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CURRENT ADVANCES IN MYCORRHIZATION IN MICROPROPAGATION

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Summary

Mycorrhization of *in vitro*-propagated plantlets is having a 'positive impact' on their posttransplanting performance. Different aspects of the technology, such as the need for improvement, screening bioassays for selection of the most effective strains, and determination of the multiple role played by mycorrhiza, are discussed. Various constraints pertaining to the utilization of this technology and their possible solutions, such as the use of mixed cultures and aspects of technology transfer are also reviewed.

Key words: micropropagation; mycorrhization; biotization; microrrhization; transfer of technology.

Introduction

In the development of sustainable crop production practices, the use of microbial inoculants as replacement for chemical fertilizers and pesticides is receiving attention (O'Gara, 1996). It is believed that delivery of microbial inoculants *via* micropropagation is one of the solutions to this problem. The total number of plants produced by tissue culture in Europe alone is around 200 million units a year, of which over 180 million are produced in commercial laboratories (Lovato et al., 1996). Similar production is being done in Eastern Asia and in North America. In micropropagation, the growth substrate is devoid of microbes and, as a result of this nutrient-rich growth substrate, delicate plants having no interaction with other microorganisms are produced (Dolcet-Sanjuan et al., 1996). Although micropropagation is an established technique for the production of elite plants, owing to transient transplantation shock plants require biological hardening before transplantation. For this reason, mycorrhizal technology can be applied.

Arbuscular mycorrhiza (AM) is a symbiotic or mutualistic association between roots of about 90% of the species of plants, including angiosperms, gymnosperms, pteridophytes and bryophytes (Mishra et al., 1980; Harley, 1991; Varma, 1991; Cazres and Trappe, 1993; Williams et al., 1994). The arbuscular mycorrhizal fungi play a significant role in insuring the health of plantlets (Gianinazzi and Gianinazzi-Pearson, 1988). Moreover, the acclimatization period of micropropagated plants can be shortened by application of arbuscular mycorrhizae (Salamanca et al., 1992).

A survey of current literature reveals that inoculation of arbuscular mycorrhizal fungi (AMF) into the roots of micropropagated plantlets plays a beneficial role (Blal et al., 1990; Schubert et al., 1990; Azcon-Aguilar et al., 1994; Declerck et al., 1994; Varma and Schuepp, 1994a,b; Gribaudo et al., 1996; Martins et al., 1996; Vestberg and Uosukaninen, 1996; Budi et al., 1998; Naqvi and Mukerji, 1998; Vosatka et al., 1999; Gange and Ayres, 1999). Reviews concerning micropropagation and mycorrhization have been published by Conner and Thomas (1981), Vestberg and Estaun (1994), Varma and Schuepp (1995) and Lovato et al. (1996).

The present review is aimed at providing some additional information including screening of mycorrhizal fungi for better performance, role of endo- and ectomycorrhizal fungi in solving problems of transient transplantation shock, and the integrated approach of utilization of microbes, such as plant growth promoting rhizobacteria (PGPRs), actinorhiza, *Agrobacterium rhizogenes*, *Rhizobium*, *Frankia*, and mycorrhizal fungi (endo- as well as ectomycorrhizae).

Multifunctionality

The AMF help the plant partner by increasing uptake of nutrients in general, and phosphorus in particular (Vestberg and Estaun, 1994). The multifunctionality of AMF has been discussed by many investigators in the recent past (Gianinazzi et al., 1990; Ponton et al., 1990; Cargeeg, 1992; Arias and Cargeeg, 1992; Varma and Schuepp, 1995; Lovato et al., 1996; Hindav et al., 1998). AMF can contribute to plant growth and survival by reducing stresses associated with nutrition, water/aeration, soil structure, pH, salt, toxic metals, and pathogens (Sylvia and Williams, 1992). Vestberg and Estaun (1994) published an excellent review on factors affecting the result of mycorrhizal inoculation (timing, medium, fertilization, inoculation, fungus-host specificity, growth substrate, etc.). The potential for biocontrol of plant diseases by AM and ectomycorrhizal fungi was reviewed by Linderman (1994) and Duchesne (1994), respectively. Varma and Schuepp (1995) enlightened the need for mycorrhization and the different role played by AMF. Lovato et al. (1996) also reviewed various functions performed by AMF as bioregulators, bioprotectors, biofertilizers, and discussed mycorrhization of micropropagated plantlets. Therefore, these aspects have not been discussed here.

Mycorrhization Techniques

A survey of the literature reveals that AM symbioses have been

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established successfully on agar medium using seedlings (Hayman, 1983) and root organ culture (Mosse and Hepper, 1975; Miller-Wideman and Watrud, 1984; Mugnier and Mosse, 1987; Becard and Fortin, 1988; Gemma and Koske, 1988; Becard and Piche, 1989; Chabot et al., 1992a,b; Elmeskaoui et al., 1995; Declerck et al., 1996a, 1998; Pawlowska et al., 1999). Vestberg and Estaun (1994) suggested that an inoculation protocol should be designed for each plant species, taking into account root growth and development rate, and number of transplants after the *in vitro* stage.

