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Review article

Tools for Support of Ecological Research on Arbuscular Mycorrhizal Fungi

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Abstract

The diverse, beneficial impacts that arbuscular mycorrhizal (AM) fungi have on plants and soils in terms of plant nutrient uptake, soil stabilization, and protection of plants roots against soilborne pathogens make these organisms important components of natural and intensively managed landscapes. Their obligate biotrophism limits their propagation in pure culture but has been a stimulus for development of a series of new molecular tools useful for identification, localization (in roots or in soil), and quantification of AM fungal activity *in situ*. This paper presents a review of the tools available to examine the fungal side of the symbiosis and suggests aspects of future development needed to bring the full benefit of these tools to bear on the task of evaluating the impact of AM fungal diversity at broad scale ecological levels.

Keywords: Arbuscular-mycorrhizal fungi, biodiversity, Glomales, glomalin, identification, quantification, root colonization, activity in soil and roots, molecular detection, immunodetection, rDNA, cDNA, taxon-specific PCR, biomass

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1. Introduction

Arbuscular mycorrhizal fungi are ubiquitous, obligate biotrophs that colonize the roots of 80% of land plants (Smith and Read, 1997). These fungi live in intimate contact with plant roots where an exchange of nutrients between plants and fungi occurs within cortical cells. The symbiosis arose between 350–460 million years ago when plants first began their adaptation from water to land (Simon et al., 1993). Evidence accumulated within the past 50 years shows that AM fungi are the most common, active and important beneficial microorganisms in soil. Fungal structures in roots also are abundant (Toth et al., 1990), and colonized root length often reaches 80%. In addition to colonizing roots, AM fungi produce large amounts of extraradical hyphae that measure, for example, 17 and 45 m/cm^3 in a tallgrass prairie and a corn field, respectively (Miller and Jastrow, 1992). AM fungi influence plant fitness, plant community structure, biodiversity, nutrient cycling, soil structure and whole plant physiology. Because the impact of these influences can be significant, it is important that arbuscular mycorrhizae and the associated fungi be integrated into broad scale ecological studies (Allen, 1991; Miller and Kling, 2000; O'Neill et al., 1991; van der Heijden et al., 1998).

The types of questions and issues that need to be addressed to examine the fungal side of the mycorrhizal symbiosis focus on three areas (see also Fig. 1): **Identification**, **Quantification**, and **Functional Activity**. At a landscape-level, for example, some of the simplest, but yet most complex questions, yet to be addressed are: Is the complement of AM fungi present in the subject area critical to the structure and function of the ecosystem? If one or more types of AM fungi were eliminated or added to the system would the system be significantly altered? To what extent are various AM taxa critically active, inside and outside roots, in response to changing environmental parameters, and are the taxa, clades, or groups an important determinant in terms of plant fitness or community structure, nutrient cycling, plant stress tolerance, soil structural changes or whole plant physiology? Answers to these questions will require tools that go beyond direct microscopic observations. For **identification**, what is needed are rapid, methods for detection of specific taxa, clades or groups so that biodiversity can be assessed, especially in the context of changing environmental parameters, such as increased atmospheric carbon dioxide.

For **quantification**, what is needed are rapid methods for point-in-time measures of root colonization and especially extraradical hyphae. For **functional activity**, what is needed are measures of enzymatic, metabolic/catabolic product accumulation or depletion associated with AM fungi *in planta* or extraradical. The ability to measure dynamics of assayed products would make functional activity meaningful. Information on

