Proc. Indian natn Sci Acad.(PINSA) **B66** Nos4 & 5 pp 223-238 (2000)

Mass Culture of Arbuscular Mycorrhizal Fungi and Their Role in Biotechnology

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(Received on 30 September 1999; after revision 13 July 2000)

Despite being aware of potentiality of arbuscular mycorrhizal fungi in agriculture, forestry and horticulture research, it is not been able to use on a commercial scale because of the biotrophic nature of arbuscular mycorrhizal fungi. For the commercial development of AM inoculants, a number of strategies has been followed time to time with their own merits and demerits. Currently, two systems are available - soil and soilless technologies. As far as soil based systems are concerned, they are cost effective with low inputs, thousands of infectious propagules can be extracted in a gram of soil. Main drawbacks associated with soil systems are the bulk amount, vulnerability of pest infestation and nutrient management. To rule out these problems, soil less technology i.e. hydroponic, aeroponic, root organ culture were developed. Hydroponic system provides nutrients to the plant in the form of thin layer on the roots which results in greater proliferation of roots, production of higher number of spores/cm of infected root. It also reduces the chances of pest infestation and isolation of roots and spores is easier. However, in aeroponic system, nutrients are provided in the form of evenly distributed fine mesh. This system reduces the risk of contamination and gives higher sporulation but it is a costly affairs. Many attempts have also been made to grow this fungus on defined and complex medium but they could only support the hyphal growth. Use of Riplasmid transformed roots is a better alternative to grow the biotrophic organism aseptically on plate because AM fungi depend on the host cell for specific metabolites.

Key Words: Mass Culture, Arbuscular Mycorrhizal Fungi, Biotechnology

Susceptibility of plants to parasites is considered a relatively rare phenomenon in nature, however, a striking exception to this rule is presented by mycorrhizal associations. Mycorrhizae, a term describing a range of mutualistic associations between soil fungi and plant roots, is no doubt the most frequent example of compatibility between plant and the microbes. The mycorrhizal habit has a long evolutionary history (Nicolson 1975, Boullard 1979) and today more than 90% of all plant taxa, ranging from thallophyte to angiosperms, form associations of one type or another with mycorrhizal fungi. Only a small number of plant species belonging mainly to Cuperaceae, Chenopodiaceae, Juncaceae and Proteaceae are reported to be non-mycorrhizal or show restricted susceptibility to the mutualists. Mycorrhizal fungi have now been reported in Cruciferae as well (Brassicaceae) however, absence of arbuscules suggests that arbuscular mycorrhizal (AM) association is non-functional and that the development of AM infection is primarily the consequence of progressive root senescence (De Mars & Boerner 1995). Amongst different mycorrhizal fungi, AM are the most important because of the wide range of benefits accrued to the host. Although, AM fungi are ubiquitous in soil, they exhibit a strong biotrophic dependence on their host plant.

While the term biotechnology has been used for the last two decades, major advances have generated new set of tools, which allow exploiting the biological resources much more meaningfully. These tools of modern biology represent an

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integration of advances and techniques that fall largely in the areas of biochemistry, cellular biology, molecular biology, genetics and microbiology. One such biological tool that is now being integrated into biotechnology is the development of commercial AM inoculants for use in agriculture, horticulture, forestry and environmental reclamation. Basic studies have demonstrated that mycorrhizal associations play an important role in plant nutrition. The plants colonized by AM fungi harbour greater amount of phosphorus and other trace elements specially when these are sparingly soluble (Abbott & Robson 1984). AM infected plants also exhibit improved resistance towards drought, environmental stress and some root pathogens (Nelson & Safir 1982, Allen & Boosallis 1983, Graham 1986, Sharma et al.1992, Goicoechea et al. 1995).

Arbuscular mycorrhizal fungi have extremely broad host range. Given this fact and their functional attributes, AM fungi hold considerable potential for use as inoculants to improve plant production in agriculture, horticulture and forestry (Ferguson 1984, Mohammad et al. 1995). Commercially important benefits that can be derived from their use include increase in plant growth and yield (Sharma & Srivastava 1991), improved crop uniformity (Aguirre-Medina & Velazco-Zebadua 1994), reduction in phosphorus and trace metal fertilizer requirements (Menge et al. 1978), reduced losses due to environmental stresses and root diseases (Sharma et al. 1992, Loth & Hofner 1995), reduced stunting in fumigated soils (Kleinschmidt & Gerdemann 1972), improved transplant establishment (Menge et al. 1978) and reduced cropping times (Saesa et al. 1987). All of these benefits translate into increased profits for farmers and nurseryman, suggesting a significant commercial potential for AM inoculants.