The inoculation technique differs depending on the substratum or the nature of the inoculum used (Trouvelot et al., 1986). Selection of quantity (Morandi et al., 1979; Daniels et al., 1981; Ravolanirina et al., 1989a; Guillemin et al., 1992; Morte et al., 1996) and quality of inoculum is an important point both for *in vitro* and *in vivo* mycorrhization (Vestberg and Uosukainen, 1996). The inoculum should not only be pure, but also be able to exhibit the desired biological effect. In the recent past, hyphae, spores, chlamydospores and mycorrhizal roots have been used as inocula by various mycorrhizologists for *in vitro*, as well as *in vivo* mycorrhization. They are also used for establishment of AMF in root organ culture (Mosse and Hepper, 1975; Strullu and Romand, 1986, 1987; Mugnier and Mosse, 1987; Ravolanirina et al., 1989b; Schubert et al., 1990; Lovato et al., 1992; Williams et al., 1992; Uosukainen and Vestberg, 1996; Azcon-Aguilar et al., 1996; Dolcet-Sanjuan et al., 1996; Declerck et al., 1996b; Morte et al., 1996; Plenchette et al., 1996).

The AMF inoculum should be sterilized properly as there are many impurities associated with it, which sometimes are not easily eradicated even after sterilization. However, healthy spores can be easily separated from the old and deterioted spores by centrifugation (Furlan et al., 1980). Only the healthy inoculum should be selected for surface sterilization. There are many procedures used for surface sterilization of inocula for establishing *in vitro* symbiosis on agar culture (Mosse, 1962; Mertz et al., 1979; Tommerup and Kidby, 1980, Macdonald, 1981; Strullu and Romand, 1986; Becard and Piche, 1992). Among these, the method suggested by Becard and Piche (1992) is widely used. Initially spores of AMF are rinsed in 0.05% Tween 20 for 1 min then treated by agitation at 4°C in chloramine T (2% solution) for two periods of 10 min. Combination of a sterile mixture of antibacterial agents, viz. streptomycin (200 mg l^{-1}) and gentamicin (100 mg l^{-1}), is used for sterilization of spores followed by four rinses (Becard and Piche, 1992). The dual cultures of root and AMF are established on solid media, particularly on White's medium (Pawlowska et al., 1999). The pregerminated spores or pieces of mycorrhizal roots showing hyphal growth are used to inoculate the sterile root system by placing them close to emerging lateral roots. The selection of agar medium is important, since some types contain inhibitory properties due to traces of heavy metals (Hepper, 1978). The concentration of sodium sulfate, phosphorus and sucrose in the culture medium is important (Becard and Fortin, 1988). Since, the MS (Murashige and Skoog, 1962) rooting medium contains these elements at high concentrations, a change of medium is essential before the tripartite culture stage (Elmeskaoui et al., 1995). Axenically infected mycorrhizal roots can also be used as inoculum to overcome the problem of contamination (Elmeskaoui et al., 1995; Declerck et al., 1996a,b, 1998, 2000; Plenchette et al., 1996).

Recently, a novel and reliable technique to establish arbuscular mycorrhizal symbiosis in micropropagated plantlets and to evaluate their effect on plant growth has been developed (Elmeskaoui et al., 1995; Declerck et al., 1998). In that technique, AMF-colonized roots cultivated in minimal medium (dual culture system) are used for inoculation of micropropagated plantlets grown on an artificial substrate, which results in a tripartite culture system (Elmeskaoui et al., 1995). The tripartite culture system with CO_2 enrichment favors proliferation of the inoculum, growth of plantlets and establishment of the mycorrhizal association. Interestingly, it allows direct *in vitro* AMF inoculation in an axenic environment. The medium used by Becard and Fortin (1988) was low in sugar and mineral nutrients in order to favor growth of the mycorrhizae. The lack of CO_2 reduces autotrophy of tissue culture-raised plantlets. In the tripartite culture system, CO_2 is essential for two reasons: it provides conditions necessary for autotrophic carbon nutrition and stimulates the development of the mycorrhizal colonization with better health (Chabot et al., 1992b).

It is hoped that the tripartite culture system will be a powerful tool for the commercial production of AM fungal hyphae and to obtain a higher percentage of mycorrhizal tissue culture-raised plantlets (Elmeskaoui et al., 1995).

Eco-friendly mycobiont with biotizing effect. AMF have increased tolerance to pollutants or environmental stress, particularly in AMF recovered from polluted soils (Dodd, 1998). If such fungi are inoculated to micropropagated plantlets, these may enhance the capacity of transplant shock tolerance and growth during the acclimatization phase. Arbuscular mycorrhiza-inoculated plantlets reduce soil erosion by ramifying the roots. Fortuna et al. (1992) evaluated transplant shock tolerance by inoculation of *Glomus mosseae* and *G. coronatum* into micropropagated *Prunus cerasifera*. After 4 wk growth, 100% survival of plants was recorded. Further, they reported that both fungi improved tolerance of plantlets after removal from *in vitro* and *in vivo* systems. Karagiannidis and Hadjisavva-Zinoviadi (1998) found that *Glomus mosseae* improved plant growth up to 11.6 times and increased grain yield up to 5.4 times as compared to non-inoculated plants. Moreover, another benefit was that *Glomus mosseae* decreased the concentration of heavy metals when inoculated in the Durum variety of wheat. Similarly, AMF might alleviate aluminum toxicity (Gervais et al., 2000).