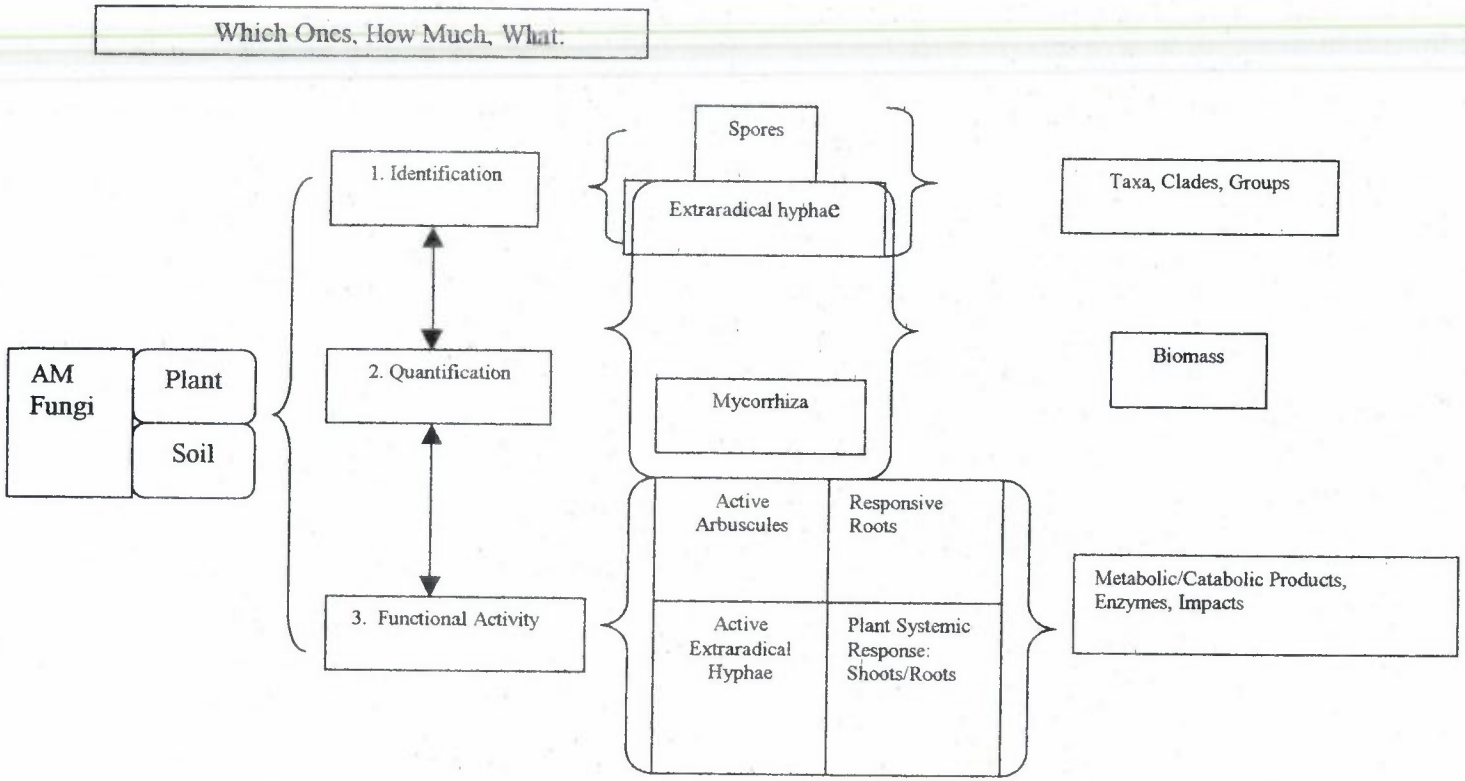

Figure 1. A diagrammatic representation of information inputs and desired outputs to assess ecological impacts of arbuscular mycorrhizal fungi.

identification, quantification and functional activity are mutually supportive and none are exclusive.

This paper will review the development of new techniques that provide some of the three types of information, and speculate on what additional tools are needed. We will also propose general approaches for tool development, and speculate on how the tools could be integrated to answer questions about the role of AM fungi at broad scale ecological levels. In general, we focus this review on effects of soil-root-fungus interaction with ecological impacts. Cell-signaling interactions between host and symbiont at the molecular level are not the primary focus of this review. This information is available in two excellent recent reviews (Harrier, 2001; Burleigh, 2000), and the examples cited here illustrate how the information might be used to develop tools that detect activity of ecological significance.

2. Measurable Parameters to Characterize AM Fungi in Soil and Roots

Quantification

The measurable components of the AM symbiosis can generally be divided into: (1) the mycorrhiza, or the AM fungal colonization of roots (arbuscules and vesicles, intraradical hyphae, and extraradical hyphae closely adherent to roots), and (2) the extraradical hyphae that extend beyond the roots several centimeters into the soil. Traditionally and presently, quantification of the extent of mycorrhization is based on microscopic examination for percent root length occupied by arbuscules, vesicles or hyphae. Quantification of extraradical hyphae is based on length or weight of hyphae per unit area or volume of soil.

Staining of sampled roots coupled with direct microscopic observation continues as the major approach used for assessing the extent of mycorrhizal colonization, based on the length of root colonized. However, Gange et al. (1999) showed how even this classical approach can produce variable results. In their evaluation of various stains and techniques used to count mycorrhizal structures in roots with 10 field-collected plant species, they found that the point-in-time extent of mycorrhizal colonization in any particular plant species depend upon the staining technique employed.