Despite the potential of AM fungi, inoculum production has been a major limitation. A complex of factors are associated with this dilemma including lack of suitable and cost effective technologies, constraints of product efficacy and market needs. In addition, while AM inoculation has had significant impact in agriculture, horticulture and forestry the efficiency has been restricted to a narrow spectrum of conditions and this too is coupled to, relatively inconsistent field performance.

Realising the fundamental importance of AM association for plant structure and function the potential of this significant fungal association still largely remains in the laboratory. This article will discuss the ways (present and future) in which current advances in biotechnology can be applied towards expanding the commercial utilisation of AM fungi in agriculture, horticulture and forestry.

Inoculum Production Strategies

Two major systems currently available for inocula production are, soil and soil-less technologies. Both the methods have their own limitations, i.e. separation of roots, spores and hyphae from substrate is difficult from soil based medium besides vulnerability to pest infestation and complex interactions related to plant nutrients especially P with the soil substrate. Hydroponics does not allow as much sporulation as is needed to result in greater spread of the organisms throughout the culture since the roots are immersed in a common flowing solution. Root organ culture whether with transformed or non-transformed roots is expensive, labour intensive and does not even approach the levels of sporulation in traditional pot cultures (Sylvia & Jarstfer 1992). However, this technology is workable with tissue culture raised seedlings since they can not withstand field stress without microbial community. Aeroponics is a comparatively better method which can eliminate the above mentioned problems but it requires a real trained personnel since every component of the working system has to be properly controlled for the desired commercial benefits. However, in the absence of other alternatives, these systems are the only ones available and various modifications are used depending upon the needs and availability of infrastructure.

Soil Based System

Since the very beginning of mycorrhizal researches, this method has been adopted for multiplying different AM strains for increasing *in situ* propagule number (Menge 1984). However, in view of the non-specificity of the AM-plant association (Harley & Smith 1983), considerable variation in AM development and its effects on host plant occurs (Smith & Gianinazzi-Pearson 1988). For example different pearl millet and maize varieties show

Table 1a Genotype dependent variation in mycorrhizal colonization of pearl millet grown at three field locations (Krishna et al. 1985)

Genotype	Soil		
	A	B	C
IP 3277	15	11	51
IP 3476	13	20	49
IP 5781	16	32	39
IP 3595	22	10	56
IP 5150	24	30	35
IP 4382	15	22	56
IP 6891	25	32	40
IP 6045	21	33	41
IP 6114	25	28	49
IP 4807	23	28	57
IP 5427	26	27	56
IP 5335	22	33	55
WCC 75	17	23	73
IP 6590	33	40	42
IP 6139	28	35	53
IP 4861	22	25	70
ICH 220	30	36	52
BJ 104	27	34	58
MBH 110	23	30	67
IP 5420	15	42	62
IP 3840	27	42	52
IP 3120	25	26	77
IP 5692	23	33	70
IP 5306	22	44	70
IP 5009	23	47	74
IP 5310	44	43	61
IP 4937	30	50	66
IP 6538	33	41	74
IP 5140	37	49	77
IP 5921	42	59	67

A = Low fertility not cultivated previous two seasons, Olsen's P 3.4 ppm; *B* = Low fertility: cultivated previous season, Olsen's P 5.0 ppm; *C* = High fertility – under long term cultivation, Olsen's P 20.0 ppm.

Table 1b Genotype dependent variation in mycorrhizal colonization of maize (Toth et al. 1990)

Maize inbred	Mycorrhizal Infection
Va 58	6.6
OH 43 N	7.7
P 37 H	5.1
H 95	3.8
H 99	11.5
W 153 R	16.4
WF 9	9.6
W 117	11.3
Oh 07 B	7.9
Oh 51 A	14.3
W 629 A	18.0
W 64 A	27.1
A 509	8.2

Table 2 Effect of different AM isloates on mycorrhizal root colonization of alfalfa (O'Bannon et al. 1980)

AM isolate	Source	Rating
G. epigaeous	Oregon	2.2
G. fasciculatus	Oregon	2.1
G. fasciculatus	Florida	2.3
G. mosseae	Oregon	2.0
G. mosseae	Florida	1.7
G. trappei	Oregon	1.8
G. monosporus	Oregon	2.2

Rating scale O = none, 1 = 1 – 30%, 2 = 31 – 60%, 3 = >60%

varying degree of root infection (tables 1a,b & 2). It has been suggested that the AM development and its influence on the host is atleast partially under genetic control (Gianinazzi-Pearson et. al. 1996), although reverse is also true. Secondly, mycorrhizal development is affected by nutrient availability in soil and with the inoculum potential of AM fungus. These three parameters are discussed below in some detail since better mycorrhization of the host reflects at greater possibility of inoculum production in return.