Generally, plants do not grow on coal-mine spoils or on any metal wastes (Johnson and McGraw, 1988). However, Mehrotra (1998) reported six spore-forming AMF including *Acaulospora scrobiculata*, *Entrophosphora colombiana*, *Glomus aggregatum*, *G. ambisporum*, *Glomus* sp., and *Scutellospora calospora* from Chandrapur, Maharastra state, India. The plants preinoculated with AMF can be used for the revegetation of mine areas (Pfleger et al., 1994). Mleczko et al. (1998) recovered ectomycorrhizae, viz. *Suillus luteus*, *Rhizopogon roseolus*, *Hebeloma mesophaeum*, and *Tricholoma scalpturatum* from zinc wastes. It seems that ectomycorrhizae are scavengers of heavy metals. Blake et al. (1993) reported chemical transformation of toxic metals by a *Pseudomonas* strain from a toxic waste site.

Nowak (1998) stated that the induced resistance response caused by inoculants is referred to as 'biotization'. *In vitro* co-culture of plant tissue explants with beneficial microbes induces developmental and metabolic changes, which enhance their tolerance to abiotic and biotic stresses. Further, he reviewed benefits of *in vitro* biotization of plant tissue cultures with microbial inoculants.

TABLE 1

ARBUSCULAR MYCORRHIZAL FUNGI USED AS INOCULANTS FOR VARIOUS PLANTLETS

Mycorrhizal species	Host plant	Reference
Glomus epigaeum	Apple	Granger et al., 1983
Glomus caledonium, *G. clarum*, *G. versiforme*	*Vitis erlandieri* × *V. vinifera* (Kober 5 BB)	Schubert et al., 1987
Gigaspora margarita (LPA 2), *Glomus mosseae* (LPA 5), *G. caledonium* (LPA 12), *G. fasciculatum* (LPA 7)	Vine (*Vitis vinifera*) and oil palm (*Elais guinnensis*)	Ravolanirina et al., 1989a
Glomus fasciculatum, *G. caledonium*, *G. monosporum*, *Glomus* sp. E3, *G. constrictum*, *G. occultum*, *G. vesiforme*	Kiwi (*Actinidia deliciosa*), grapevines	Schubert et al., 1990
Gigaspora margarita	*Coffea arabica* L.	Souza et al., 1991
Glomus sp. (LPA 21), *G. intraradices* (LPA 8)	Grapevine rootstocks, pineapple (*Ananas comosus*)	Lovato et al., 1992
Glomus aggregatum, *G. deserticola*	*Prunus avium*, *Spiraea vulgaris*, *Syringa japonica*	Arines and Ballester, 1992
Glomus deserticola, *G. mosseae*	Avocado (*Persea americana*)	Azcon-Aguilar et al., 1992
Glomus fasciculatum, *G. intraradices* (LPA 8), *Glomus* isolate (LPA 21)	Date palm (*Phoenix dactylifera*)	Bouhired et al., 1992
Glomus fasciculatum, *G. mosseae*, *G. intraradices*	Apple (M 9, M 26, golden)	Branzanti et al., 1992
Glomus mosseae, *G. coronatum*, *G. caledonium*, *G.* sp. strain A6	Plum rootstock (*Prunus cerasifera*)	Fortuna et al., 1992
G. sp. (LPA 21), *G.* sp. (LPA 22), *G. clarum* (LPA 16), *G.* sp. (LPA 25), *Scutellispora pellucida* (LPA 20)	Pineapple (three varieties) (*Ananas comosus*)	Guillemin et al., 1992
Glomus mosseae	Banana (*Musa acuminata*)	Jaizme-Vega, 1992
Glomus fasciculatum	*Tetraclinis articulata*	Honrubia and Morte, 1992
Glomus fasciculatum	*Anthyllis cytisoides*, *Spartium junceum*	Salamanca et al., 1992
Glomus sp. strain A6	Apple and peach rootstocks	Sbrana et al., 1992
Glomus sp. strain E3	Kiwi (*Actinidia deliciosa*)	Schubert et al., 1992
Glomus fasciculatum	*Platanus acerifolia*	Tisserant and Gianinazzi-Pearson, 1992
Glomus intraradices Finn 98, *Glomus* sp. Finn 128, *G. geosporum*	Strawberry (*Fragaria ananassa*)	Williams et al., 1992
Glomus intraradices, *G.* sp. V21/88, *G. macrocarpum*, *G. mosseae* V11 b, *G. mosseae*, *G.* sp. V4	Strawberry (*Fragaria ananassa*, *F. virginiana*, *F. vesca*)	Vestberg, 1992
Glomus fasciculatum	Avocado (*Persea americana*)	Vidal et al., 1992
Glomus intraradices	Hortensia (*Hydrangea*)	Varma and Schuepp, 1994b
Glomus intraradices	Strawberry (*Fragaria ananassa*)	Elmeskaoui et al., 1995
Glomus deserticola	*Annona cherimoya*	Azcon-Aguilar et al., 1996
Glomus fasciculatum	*Tetraclinis articulata*	Morte et al., 1996
Glomus sp.	*Pyrus communis*	Rapparini et al., 1996
Glomus mosseae V 57 (BEG 29), *G. hoi* V 98 (BEG 48), *G. caledonium* V 126b, *G. fistulosum* V 128 (BEG)	Greenhouse rose 'Mercedes'	Vestberg and Uosukainen, 1996
Glomus intraradices, *G. fasciculatum*, *G. caledonium*, G. monosporum, *G.* sp. E3	*Prunus avium*	Cordier et al., 1996
Glomus mosseae, *G. intraradices*	Walnut (*Juglans regia*)	Dolcet-Sanjuan et al., 1996
Glomus claroideum, *G. fistulosum*	Crab apple cv. Marjatta	Uosukainen and Vestberg, 1996
Gigaspora rosea, *Glomus mosseae*	*Allium cepa*	Rancillac et al., 1996
Glomus fistulosum	Strawberry (*Fragaria vesca*)	Cassells et al., 1996
Glomus mosseae	Grapevine (Kober 5 BB) *Vitis berlandieri* × *V. riparia*, *Actinidia deliciosa*, *Malus pumicola*	Gribaudo et al., 1996
Glomus versiforme, *G. intraradices*, *G. fasciculatum*, *G. macrocarpum*	Banana and sugarcane	Declerck et al., 1994a
Glomus deserticola, *G. clarum*, *G. fasciculatum*	Cassava (*Manihot esculenta*)	Azcon-Aguilar et al., 1997
Glomus fasciculatum	*Leucaena leucocephala*	Naqvi and Mukerji, 1998
Glomus fasciculatum, *G. macrocarpum*	*Leucaena leucocephala*	Puthur et al., 1998
Glomus mosseae	*Casuarina equisetifolia*	Mark et al., 1999