Hyphal length in soils is quantified by microscopic evaluation using grid-line intersect methods after separating fungal hyphae from soil (Sylvia, 1992; Jakobsen et al., 1992; Rillig et al., 1999). Methods used to prepare soil for extraction can influence results. For example, grinding soil in a mixer (Jakobsen et al., 1992) as opposed to shaking to release hyphae (Rillig et al., 1999) can

make a difference in the ability to subjectively separate AMF from non-AMF hyphae. Even after this separation, correct recognition of AMF in the mix of various hyphae obtained is problematic, tedious, and can be exceedingly difficult for specialists as well as inexperienced workers.

Mycorrhiza biomass

Biomass estimates of mycorrhiza and extraradical hyphae using specific chemical components, such as specific fatty acids (Olssen et al., 1995) depend upon calibration per unit mycorrhiza or hyphae. The method of Olsson et al. (1995) to assess mycorrhizal biomass is based upon the ability to detect and identify hyphal neutral lipid and phospholipid fractions of fatty acids by gas chromatography. Extraction of fatty acids and conversion to free fatty acid methyl esters is a procedure used in many laboratories for the identification of bacteria (Sasser, 1990; Welch, 1991). It requires specialized equipment, instruments and expertise. Protocols for AM hyphae phospholipids are modifications of these procedures. Olsson et al. (1995) tested a method using irradiated soil and a T-shaped growth unit with one lateral compartment with mycorrhizal hyphae and one compartment with both roots and hyphae. Plants were inoculated with an isolate of an unidentified *Glomus* sp. or *Glomus caledonium* (Nicol. & Gerd.) Trappe & Gerd., and values for marker phospholipid fatty acids were obtained. The authors concluded that the combination of fatty acids 16:1 omega 5 and 20:5 can be used as an indicator of (1) AM fungi in well-controlled experiments, and (2) the relative distribution of intra- and extraradical hyphae. For use with field collected root and soil samples, some additional preliminary characterizations would be required and would need to include calibration of 16:1 omega 5 and 20:5 fatty acid content to some measure of colonized root and/or hyphae. The authors extended fatty acid concentration to fungal biomass C with reservations about the use of reported values for phospholipids and fatty acids for these estimates. They more strongly recommended the use of ATP values to estimate biomass C. Their experimental design permitted them to calculate a difference in ATP between compartments with and without hyphae and, thereby, to obtain an estimate for biomass C from a published ATP:C conversion factor. The authors also used hyphal length to estimate extraradical mycorrhizal biomass C by using measured hyphal length and radius, an assumption for hyphal density, an estimate to convert density to dry weight, and an estimate of fungal dry weight C.

Analysis for chitin [poly-β-(1,4)-N-acetylglucosamine], a component of the cell wall of mycorrhizal fungi, also has been used to measure biomass in colonized roots (Bethlenfalvay et al., 1981) and in extraradical hyphae

(Pacovsky and Bethlenfalvay, 1982). The assay relies on colorimetric detection at 650 nm of the chitin degradation product, N-acetylglucosamine; results are expressed in terms of μg of glucosamine per unit dry weight of root, soil, or extraradical hyphae, depending on the focus of the analysis. This method has been used primarily in comparative pot studies, and may require some additional work to ensure that the gluosamine content of root colonized fungal biomass is equivalent to that of extraradical hyphae biomass, a possible inconsistency noted by Hepper (1977) when she evaluated the method.

Functional activity

Activity of AM fungi as used here refers to enzymatic reaction, or metabolic/catabolic product accumulation/depletion, *in planta* or extraradically, at either a systemic, root localized, or soil level. Clearly, the relationship between activity of an AM fungus and its population density, or the number of spores produced in soil are not necessarily related, as is the case for numerous other soil fungi. So spore abundance data are inappropriate measures of activity. Moreover, because several different AM fungi can simultaneously colonize an individual host (Clapp et al., 1995; Helgason et al., 1998; van Tuinen et al., 1998), and they may interact uniquely with that host, the total activity may be different from that of individuals. Thus, to explain how the population of AM fungi works in complex associations involving many AM fungi with a variety of plants encountered in ecological studies requires some new approaches that either integrate an endomycorrhizal-specific activity across the whole group of these fungi or identifies a function or product that is clearly distinct for a defined taxon, clade, or family.