Host

Dependency of those plant species with relatively less endosperm tissue on AM fungi is well documented (Harley & Smith 1983). Hayman (1982) showed a doubling of the growth of *Stylosanthes* by AM in a soil with 40 ppm Olsen P where normally mycotrophic leucerne did not respond. Azcon and Ocampo (1981) reported different degree of mycorrhizal infection in 13 varieties of wheat under similar soil conditions. In an interesting observation, Vierheilig and Ocampo (1990) reported that wheat cultivars inoculated twice resulted in better infection. A number of papers have since appeared on this aspect and the subject has been reviewed (Hayman 1982, Hall 1988, Bethlenfalvay 1992). Host plants that can be propagated from seeds are preferable than cuttings since seeds can be more readily disinfected than the cuttings. In order to obtain higher sporulation of AM fungus in soil, it is important to choose a crop with profused root growth so that chances of infection increase; use of monocots like maize, sorghum etc is a common practice for soil based inoculum production. Mehrotra (1996) reported *Paulownia* as a host to strengthen containerized seedling production in forest nurseries. Thus, it is important to select the nurse plant carefully on the basis of such criteria such as rapid infectibility by AM species, ability to produce abundant root biomass within short time and inherent resistance to diseases and insects.

Trap culturing

For the isolation of AM fungi, it is important to multiply in bulk. The following protocol is generally used for this purpose: First of all the site of interest is marked depending on the requirement. The susceptible plants mainly grasses, cereals, legumes are taken out with the root and rhizospheric soil without disturbing the extramatrical mycelium. The shoot of the plant can be removed and placed in the earthern/plastic pots. The sterilized seeds of cereals, vegetables, ornamental plants, legumes can be sown in these soil. Two consecutive cycles of 3 months each are required to get better sporulation if the soil is not very rich in mycorrhizal spores. Spores then can be isolated, identified and sterilized and subsequently used for infecting the germinated seeds of grass or maize or vegetables. As soon as the roots of the germinated seeds are infected by the spores, these are then transferred carefully to the sterilized soil – sand (1:1). Initially watering should be done cautiously. Three to four cycles of 3 months each are enough to get sufficient number of infective propogules.

Soil/Nutrients

It is important to consider the physical and chemical status of the soil, besides nutrient concentration in the growing medium because both affect AM growth. It has been established beyond doubt that a nutrient poor soil favours mycorrhization of the root system (Bethlenfalvey 1992). The amount and solubility of P affects mycorrhization (Read & Smith 1997); a range of 0.01 to 0.02 mg P ml^{-1} is optimum not only to support plant growth but also for AM infection. Although the role of acquiring N compounds from soil by AM fungi is still not very clear, available evidence suggests that these fungi increase the uptake of NO_3^- and NH_4^+ from soil. Habte (1990) reported that ammonium form was particularly toxic and levels that were lower than 400 ppm N, did not have any adverse effect on AM colonization.

The optimum moisture for AM growth in soil has been reported between 0.1 to 0.2 bars (Habte 1990). Photosynthate supply, root exudation, light and soil temperature are to be considered critically for effective mycorrhization and greater sporulation.

It is essential to disinfect all the components of culture system prior to initiation of a culture of AM fungi; this includes containers, spores and soil. Host disinfestation is generally accomplished more easily than disinfestation of the soil. In general, washing of spores with chloramine T (2%) and streptomycin (200 ppm) is sufficient to

achieve the desired results (Schenck 1982), however it is essential to standardise the method since each fungal strain varies by way of spore wall structures and thus this could be a source of non-pathogenic contaminating micro-organism. The objective of soil disinfestation is to kill any existing AM fungi, pathogenic organisms and weeds. Formaldehyde and methyl bromide are most frequently used chemicals for soil sterilization; these have been shown to be partially effective in killing the pathogens but are not known to alter the chemical constituents of the soil. Steam sterilization, however, alters the chemical composition of the soil. Plazzo et al. (1994) demonstrated an adverse effect of soil sterilization on *Citrus aurantium* L. Therefore, a suitable technique has to be developed to realize maximum benefit after soil sterilization. Sylvia and Jarstfer (1994) pasteurized soil by heating at 85°C twice for 8hr, 48hr apart to achieve balanced chemical status of the soil.