Screening for Efficient Mycorrhizal Strains

Mycorrhizologists rarely provide the rationale for selection of particular AM endophytes for growth promotion of plants (Bagyaraj, 1992). Research over the past decade has demonstrated that most species of *Glomus*, including *G. mosseae*, *G. fasciculatum*. *G. etunicatum*, *G. tenue*, and *Gigaspora margarita* were selected for inoculation to the desired host plants due to their easy availability (Table 1).

In the past two decades, remarkable progress has been made in establishing plant host–endophyte associations. But, endophytes vary in their efficiency in enhancing plant growth as they show functional host-specificity (Clarke and Mosse, 1981; Plenchette et al., 1982, 1983; Doud-Miller et al., 1985; Schubert and Commarata, 1986; Guillemin et al., 1992; Lemoine et al., 1992; Vestberg and Estaun, 1994; Sylvia, 1998; Rajan *et al*., 1999). To overcome this problem, AM fungal inoculants should be screened for their efficacy, and later the species/strains showing high

potential for nutrient uptake should be selected before inoculating the plants (Abbott and Robson, 1978; Haas and Krikun, 1985; Abbott et al., 1992; Azcon-Aguilar et al., 1997; Naqvi and Mukerji, 1998; Puthur et al., 1998). Sieverding (1989) suggested that it would be more interesting to find one isolate that is effective with a wide range of plant species, since interactions can occur between different isolates in mixtures. Ravolanirina et al. (1989a,b) proposed a procedure for effective inoculation of micropropagated vine and oil palm with *Gigaspora margarita*, *Glomus mosseae*, *G. caledonicum* and *G. fasciculatum*. Schubert et al. (1990) also screened AMF and found *G. constrictum* to be less effective in increasing growth of *Actinidia deliciosa* than *G. caledonium*, *G. occultum* and *G. versiforme*. Azcon-Aguilar et al. (1992) evaluated the influence of *Glomus deserticola* and *G. mosseae* on the growth and development of micropropagated plantlets of avocado (*Persea americana* Mill) and reported that the former increased shoot height, leaf number and vigor of the plantlets more than the latter. Arines and Ballester (1992) applied *G. aggregatum* and *G. deserticola* for mycorrhization of micropropagated plantlets of *Prunus avium* and found 100% plant survival. The influence of inoculation of *Glomus fasciculatum* (LPA 7), *G. intraradices* (LPA 8) and *Glomus* isolate from a tropical soil (LPA 21) on the growth of *Phoenix dactylifera* (date palm) was studied by Bouhired et al. (1992). They reported mycorrhizal inoculation as an essential factor for increasing growth of *Phoenix dactylifera*. After the screening of more than 80 Finnish inoculants, Williams et al. (1992) selected only two *Glomus* species, Finn 98 (*G. intraradices*) and 128 (*Glomus* sp.), for inoculation of micropropagated plantlets of strawberry, which form an important planting material in the UK and Finland. *Glomus geosporum* from the Kent collection was chosen as a broad range AMF to include within the trial. The Finnish AMF isolates significantly increased shoot dry weights more (47 and 46%) than the Kent isolate, i.e. *G. geosporum* (26%). Similarly, Vestberg (1992) evaluated *G. intraradices* and *G. mosseae* for inoculation of strawberry and reported that the latter was found to be the most efficient fungus, as it increased shoot growth several-fold. Lemoine et al. (1992) screened seven ericoid mycorrhizal fungi against microplants of nine cultivars of *Rhododendron hybrida* and found that use of defined disinfected substrata, combined with specific mycorrhizal fungal strains, is essential for guranteeing an optimal production of outplanted *Rhododendron* microplants at nursery level.