The first reports on enzymes among AM fungi focused on acid and alkaline phosphatases (Gianinazzi-Pearson and Gianinazzi, 1976, 1978) and characterized the identification of an alkaline phosphatase specific to mycorrhizal infection. In a subsequent series of studies, isozymes of esterase, glutamate oxaloacetate transaminase, and peptidase, were shown to have some limited use in localization of specific mycorrhizal activity in roots colonized by *Glomus calendonium, G. mosseae* (Nicol. & Gerd.) Gerd. & Trappe, and *G. fasciculatum* (Thaxter sensu Gerd.) Gerd. & Trappe E3 (Hepper et al., 1986, 1988; Rosendahl et al., 1989). The apparent endomycorrhizal specificity of these enzymes has yet to be used to prepare specific serologically- or genetically-based diagnostic tools although recent advances (Hahn et al., 2001; Harrison et al., 1996) should make it possible. Tisserant et al. (1993) focused on localization and improved detection of alkaline phosphatase in endomycorrhizàe by use of Fast Blue RR salt (FB). Later, the FB technique was proposed as a method for measuring viability of AM fungi in colonized roots

(Gianinazzi-Pearson and Gianinazzi, 1995). Recently, Aarle et al. (2001) described a new, highly sensitive technique for localization of acid or alkaline phosphatase in hyphae and colonized roots. Although the technique is not specific for AM fungal phosphatase at present, it may be possible to couple the fluorescently-labelled enzyme substrate probe with antibody that is specific for AM fungi to refine the assay. In any case, additional developmental work would be needed to adapt the technique for use in broad scale studies.

The main source of information on AM fungal metabolism and by-products is limited to dual culture and host plant interaction studies because these fungi are obligate symbionts. Establishment of a compatible AM symbiosis clearly involves a controlled host response to the fungal colonization as evidenced by the intracellular growth of the fungus in the root cortex and the concurrent production of a defense response and accompanying metabolites (Morandi et al., 1984; Harrison and Dixon, 1993; Volpin et al., 1994; Gianinazzi-Pearson et al., 1996) which is subsequently suppressed (Volpin et al., 1995; Kapulnik et al., 1996). The signaling and regulation steps involved in such defense response events is still poorly understood and therefore not amenable for use at present in broad scale assays at field or landscape levels. In the future, as more is learned about the defense response molecules elicited in response to establishment of AM fungi in roots, it may be possible to develop a diagnostic tool that targets a key moiety common to all endomycorrhial interactions.

Methods to measure activity and biomass simultaneously

A significant effort has been made to develop tools to broaden the acquisition of information about AM fungi in soil as opposed to the host. These approaches address issues that arise from the interaction of the extramatrical hyphae and their environment, the soil. These are discussed briefly along with their limitations.

To quantify hyphal biomass and activity simultaneously, Sylvia (1992) used a colorimetric method to detect dehydrogenase in hyphae. Limitations to this approach occur in the separation of hyphae from soil, and especially in reliably distinguishing hyphae of AM fungi from other fungi, and in performing the assay in an efficient and timely manner.

In an effort to provide a tool to quantify AM fungi from a biomass and activity perspective, monoclonal antibody (MAb) technology was used to produce an antibody against an unknown surface antigen on fresh spores of *Glomus intraradices*. The antigen that stimulated the MAb was subsequently characterized as an insoluble glycoprotein that coats hyphae and fresh spores of *G. intraradices* (Wright et al., 1996) as well as all other members of the Glomales, including the ancient Paraglomaceae and Archeosporaceae. The

name glomalin was ascribed to the hydrophobic glycoprotein. The MAb was an invaluable tool used in the development of a method to solubilize glomalin and to quantify its presence in a wide range of field soils (Wright et al., 1996; Wright and Upadhyaya, 1998). The ability to readily extract and quantify this endomycorrhizal-specific by-product in field soils has revealed much about the ecological importance of AMF in soils relative to aggregation, stability, and soil health. Glomalin is present in temperate soils in amounts of 1 to 15 mg/g (Wright and Upadhyaya 1996, 1998) and is correlated with aggregate stability (Wright et al., 1999).

The relationship between glomalin and AM fungal biomass is 17–63 μg glomalin/mg of extramatrical hyphae (dry weight basis) for six isolates and appears to depend upon the isolate (Wright et al., 1996). However, this does not account for the very large amounts of glomalin shed from hyphae and deposited on soil particles, roots, and plastic traps in pot cultures, as occurred when single clover plants were grown for 3 months in large sand-based pot cultures that were fed with nutrient solution from the top. Distribution of glomalin in the top 16 cm of the root-free hyphal compartment of a pot was: 82 μg (15%) from free hyphae, 173 μg (31%) free-floating on the surface of rinse water (unattached to hyphae), and 297 μg (54%) attached to plastic horticultural mesh (Wright and Upadhyaya, 1999) inserted 16 cm below the sand surface (unpublished data). Hyphae and glomalin apparently released from hyphae were trapped by the horticultural mesh. Thus, the majority (85%) of glomalin produced during the period had separated from the hyphae and the amount that remained attached to the extramatrical hyphae represented a small percentage of the total production.