The containers used for pot cultures should be shielded from contaminated soil, splashing water and crawling insects. In addition, specific isolates of AM fungi should be kept well separated to reduce cross-contamination. The container size should match the potential volume of the root system within practical space constraints; large containers have been shown to result in higher spore population (Ferguson & Menge 1982).

The quality of light and irradiance, soil water content, and temperature markedly influence root colonization and spore production (Menge 1984, Price et al. 1995). Non-shaded greenhouses or high intensity metal-halide and sodium vapour lamps used for supplemental light give good results, provided that adequate cooling is maintained (Sylvia & Jarstfer 1994). A moderate watering regime often supports optimal spore production (Nelson & Safir 1982). Our own experience suggests a well drained container and soil as an appropriate system. Since most AM fungi sporulate between 15-30°C, a warm environment supports better propagule number (Schenck & Smith 1982). Soil temperature is generally considered to be more important than air temperature for AM development (Habte 1990). Use of pesticides is quite common in the production of soil based AM fungal inocula (Graham 1986). However, several fungicides affect the sporulation adversely (Trappe et al. 1984) while others are reported to be synergistic (Sreenivasa & Bagyaraj 1989). The use of systemic fungicide propamocarb hydrochloride (propyl [3-(diemethyl-amino) propyl carbamate] was specially useful for the protection of inoculum against *Pythium*, which is one of the commonest contaminants. Our experience shows that a carbendazim product is better than others (Chaturvedi et al. 1989). Storage of AM fungal inoculum has also been a problem. Douds and Schenck (1990b) have suggested that the inoculum can be dried *in situ* with the host and frozen at -70°C. Addy et al (1997) showed that AM hyphae kept under frozen condition were effective even after one month.

Phosphorus and nitrogen fertilization are strain dependent for AM colonization and sporulation. Douds and Schenck (1990a) reported an increase in sporulation by increasing N-fertilizer; however, the sporulation of *Glomus intraradices* was severely affected by N-fertilization. Phosphorus fertilization, a major concern for inoculum production, severely affects germ tube growth and sporulation (Same et al. 1983, Suriyapperuma & Koske 1995). Our own experiences suggest that the pots, provided with 4ppm P or simple tap water produced more spores of *Glomus macrocarpum* on maize than the other nutrient regimes. More research is however, needed in the direction of the management of the level of micronutrients specially Ca, B, Zn, S, Fe which influence plant growth and hence, mycorrhizal development in roots. Secondly, a proper harvesting time for the host plant is also essential so that maximum spore production can be achieved in a short duration rather than waiting for the maturation of the crop. Vilarino et al. (1992) demonstrated better sporulation and higher amount of extramatrical hyphae in red clover when the aerial parts of the host were removed. They have suggested that the frequency of removal of aerial parts and environmental condition are important in achieving maximum inoculum production. Application of synthetic formononetin to soil with AM fungus increased the root

colonization and density of arbuscules and vesicles (Silva-Jr. et al. 1997). Use of formononetin appears to be an interesting approach for the production of soil based AM inoculum.

Vermiculite, perlite, sand or a mixture of these with only a small proportion of soil have generally been used for multiplication of AM fungal inocula. A new phase in inoculum production began with the use of calcinied montmorillonite clay as a supporting medium. This approach, used in conjuction with complete Hong Ashton mineral solution, proved a good substrate for plant growth and abundant mycorrhizal infection, eliminating the need for soil in the growth medium; another material used was expanded clay called 'Blahton' (Dehne & Backhaus 1986). After sterilization plants were raised and fed with complete nutrient solution containing (in mg/l) N 800, P_2O_5 800 and K_2O 600, applied twice weekly. After three months growth of host plants and root inoculum the Blahton particles were strongly colonized by AM fungi. After air drying and storage at room temperature for one year, they retained 75–100% infectivity (Dehne & Backhaus 1986).

In order to minimise large scale use of inocula especially in field crops , efforts have been made to build up the native consortium of AM fungi by manipulating the management practices used in the crop production . However, it is important to assess the impact of these practices on AM fungi for better manipulation and hence greater advantage from the symbiont to the host plant.