A significant contribution has been made by Guillemin et al. (1992) in that field. They screened several AMF for establishment of symbiosis in micropropagated pineapple plantlets, and reported that Queen and smooth cayenne pineapple plants associated with *Glomus* species (LPA 21) presented better growth than those infected with the other AMF, and the best growth was obtained for the Spanish variety by inoculating plants with *Glomus* sp. (LPA 25). Fortuna et al. (1992) reported the infectivity and effectiveness of different species of AMF, viz. *Glomus mosseae*, *G. caledonium*, *G. coronatum* and *Glomus* strain A6, in micropropagated plantlets of plum rootstock (*Prumus cerasifera* Ehrh, clone Mr S 2/5). Further, they evaluated the most and the least infective fungi, *G. mosseae* and *G. coronatum*, respectively, for enhancement of growth of tissue culture-derived *Prunus cerasifera*. Verma and Jamaluddin (1995) found a low percentage of infection in seedlings of teak (*Tectona grandis*) when inoculated with *G. fasciculatum*. On the other hand, the mixed inoculum of AMF was found to be more effective for enhancement of growth and biomass of teak.

As a matter of fact, the efficacy of ectomycorrhizal fungi was not considered earlier by mycorrhizologists for inoculation of *in vitro*-produced microplants. Therefore, the information pertaining to application of ectomycorrhizal fungi is scanty. However, Gay et al. (1992) used ectomycorrhizal fungi as a tool to enhance rooting of micropropagated cuttings of *Pinus halepensis* and remarked that an indole-3-acetic acid (IAA) overproducer mutant of ectomycorrhizal fungi, such as *Hebeloma hiemale* and *H. cylindrosporum*, could enhance the rooting of cuttings of *Cerasus avium* and *Prunus cerasus* up to 95%, which are generally non-ectomycorrhizal. Therefore, these mutants could be used to promote *in vitro* rooting of cuttings of ectomycorrhizal and even non-ectomycorrhizal plants. Martins et al. (1996) also reported *Amanita muscaria*, *Laccaria laccata*, *Piloderma croceum* and *Pisolithus tinctorius* to be useful on acclimatization of micropropagated plantlets of *Castanea sativa* Mill. They found a beneficial effect on growth of mycorrhized plants of *Castanea sativa* as well as on physiological parameters and weaning results. *Pisolithus tinctorius* was most effective in colonizing roots of both micropropagated plants and seedlings, whereas *A. muscaria* and *L. laccata* only colonized a few feeder roots of some plants and *Piloderma croceum* did not form mycorrhizae. Grange et al. (1997) studied the effect of different genotypes of *Hebeloma cylindrosporum* on *in vitro* rooting of micropropagated cuttings of *Prunus avium* and *P. cerasus*, and found that the rooting percentage of cuttings cultivated in the absence of IAA was enhanced by all strains of *H. cylindrosporum*, ranging from 50% to 60% with IAA-overproducing mutant D 111 or the wild-type dikaryon D1, to 100% in the presence of mutants 331 or D 117. They reported that *P. cerasus* exhibited higher rooting ability than that of *P. avium*. The survival percentage was also increased from 30 to 100%. Mycorrhizal activity of different strains of *Paxillus involutus* with seedlings of *Pinus sylvestris* cultured under aseptic conditions was evaluated by Rudawska and Kieliszewska (1997). They found that fungal strains that were characterized in pure culture conditions by high IAA-synthesizing activity induced more fine roots and significantly higher numbers of mycorrhizae than strains which showed a low activity of IAA synthesizing enzymes. The increased ectomycorrhiza formation was generally accompanied by an increased growth of seedlings. Reddy and Satyanarayana (1998) screened five ectomycorrhizal fungi, viz. *Cenococcum geophilum*, *Laccaria laccata*, *Paxillus involutus* and two isolates of *Pisolithus tinctorius*, to inoculate micropropagated plantlets of *Populus deltoides* (G 48), and found that *P. involutus* formed mycorrhizae with plantlets of *P. deltoides* while others failed, though they colonized the substrate extensively. The plantlets colonized with *P. involutus* showed significantly enhanced growth and dry weights.