Additional effort at detecting glomalin production activity of AMF fungi has involved the use of plastic horticultural mesh traps in sand cultures (Wright and Upadhyaya, 1999). Glomalin was extracted from the mesh traps and quantified on the basis of the area of mesh exposed. There are limitations to plastic traps for use in soil. Traps that remain in soil for long periods of time must exclude colonized plant roots that would increase variability of deposition and also must exclude soil particles that contaminate traps with previously deposited glomalin. The immunoassay to examine horticultural mesh visually is described by Wright (2000).

Glomalin constitutes 4–5% of total soil C in contrast to the contribution of soil microbial biomass (chloroform fumigation method) of 0.08–0.2% of total soil C (Rillig et al., 2001). Based on ^{14}C-dating technique the residence time range for glomalin is estimated as 6–42 years (Rillig et al., 2001). Factors involved in its degradation are currently not known. However, extraction and quantification of glomalin provides a unique approach to measuring biomass and activity of AM fungi in soils.

3. Identification

Landscape level studies require identification of fungi that reflect biodiversity, changes in biodiversity, and abundance. At the beginning of the 1990s tools for identification did not meet the need to make assessments at different ecological levels. During the 1990s advances were made in describing spore ontogeny and in two types of molecular probes – immunologic and genetic. These advances supported development of new tools for identification including identification of hyphal colonization in roots.

Classical

Examination of spores for size, shape, color, basal structure, ornamentation and wall structure (Morton and Benny, 1990; Rosendahl et al., 1994) remains the primary means of identification for the majority of AM fungi, even with recent molecular advances and taxonomic revisions at the family level (Morton and Redecker, 2001). Fresh spores are required for morphology-based classification, and often spores in soil are degraded beyond the point of being useful to taxonomists. Differentiating taxa by their spore features requires extensive skill and practice that is challenging even for experienced specialists. The difficulty in identification of spores is further complicated by the large numbers of described taxa, e.g. more than 124 *Glomus* species alone.

Immunologic

The serologic and serotaxonomic approaches applied to endomycorrhizal fungi were recently reviewed (Hahn et al., 2001). Antibodies against AM fungi for identification of species have had limited application because, in general, antigens from these fungi do not elicit reasonably specific antibodies (Hahn et al., 2001). Monoclonal antibody (MAb) technology has been used to produce antibodies for species identification (Hahn et al., 1993; Wright et al., 1987). A MAb highly specific for *Glomus occultum* was produced (Wright et al., 1987). Recent placement of *Glomus occultum* in the new family Paraglomaceae (Morton and Redecker, 2001) was based partly on immunological evidence that unique antigens stimulated antibodies not cross-reactive with other members of the Glomales. A genus-specific MAb against *Gigaspora* was developed (unpublished data), but numerous attempts to produce MAbs specific for other genera have failed.

Molecular genetic

Availability of PCR, sequencing, and other molecular genetic techniques

Table 1. PCR primers for identification of strains, species, genera, or families of arbuscular mycorrhizal fungi as spores or *in planta*

Primer pair nucleotide sequences (all 5'-.....-3')	Family-genus or species of AMF detected	DNA target region, product size (bp)	Reference
CTG CCG CCA CTG GAA CAT GAT TTT G CTG CCG CCA CCA GAA ATC GAA CCG	*Gi. margarita*	Not specified – RAPD	Abbas et al., 1996
CTG CCG CCA CCC CTA TTT TAA TCT AGC CTG CCG CCA CTG TCG GAA TA	*G. mosseae*		
$(GTG)_5$	*G. mosseae*	Not specified – microsatellite	Longato and Bonfante, 1997
Myc1 TCA TAA AAT TTT AAT TGG TG Myc2 TAA TTT TCA GGT AAA GAT GG	*Sc. castanea, Gi. rosea, G. caledonium, S. pellucida A. laevis*	Not specified – Repetitive element (1058 bp, 569 bp)	Zézé et al., 1996, 1999
Real-time fluorogenic PCR P1 TGC AAC GGA TAC CCT TCA GG P2 AAT TCG TTC TTA CCT TTT GAC CGT AAT F1 AGC AAG CTT TCA ACA TCA AGG CAA CGT ATC A	*G. mosseae*	28S (109 bp)	Böhm et al., 1999
GMOSS1 CTG ANG ACG CCA GGT CAA AC GMOSS2 AAA TAT TTA AAA CCC CAC TC	*G. mosseae*	ITS (~400 bp)	Millner et al., 1998
GETU1 GTA TTC AAA ACC CAC ACT GETU2 CTC ATC AAG CAA TTA CGA	*G. etunicatum*	ITS (~400 bp)	Millner et al., 2001a
GOCC56 CAA CCC GCT CKT GTA TTT GOCC427 CCA CAC CCA KTG CGC	*G. occultum*	ITS (~370 bp)	Millner et al., 2001b
GBRAS86 TGT AT TGG ATC AAA CGT C GBRAS388 CGC TAT TCA TTG TGC ACT	*G. brasilianum*	ITS (~200 bp)	

