoresis. Proteins or polypeptides from spores or sporocarps of different fungi show a large variability in both quantity and electrophoretic patterns. All species examined possess a distinct polypeptide profile, but, interestingly, one major band, between 21.5 and 31 kDa, appears to be common to all of them, independent of the species or genus affinity (Giovannetti and Lioi 1990). Isozymes detected in glomalean spores display clear variations between species amongst geographically different isolates (Rosendahl and Sen 1992; Shankar and Varma 1993). In contrast, alkaline phosphatase does not show a high degree of polymorphism between different glomalean species (Gianinazzi et al. 1992).

Immunological Characterizations

Serological approaches to the identification of glomalean fungi have met with problems of antibody aspecificity (Sanders et al. 1992). However, the finding of serologically specific protein fractions creates the possibility of

obtaining specifically targeted antibodies to identify fungi (Wright and Morton 1989). The difficulty in obtaining specific antisera against structural antigens is based on the fact that the large insoluble mycelia and spores are difficult to convert into a homogeneous suspension without considerable loss. Production of monoclonal (mAb) and polyclonal antibodies (pAb) against cell wall structures of arbuscular fungal hyphae has been demonstrated by Göbel et al. (1995). The availability of selective antibodies greatly facilitates the construction of qualitative and quantitative assays for different species of AM fungi. This approach provides the basis for future work in ecological studies of different fungal isolates introduced into the environment and the molecular identification of fungal taxa (Perotto et al. 1992; Hahn et al. 1995).

Immunogold labelling with silver enhancement on semithin sections is a method combining the advantage of immunofluorescence with further signal enhancement (Hahn et al. 1993). This method is sufficiently sensitive to visually evaluate the presence of hyphae. However, if a quantitative approach is desired, an ELISA format should be considered, using enzyme-labelled Abs.

Recently, the specificity of the antisera raised against intact spores of *Glomus mosseae* was checked in ELISA against the homologous antigens. The antisera recognised *G. mosseae* specifically up to 1:6000 dilution as compared to the preimmune serum (Verma 1996). For the cross-reactivity of the antisera with different species of the genus *Glomus* and three species of *Scutellospora*, an ELISA was performed. The antisera recognised maximally the Ag against which it was raised, i.e., *G. mosseae*; however, with other *Glomus* species the reaction was comparatively very low. The antisera gave much lower optical absorbance with *Scutellospora* species, except *S. gilmorei* (unpubl.). These results suggest that, although morphologically different, *S. gilmorei* is antigenically more similar to *Glomus*, in contrast to other species of *Scutellospora*.

DNA Analysis

Clearer taxonomic considerations require the establishment of large genomic libraries, and this has posed problems because AMF are considered to be biotrophic obligate symbionts. Spores of glomalean fungi are multinucleate, and estimations of number of nuclei per spore indicate that this varies considerably (745–20 000). Likewise, the amount of DNA per nucleus has been found to differ considerably in *G. versiforme* and *G. margarita* (Bianciotto and Bonfante 1992).

Therefore, DNA must be extracted from spores in order to exclude contamination from plant DNA. This has yielded DNA that can be used for PCR analysis (Lanfranco et al. 1995) but is not suitable for digestion by restriction enzymes. Zeze et al. (1994) have developed a method for ex-

tracting restrictable DNA from AMF spores and obtained the first AMF plasmid library. Whilst such plasmid libraries are useful for the non-targeted isolation of specific and non-specific DNA fragments that can be used to generate probes for analysing AMF genomes, their size limits their use for targeted isolation of complete genes.

Franken and Gianinazzi-Pearson (1996) described the establishment of phage genomic libraries from *Glomus mosseae* and *Scutellospora castanea*. The number of rDNA clones per library indicates that these libraries can also be used to isolate gene with low copy numbers. Differences between the 18S and the 5.8S rRNA genes were few and within the range of variation found for other fungi. This work underlines the importance, in establishing phylogeny, of not only looking at one region of the genome, but also taking other sequences, as well as isoenzyme data or morphological features, into account.

The taxonomic considerations for the ectomycorrhizae are much more defined and orderly (see Agerer 1995). A literature survey revealed that either symbiont, fungus or plant, can influence the shape of the ectomycorrhiza. Although the plant genus is the most important component, some fungal relationships can control, at least in part, the final form. The influence of environment, e.g. soil conditions, is small or even absent, whereas age-dependent differences occur (Agerer 1987–1993). Zak (1973) rightly pointed out that the taxonomic position of some ectomycorrhizal fungi will be modified as further identification, description, and catologuing of natural mycorrhizas add to the knowledge of species differentiation. Moreover, it can be added that fungal relationships very likely can be enlightened if, in addition to fruit body, characteristic features of ectomycorrhizas are also taken into consideration.

Microorganisms Associated with Spores and the Hyphae

Investigating the presence of different symbionts associated with the roots of eucalypts, unique populations of bacteria and/or actinomycetes were found in the mantle of mycorrhizas. It is not conceivable that differences in hyphal exudates by various symbionts, together with host root exudates, may have influenced the development of these populations. Compared with non-mycorrhizal roots, the number of bacteria and/or actinomycetes was four to five times higher in the mycorrhizosphere than in the rhizosphere (Malajezuk 1979). In addition to occupying the rhizosphere and the rhizoplane, soil microorganisms also actively penetrate and colonize the cortical tissues. To some degree, these three microhabitats should be regarded as a single microbiological milieu with no sharp demarcations between them. Bacteria could penetrate several layers of cells; avenues of penetration included preexisting pits in the cell walls of cortex and endodermis, and perforations and channels in epidermal and

cortical cell walls in sand dune grasses. These perforations appeared to be the result of bacteria lysing holes in the cell wall. This could reflect the relative susceptibility of this region to bacterial lysis, or an enhanced availability of nutrients in these junctions between cells.