These fungi can be utilized effectively for enhancement of growth of the micropropagated plantlets as they are easily available facultative biotrophs. The basic advantage of these fungi is that they can be cultured axenically on artificial medium. However, a thorough screening is required for their efficacy before using them on a large scale.

Bottlenecks

Although, the technique of mycorrhization is of utmost importance for the growth and development of the micropropagated plantlets, there are some problems in establishment of mycorrhizal–

host symbiosis *in vitro*: (1) contamination of inoculum, (2) behavior of the host *in vitro*, and (3) obligate nature of the endophyte.

There have been many problems pertaining to the sterilization and germination of spores in agar medium. Becard and Piche (1992) recommended centrifugation during sterilization of spores. On the contrary, Rancillac et al. (1996) stated that after centrifugation, germination capacity of spores decreases due to a Percoll negative effect and contamination increases. Therefore, its better to sort out spores manually even if it is a time-consuming process. Spore germination ability of AMF increases by increasing agar concentration to 1.5 g l^{-1}. Furthermore, a double disinfection is better for suppressing contamination.

Properly sterilized spores of AMF play a prime role in establishment of healthy symbiosis. The main hurdle in establishing *in vitro* strains of mycorrhizae on host plants is the starting point: 'How to obtain spores without contamination? Really, it's not easy to overcome this bottleneck', remarked Rancillac et al. (1996). Concurrently, Sylvia (1998) explained obstacles in inoculation. In fact, there are mainly two points to be considered carefully concerning the quality of spores: (1) ability of quick germination and (2) development of the mycelial network responsible for penetration of root cells. Both the criteria are difficult to be fulfilled by AMF due to their obligate nature.

The spores of AMF failed to germinate on half-strength MS medium used for grapevine micropropagation (Schubert et al., 1987). On the other hand, the spores germinated readily on water agar, as recorded by Hepper and Smith (1976). On MS medium, the spores neither germinated nor established symbiosis. The medium exhibited adverse effects so much that even the pregerminated spores stopped growth. However, this was probably not due to high concentrations of metal cations, which are known for their toxicity to AMF (Gildon and Tinker, 1983), as in an experiment performed by Schubert et al. (1987) on AMF colonization in *Vitis berlandieri* × *V. rifaria*, the concentration of heavy metals like Mn, Zn, and Cu in the agar medium was reduced to values indicated as non-toxic by Hepper (1978). Moreover, phosphorus was not added in amounts which should inhibit germination (Pons and Gianinazzi-Pearson, 1984). The reason for failure of germination must be due to lack of CO_2 in nurse cultures, which is needed for proper growth of plantlets and inoculum as well.

An example of successful AMF inoculation in an agar medium designed for micropropagation is that of cherry plants inoculated with *Gigaspora margarita* in an enriched agar medium containing sterile soil (Pons et al., 1983). Evidently, in such a situation it is difficult to analyze the effect of the different components of the nutrient solution on mycelial growth, as the soil organic matter and clay may adsorb part of them, thus making fungal development possible. It is conspicuous that *in vitro* inoculation of micropropagated plantlets does not seem a feasible practice without changes in the methodology of propagation, e.g. modification of nutrient media. To overcome this problem, Elmeskaoui et al. (1995) developed an *in vitro* system for culturing the *Glomus intraradices* with Ri T-DNA-transformed carrot roots or nontransformed tomato roots as a reliable method to obtain AMF in micropropagated plantlets and to determine their influence on growth. After root induction, micropropagated plantlets grown on cellulose plugs (sorbarod) were placed in contact with the primary mycorrhizae in growth chambers enriched with 5000 ppm CO_2 and fed with a minimal medium. After 20 d of tripartite culture, all plantlets placed in contact with the primary symbiosis were colonized by the AM fungus. As inoculum source, 30-d-old arbuscular mycorrhizal-transformed carrot roots had a substantially higher infection potential than 5-, 10- or 20-d-old AM. Colonized plantlets had more extensive root systems and better shoot growth than control plants. The AM symbiosis reduced the plantlet osmotic potential. This response may be a useful pre-adaptation for plantlets during transfer to the acclimatization stage (Elmeskaoui et al., 1995). However, the problems have been solved partially, if not wholly, by the recent contribution of Declerck et al. (1998), who developed monoxenic culture of the intraradical forms of *Glomus* sp. They isolated AMF, viz. *G. versiforme*, *G. intraradices*, *G. fasciculatum* and *G. macrocarpum*, from the rhizosphere of banana and sugarcane from a tropical ecosystem and successfully cultured them *in vitro* in association with genetically transformed roots of carrot. Several thousand fungal propagules were obtained for *G. versiforme*, *G. intraradices* and to a lesser extent for *G. fasciculatum*, whereas few spores were obtained for *G. macrocarpum*. The intraradical forms of the fungi as mycorrhizal root pieces and single isolated vesicles constituted excellent sources of inoculum for the establishment of *in vitro* cultures and for the continuous culture of the species (Declerck et al., 1998). However, this process seems to be time consuming, tedious and artificial as compared to *ex vitro* inoculation of AMF after transplanting into pots, which is an easy, natural and feasible process, and shows no particular technical problems. Abundant infections may be obtained with 40–50 spores per grapevine plantlet, with an easily implementable method which does not require the sophisticated equipment necessary for *in vitro* inoculation.