Table 1. Continued.

Primer pair nucleotide sequences (all 5'-.....-3')	Family-genus or species of AMF detected	DNA target region, product size (bp)	Reference
GiITS1 TGA GGT Att TTA TAC CTC TTG GiITS2 ACG CTT CAC ATT ACA TAA CC	*Gi. margarita*	ITS (298 bp)	Lanfranco et al., 1999
GiITS1 + GiR2 ATCACGAACTAAATTACTTGG GiITS1 + GiR3 ATCACCACCTACTTGGTAG	*Gi. gigantea* + *Gi. rosea* *Gi. rosea*	ITS (375 bp)	Lanfranco et al., 2001
Primary PCR: LSU061 AGC ATA TCA ATA AGC GGA GGA LSU599 TGG TCC GTG TTT CAA GAC G Secondary – Nested PCR: LSURK4f LSURK7r	Eukaryotic organisms *Glomus, G. intraradices, G. mosseae* in single stranded conformation polymorphism	28S rDNA	Kjøller and Rosendahl, 2000
Primary PCR: GeoA1 GGT TGA TCC TGC CAG TAG TC ART4 TCC GCA GGT TCA CCT ACG G Secondary – Nested PCR: GeoA2 CCA GTA VTC ATA TGC TTG TCT C Geo11 ACC TTG TTA CGA CTT TTA CTT CC	All glomalean families and *Geosiphon pyriforme*	18S rDNA (1760 bp)	Schwarzott and Schüssler, 2001

Table 1. Continued.

Primer pair nucleotide sequences (all 5'-.....-3')	Family-genus or species of AMF detected	DNA target region, product size (bp)	Reference
Primary PCR: * ** ITS1 TCC GTA GGT GAA CCT GCG G NDL22 TGG TCC GTG TTT CAA GAC G	Primary – all fungi	28S rDNA (~750 bp)	Van Tuinen et al., 1998*
Secondary – Nested PCR LR1 GCA TAT CAA TAA GCG GAG GA* **	Secondary		Jacquot et al., 2000**
CCT TTT GAG CTC GGT CTC GTG*	*G. mosseae* 5.21, 5.23		
5.23 CGGTTAGTCAACATCG**			
AAC TCC TCA CGC TCC ACA GA*	*G. intraradices* 8.22; 8.23		
8.23 GTT CGG TTG ATC AGA TCC GCT**			
GAA TCA CAG TCA GCA TGC TA*	*Gi. rosea* 23.22, 23.23		
GCT ATC CGT AAT CCA ATA CTG**			
Primary PCR: NS5 AACTTAAAGGAATTGACGGAAG ITS4 TCCTCCGCTTATTGATATGC	Primary –Universal	18S, ITS (~1200 bp)	Redecker, 2000
Secondary – Nested PCR:	*Glomus clarum, G. coremioides,*	18S	
ITS1F CTTGGTCATTTAGAGGAAGTAA	*G. intraradices, G. mosseae,*		
GLOM1310 AGC TAG GCT TAA CAT TGT TA	*G. sinuosum, Glomus etunicatum,*	ITS	
LETC1670 GAT CGG CGA TCG GTG AGT	*G. claroideum*		
ACAU1660 TGA GAC TCT CGG ATC GGG	Acaulospora, Entrophospora	ITS	
ARCH1311 TBC TAA ATA BCT AGG CTGY	Archaeospora, Paraglomus	18S	
GIGAS5.8R ACT GAC CCT CAA GCA KGT G	Gigasporaceae	5.8S	

Table 2. Genes involved in up- or down-regulation during mycorrhization or metabolic products specifically induced in either the host or the fungus in response to the establishment of the symbiosis