The occurrence of apparently non-pathogenic bacterium-like organelles (BLOs) in the cytoplasm of fungi is a recognised phenomenon (Scannerini et al. 1975; Wilson and Hanton 1979; Macdonald and Chandler 1981; Schmid and Oberwinkler 1993; Dalpe 1994). The cell wall contains chitin as main stuctural component, as shown by the autofluorescence characteristics. In addition, evidence suggests the presence of other polysaccharides, such as those with vicinal hydroxyl groups, and lipids, both common components of fungal cell walls (Jabaji-Hare et al. 1986). Varma and coworkers (1981) have reported several bacterial groups within endogone spores (*Glomus macrocarpus* var. *macrocarpus* from desert habitats) which were successfully cultured on the synthetic media. The potential importance of microbial activity in cortical tissues to plant nutrition and root decomposition has still to be explored.

The Rhizosphere or "Mycorrhizosphere" – Impact and Interactions

The major substrate for microbial activity in the rhizosphere or on the rhizoplane is organic carbon (rhizodeposition) released by plant roots. This organic C varies from simple organic molecules to mucilage and cells and tissues sloughed off during root growth (Rovira et al. 1983). The simple, low molecular weight compounds consist mainly of sugar, amino acids, organic acids and phenolics. The presence of AMF in the root may change root exudation in the colonized plants. The beneficial effects of colonization by mycorrhizal fungi may not always outweigh the cost to the host, which in terms of extra carbohydrates translocated to the roots is approximately 6–10% of the net fixed; and in some cases, the C diverted to the fungi is sufficient to decrease plant growth (Bethlenfalvay et al. 1982; Espinoza-Victoria et al. 1993; Quintero-Ramos et al. 1993; Varma and Schuepp 1994a; Azaizeh et al. 1995).

Even though the evidence is clear that microbial shifts do occur in the "mycorrhizosphere", most studies have not considered these changes relative to biological control of diseases, so few data are available to support such a mechanism. The concept of the "mycorrhizosphere" implies that mycorrhizae significantly influence the microflora of the rhizosphere by altering root physiology and exudation. In addition, extraradical hyphae of mycorrhizal fungi provide a physical or nutritional substrate for bacteria. This leads to both qualitative and quantitative changes in the "mycorrhizosphere" soil of the mycorrhized plants, compared to the rhizosphere soil of nonmycorrhized plants. These microbial

shifts were clearly time-dependent and dynamic, changing as the plants developed (Pfleger and Linderman 1994; Allen et al. 1996). The total population of bacteria in the "mycorrhizosphere" soil of mycorrhized plants was greater than in the soil of nonmycorrhized plant roots. The effect of mycorrhizae on other microbial groups of bacteria, including nitrogen-fixing bacteria, actinomycetes, morphological and physiological groups of bacteria (Gram-positive and -negative bacteria, spore formers, urea hydrolyzers, and starch hydrolyzers) varied with each fungal species (Linderman 1994).

Fungal symbionts produce extracellular hyphae (Tommerup 1992) that may extend several centimeters out into the soil and exude organic materials that are substrates for other soil microbes. These hyphae-associated microbes frequently produce sticky materials that cause soil particles to adhere, creating small aggregates that impart structure to soil, allowing for improved aeration, water percolation and stability (Tisdall 1991; Varma 1995a,b). Mycorrhization alters the selective pressure on the population of soil microorganisms, some of which can antagonise root pathogens (Hashem 1995; Liu 1995).

Very little is known about the interactions that occur between fungal and bacterial plant growth-promoting organisms (PGPRs), either in the soil or at the root surface. Beside long-distance interactions (molecular signals and or soluble components), direct cell-to-cell interactions may be an important factor in the soil. *Rhizobia* and pseudomonads are capable of adhering to AM fungal structures. The number of bacteria found on the fungal spores and hyphae differ, however, depending on the strain used (Bianciotto et al. 1996). The cell walls of mycorrhizal fungi represent a suitable surface for bacterial attachment and colonization. There could be synergistic or antogonistic effects caused by soil bacteria on the mycelial growth of arbuscular fungi.

Interactions with Fauna

The topic of interaction of AMF with nematodes and insects is in a greatly confused state, with a variety of isolated observations from which no useful generalizations can be made. Diversity of interactions between AM fungi and nematodes have been reported, and there is general consensus that mycorrhizae have reduced the severity of disease caused by plant nematodes (Hussey and Roncadori 1982).

The collembola, *Folsomia candida*, has been observed eating the external hyphae of the mycorrhizal fungus, and thereby reducing the effectiveness of the mycorrhiza on leeks. It was further suggested that this feeding activity of the soil microfauna may be one of the reasons why it is much easier to demonstrate increases in yield due to mycorrhiza in pots containing sterilized soil than it is in the field. The locally enhanced