It has been observed that often much attention is being paid towards the quality of AMF and, thus the host plant partner is neglected. Rancillac et al. (1996) stressed the need for improvement of the behavior of the host plant. They found a better behavior only with precultivated onion bulbs with a higher concentration of sucrose indicated by longer roots and shoots. However, more thorough research is required in this direction. The use of AMF for practical application is limited by our inability to cultivate them in axenic culture (Safir, 1994). Regarding the axenic culture of the mycobiont, research is still in progress in various laboratories, and success is yet to be achieved.

Tackling the Problem

Mycorrhization to microrrhization. An integrated inoculum approach for enhancement of the growth of the micropropagated plants would be more beneficial as some other microbes will certainly promote the growth of the plant. Lovato et al. (1996) have also concluded that a perspective for the near future should be the development of integrated technologies. 'Not only the mycorrhizal fungi, but also other organisms capable of promoting plant growth or protection, such as, symbiotic or associative bacteria, plant growth promoting rhizobacteria (PGPR), pathogen antagonists, or hypovirulent strains of pathogens would be incorporated into the substrate for micropropagated plant production', they futher added.

Bianciotto et al. (1996) have stated that AMF are a vehicle for the colonization of plant roots by soil rhizobacteria. Von Alten (1998) emphasized use of mycorrhizae-helping bacteria (MHB) for enhancement of growth of the plants. He reported that rhizosphere strains of *Bacillus mycoides* and *Pseudomonas fluorescens* promoted

AMF formation in various crop plants by improving susceptibility of roots to AMF. The role of MHB was studied in detail by Garbaye et al. (1996). The same paper discussed the mechanism involved between interaction of *P. fluorescens* and AMF. Evidently, MHB can also be inoculated into the roots for giving an opportunity to AMF for efficient, profuse and rapid colonization. Vosatka et al. (1992) evaluated the effect of the rhizosphere bacterium *Pseudomonas putida*, AMF, and substrate composition on the growth and development of strawberry. Similarily, Gryndler et al. (1993) reported the possible use of AMF and other rhizosphere microflora in micropropagated plant production. Recently, Siddiqui and Mahmood (1998) found that combined use of *Glomus mosseae* and *Pseudomonas fluorescens* caused greater increase in plant growth of tomato as compared to individual application of both the organisms. Moreover, inoculation of both organisms in combination resulted in much reduction of galling and reproduction of nematodes than when used singly. Andrade et al. (1998) studied plant-mediated interactions among *Pseudomonas fluorescens*, *Rhizobium leguminosarum* and AMF on pea. They found that *P. fluorescens* F 113 enhanced nodulation by *Rhizobium* fourfold. In addition, the nodules produced were much larger and highly pigmented.

With the emergence of molecular biology, sophisticated techniques of recombinant DNA technology are being applied by the molecular biologists to develop more efficient strains of inoculants, particularly for enhanced biocontrol potential. Further, under the EU BIOTECH IMPACT project involving close collaboration among 17 centers, the development of biocontrol strains of *P. fluorescens* with enhanced biocontrol efficiency has been pursued (O'Gara, 1996). The main objectives of genetic modification include antifungal metabolites, such as 2,4-diacetylphloroglucinol, salicylic acid, and iron-chelating siderophores. The genetically modified strain of *P. fluorescens* possesses all the antimycotic metabolites in increased quantity. It exhibits enhanced antifungal activity *in vitro* and has higher potential to fight against fungal invaders, like *Pythium ultimum*, which is a common root pathogen. Such strains of microbes having strong potential could be utilized for effective 'microrrhization' (a new term is being proposed here to designate combined use of inoculants of mycorrhiza and bacteria).

Agrobacterium rhizogenes causing hairy-root syndrome in dicots has been the center of attraction recently for most biotechnologists. The technology is being applied successfully for the promotion of growth of roots and their effective mycorrhization (Mugnier and Mosse, 1987; Becard and Fortin, 1988; Declerck et al., 1998). The bacterium *A. rhizogenes* is also a genetic vector. It causes the transfer and integration of part of its DNA into plant cells. This results in the production of genetically transformed hairy roots at the infection site. The fast-growing roots offer an ideal and easy opportunity for mycorrhization *in vitro*. *Agrobacterium rhizogenes* was successfully established in grapevine (Gribaudo and Schubert, 1990; Gribaudo et al., 1995) The 'hairy-root technology' is gaining momentum (Mencuccini et al., 1991, 1999). Becard and Piche (1993) have aptly remarked that essentially, transformed root systems colonized by AMF spores could be, for mycorrhizologists, the 'white mouse' for analytical studies involving AMF and their responses to different hosts and environmental stimuli.