Gene (G) or product (P)	Induced activity	Host	Tissue/site of activity	Reference
G	Lipid tranfer protein	*Oryza sativa*	Root	Blilou et al., 2000
G	Phenylalanine ammonialyase		Root	
P	Salicylic acid		Root/arbuscules	
P	Plama membrane ATPase	*Nicotiana tabacum*	Root/arbuscules	Gianinnazzi-Pearson et al., 2000
G	Pma 2, Pma 4		Root/arbuscules	
G	Plama membrane ATPase GmHA1-GmHA5	*Glomus mosseae*	Hyphae	Ferrol et al., 2000
P	Induced glycosylated C13 cyclohexanones	*Nicotiana tabacum* *Lycopersicon esculentum*	Root	Maier et al., 2000
G	Aquaporin (Mtaqp1), tonoplast intrinsic protein	*Medicago truncatula*	Root	Krajinski et al., 2000
P	Endoxyloglucanases	*Glycine max*	Root	Garcia Garrido et al., 2000
G	Chitinase Class III, Mtchitinase III-2 & III-3	*Medicago truncatula*	Root	Salzer et al., 2000
G	GINMYC1, putative hormone signal gene GINMYC2, N-acetylglucoasmine transferase, GINHB1, homeodomain and leucine zipper, putative regulatory gene	*Glomus intraradices*	Hyphae	Delp et al., 2000
G	Unknown	*G. intraradices*	Hyphae	Sawaki and Saito, 2001

enabled development of new tools that have been applied to the taxonomy and phylogeny of mycorrhizal fungi (Simon et al., 1993; Simon, 1996; Bago et al., 1998; Redecker et al., 1999, 2000a, 2000b).

Progress in taxonomy and sequencing led to PCR-based methods for identification of specific taxa and groups of related species (Jacquot et al., 2000; Lanfranco et al., 2001; Millner et al., 1998, 2001a, 2001b; Redecker et al., 1997; Redecker, 2000). A variety of adapted PCR techniques were applied at first to study AMF in containerized, single isolate studies (Abbas et al., 1996; Classen et al., 1996; Di Bonito et al., 1995; Lanfranco et al., 1995; Redecker et al., 1997; Sanders et al., 1995) and eventually in field studies (Harney et al., 1997; Helgason et al., 1998; Daniell et al., 2001). Variation within rDNA ITS sequences can also be quite large for some collections, e.g., three field spores of *G. mosseae* were at least 2–5 times more genetically diverse than one single spore and a group of *Gigaspora margarita* spores (Antoniolli et al., 2000).

The quantitative aspects of these detection methods have not been addressed in depth and will be a critical aspect for future work in order for the full potential of these tools to be realized in field and landscape level studies.

Table 1 shows the AM fungal taxa, groups, genera, and families and the corresponding PCR primers used to detect them. A range of PCR approaches have been reported. Most of the successful strategies for detection of AM fungi in roots require use of nested PCR in standard single primer-pair or duplex-pair reactions. Only one report features a real-time fluorogenic approach to PCR (Böhm et al., 1999) and this was for only *G. mosseae*.

Likewise, multiplex PCR has yet to be applied to AM fungi in a system directly usable for field scale studies. The potential exists to use several sets of primers, such as those that detect 1) all glomalean fungi, 2) Archaeosporaceae and Paraglomaceae, 3) *Glomus* clusters, 4) Gigasporaceae, 5) Acaulosporaceae, and then, if additional detail is desired, a limited set for specific taxa (Millner et al., 1998, 2001a, 2001b; Redecker, 2000; Schwartzott and Schüssler, 2001). Large numbers of samples required for field and landscape scale studies would likely make single-stranded conformational polymorphism analysis and single tube, single primer-pair reactions inefficient and cumbersome, so some adaptation of format to multiplex reactions and adaptive use of micro-arrays would help facilitate analyses.

Recent emphasis of molecular studies have focused on unraveling the mechanisms (1) by which the fungal colonization is constrained by the host, (2) by which transport of carbon, nutrients, and energy occur, and (3) signals of early plant-fungal interaction.

cDNAs and gene products

In an examination of the apparent transition from incompatible to long-term compatible interaction between fungus and plant root, Lambais and Mehdy (1995) studied defense-related gene activity in AM colonized and uncolonized roots. They found differential mRNA expression of cell-wall degradation and isoflavonoid phytoalexin genes: β-1,3-glucanase, two other glucanases, and chalcone isomerase activity were observed, along with a transient induction of endochitinase, followed by suppression, under low and high phosphorus (P) conditions. They also noted an accumulation of defense-related mRNAs and acidic and β-1,3-endoglucanase mRNAs around cells containing arbuscules, regardless of soil P condition. As more information of this type is accumulated for other AM fungi, it may be possible to develop diagnostic tools that focus on the genes or the messenger RNA involved in the defense-related response.