Besides the hairy-root technology, progress has been made towards inoculation of actinorhiza. Recently, Mark et al. (1999) developed an *in vitro* culture of AMF and *Frankia* for inoculation of *Casuarina equisetifolia*. However, there is a greater need to study the *in vitro* and *in vivo* interactions taking place among inoculants so that their antagonistic/synergistic effect may be evaluated before application of this technology on a large scale.

Search for New Growth Boosters

In order to obtain enhanced growth of the micropropagated plants and save them from transplant shock, dark septate endophytes (DSE) could also be screened to search for growth-promoting endophytes. DSE are sterile or conidial fungi belonging to the Fungi Imperfecti or Deuteromycotina. They have been reported for nearly 600 plant species representing about 320 genera and 100 families (Jumpponen and Trappe, 1998). An additional advantage of these fungi is that they are root-colonizers and can be cultivated easily on artificial culture medium. Recently, Ari and Trappe (1998) discussed the ecological role of different DSEs. Sieber et al. (1998), working on DSE in general and DSH (dark septate hyphomycetes) in particular for the past two decades, suggested that fungal endophytes are ubiquitous in trees, and therefore they should be screened for obtaining more and more DSH. Eventually, the most efficient strains of these fungi should be selected for enhancing plant growth.

A New Hope

Recently, Verma et al. (1998) discovered *Piriformospora indica*, a new fungal endophyte, which belongs to hyphomycetes (Basidiomycota) from sandy desert soil of Rajasthan in north-west India. It can easily be cultivated on various synthetic media (Verma et al., 1998; Varma et al., 1999). The molecular analysis of the fungus revealed that it is closely related to the *Rhizoctonia* group. The typical pear-shaped chlamydospores were found to be efficient in successfully colonizing plants like maize, tobacco and tomato in pot cultures. The hyphae generally colonize the surface of the roots and later (about 2 wk), the cortex of the plant. The fungus seems to be promising due to its rapid root-colonizing capacity and cultivable nature (Varma et al., 1999). A number of experiments are being done in different parts of India on the capacity of colonization of the fungus on tropical timber-yielding plants, fodder-yielding tree species and medicinal plants.

Bringing the Technology from Laboratory to Field

The microbial technology is receiving increased attention from industry, for instance, Cargeeg (1992) developed Vaminoc™, a new horticultural inoculant which contains AMF. Similarly, the 'Biorize' company of Dijon, France has set up its own strategy to demonstrate the wider use of AMF inoculum in the nursery. It was established in 1995 by Blal (personal communication). Blal (1998) produced inoculum of AMF referred to as 'Endorize', which can be used easily with convincing success in various fields of plant protection, such as acclimatization of micropropagated plants in soilless media, transplanting into disinfected and non-disinfected soils, compost-grown crops, etc. Recently, a new mycorrhizal powder, 'Bio Blend' has been launched by Bio-organics of Camarillo, California. It can be used on a variety of fruit and nut-trees as well as grapevines, a high dollar crop (Eddy, 1999).

The 'know-how' of the technology should also be transferred to

other developing countries so that eventually they could become independent and self-sufficient in microbial technology.

Conclusion and Future Directions

At the end of the 20th century, we have been able to develop modern biotechnologies concerning micropropagation and mycorrhization with proven impact. These technologies coupled with bacterization would be more meaningful to 'biotize' *in vitro*-propagated plantlets for transient transplant shock during acclimatization (Nowak, 1998). The modern 'hairy-root technology' has played a significant role for establishment of AMF with transgenic host plants. The use of dual technology is promising as micropropagated plantlets are suitable platforms for understanding the mystery of host–endophyte interaction, excessive production of secondary metabolites, heavy metal tolerance, bioprotection, bioremediation and growth-promoting activity. This will also help to unravel impediments for the growth and development of AMF in culture. In order to establish effective host–AM symbiosis, there is an urgent need to screen various AMF capable of rapid root infection and efficient transfer of nutrients. An integrated inoculum approach by using mixed cultures of microorganisms would be more effective than a single fungus. Moreover, an inoculation protocol should be developed for each plant species. There is a resurgence of interest in pharmaceuticals due to high demands for plant-based products, like Taxol® and Artemisinin®, which are the current generation of drugs. The microbial technology of mycorrhization of tissue culture plants would provide new insights into the excessive production of biologically active secondary metabolites. The technology can also be applied for the conservation of rare and endangered plants in general, and medicinal plants in particular, by *in vitro* propagation and inoculation of growth-promoting microbes. Usually, much attention is paid to the improvement of the quality of the fungal partner, while the behavior and improvement of the plant partner is overlooked. Considering the importance of this aspect, it will be more fruitful to improve the quality and behavior of the plant partner for establishment of healthy symbiosis. The technology offers an opportunity to apply molecular approaches to understand host–symbiont interactions, secretion of flavonoids, and signal-transduction pathways. There has been increasing interest among the users. However, generation of awareness among the public is necessary to achieve the target. The transfer of the technology from laboratory to the field will be a pragmatic approach for the benefit of the cultivators. It is believed that in the 21st century, the technology will flourish with its high promise and far-reaching positive consequences.

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