Martin-Laurent et al. (1998) reported on the production of a mycorrhization protein, PSAM1, induced and located in the cytoplasm surrounding young arbuscules in pea roots early in the AM interaction. A number of additional reports (Table 2) focus on identification and characterization of a variety of plant gene responses specific to mycorrhizal interaction. Most of these genes, or the corresponding gene products, however, are localized not simply in the colonized root, but specifically in the cells that are occupied by arbuscular structures. Using these genes and gene products to detect and quantify mycorrhizal activity at realistic scales in field and landscape studies remains unexplored. In order to use such products as markers of mycorrhizal activity, methods are needed to adequately sample, process, and quantify detected activity in root samples so that the localized products of mycorrhizal activity are adequately obtained, represented, and calibrated to actual occurrence. As yet, no plant systemic response distinctively associated with the endomycorrhizal state has been reported. Nevertheless, the potential diagnotic value of such a response merits the attention of investigators as they proceed to unravel the genetic code that enables AM fungi to form their symbiotic relationships with the vast array of hosts.

4. Future Paths

The pursuit of more rapid and specific means for detection and identification of AM fungal activity and hyphae is dependent on continued development and integrated use of molecular tools of a wide variety. An analogous situation has occurred with other obligate biotrophic, plant, animal and human pathogens. With such organisms, methods to detect their presence and in some cases viability, even for specific strains at very low concentrations, have been

developed, e.g., *Cryptosporidium, E. coli, Fusarium, Gibberella zea,* plum pox virus, *Tapiesia* spp. and *Salmonella* (Baumner et al., 2001; Dyer et al., 2001; Esch et al., 2001; Lee et al., 2001; Lin and Tsen, 1999; Rappelli et al., 2001; Schner et al., 2001; Szemes et al., 2001). High degrees of detection specificity have been achieved for some AM fungi, however, activity, i.e., viability of the target, and quantitation, including root volume need additional testing or a different approach to sampling for field and landscape level experiments. Presently, the limits of detection and efficiency of extraction from roots of AM fungi *in planta* have not been well documented for the various molecular methods reported.

Many reports indicate that robust methods are now available for direct isolation and preparation of DNA/RNA from in situ root- and soil-inhabiting microbes such that humic substances and other constituents will not quench the PCR. The next step will involve developing and calibrating quantitatively the presence and activity of mycorrhizal fungi detected with the new molecular tools to physical and spatial distribution patterns in the field.

5. Sampling Issues

At present, methods for identification of AM fungi are clearly distinct from those used for determining activity. Identification of specific fungi *in planta* produces results in terms of 'detect-no detect' rather than in quantitative or activity terms. Methods for quantitation have been explored (Edwards et al., 1997; Simon et al., 1992) and assays for enzyme (Aarle et al., 2001) and secretory activity (glomalin) (Wright et al., 1996) have been developed. In the future, techniques for identification of AM fungi might be linked to methodology appropriate for detection of active hyphae or production of specific gene products. Adaptation of the technique to field soils and plant roots/shoots will be a particularly useful addition to the mycorrhizast's toolbox. Construction of cDNA libraries from mycorrhizal roots coupled to subsequent differential screening will continue to be a useful approach to isolate genes expressed during mycorrhization. A small-scale level technique, *in situ* hybridization, can aid identifications of taxa or possibly presence of specific activity genes in sections of colonized roots.

However, the scale and current format of *in situ* hybridization assays may limit the use of this approach in landscape and broad scale studies. The need for extensive sampling and replication in ecological studies points to the need for purposeful development of identification and activity assessment tools appropriate for use at such scales.

6. Summary

Rapid methods for identification and activity are needed to evaluate the impact of AM fungi at broad scale ecological levels; and they need to be linked to each other to evaluate the role of specific taxa or groups. Detection of taxa presently is accomplishable with PCR, but it needs to be adapted to accommodate rapid analysis of large numbers of samples if it is to be useful for landscape studies. Activity assessment presently is accomplishable with glomalin and enzyme assays, but it also needs to be adapted to accommodate landscape-scale studies and to include up-regulated genes and their products. This would require identification and purification of target products, and evidence that the target is universally produced or excreted by AM fungi. The advent of increasingly smaller technologies for microbial identification, e.g., DNA microarrays and real-time PCR, as well as electronic sensor technology for enzyme-linked immunosorbent assays offers an unprecedented opportunity for development of rapid, real-time information on mycorrhizal presence and activity in roots/soils.

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