Soil-Less Media

To eliminate contamination from soil organisms and for better control of physical and chemical properties, AM fungi have been cultured in soil-less media; such techniques provide greater uniformity in the composition of the product and facilitate better aeration than soil.

At least fifteen species of AM fungi from four genera have been cultured in soil less media (table 3). The ideal soil-less mixture should hold sufficient water for plant growth and simultaneously permit good aeration. Bark, calcined clay, expanded clay and perlite provide good aeration. Peat and vermiculite hold more water than these materials but allow air to penetrate better than sand. Dry perlite and vermiculite are very light while sand has high bulk density.

The availability of P in most soil-less media requires judicious management of nutrients. Frequent addition of dilute soluble nutrient solutions (Waterer & Coltman 1988, Douds & Schenck 1990a), incorporation of time released fertilizer (Coltman et al. 1988) or use of less-available forms of P (Thompson 1986) are the general strategies tested for nutrient management in soil-less media during *Glomus aggregatum* culturing.

Nutrient Film Technique

The possibility of pathogenic organisms contaminating mycorrhizal inoculum is an extremely serious problem when multiplying AM

Table 3 *Soil less media used in the culture of arbuscular mycorrhizal fungi (Jarstfer and Sylvia, 1992)*

Material	AM Fungi
Bark-shredded douglas fir	*Glomus fasciculatum*
Calcined Mont-morillonite clay	*G. Macrocarpum, G. monosporum, G. versiforme, Scutellospora calospora*
Expanded clay aggregates	*Acaulospora laevis, G. constrictum, G. fasciculatum, G. intraradix, G. macrocarpum, G. mosseae, Scutellospora pellucida*
Peat	
Hypnum	*G. fasciculatum*
Sphagnum vermiculite + pumice (2:1)	*A. spinosa, G. fasciculatum* +perlite and *G. mosseae, G. intraradix, G. fasciculatum, Gigaspora mgarita, G. teune*
Perlite	*G. fasciculatum*
+ soilrite (1:1)	*G. Fasciculatum*
Sand	
Basaltic	*G. aggregatum*
River	*A. spinosa, G. Fasciculatum, G. mosseae*
Silica	*G. fasciculatum, G. mosseae*
+ vermiculite (3:1)	*G. mosseae, G.etunicatum, G.fasciculatum*
+ grit (2:1)	*G. mosseae, G. fasciculatum*
Vermiculite	*A. spinosa, G. fasciculatum, G. mosseae*

fungi in semi-sterile conditions in greenhouse. Although any host used in the nutrient film technique (NFT) should be first grown in the soil substrate with AM inoculum to get the initial infection, NFT is one of the probable answers which can eliminate the problem of contamination; also, the biomass of roots produced in NFT is quite high and, therefore, it appears as an alternative to production technologies for AM fungi.

NFT is a specialised technique developed for the commercial production of crops such as tomato, lettuce, merigold, napier and cucumber. It entails continuous recycling of a large volume of nutrient liquid in a film which flows over the roots of plant. MacDonald (1981) used a compact autoclavable hydroponic culture system for the production of axenic AM fungus between *Trifolium parviflorum* and *Glomus caledonicum*. Since then, a number of papers have been published in this area of research, (Mathew & Johri 1988, Howeler et al. 1982, Mosse & Thompson 1984, Elmes et al. 1984, Elmes & Mosse 1984).

Besides the variation of techniques used in aseptic inoculation of AM fungi to the host plant, the major concern in the NFT system is the concentration of nutrients. The preferred values for the various nutrient elements vary from one particular mycorrhizal system to another depending particularly on the size and other features of the plant. For example, maize requires a higher level than beans whilst beans require a higher level than lettuce. However, the following values in ppm are considered as reasonable representative of the NFT system. N, 110; Mg, 48; K, 200; Ca, 160; S, 200; P, 30; Fe, 3; B, 0.5; Mn, 0.5; Zn, 0.05; Cu, 0.02; Mo, 0.01; Co, 0.01. These values also depend on the size of the container and the population of plants in the system. Another important factor is the compromise between plant growth and mycorrhizal infection since water logged conditions affect mycorrhizal growth adversely (Tarafdar 1995). It is therefore, necessary to maintain the nutrients in the form of thin film (5mm - 1cm); form of the nutrient elements also affects mycorrhizal infection and therefore it is essential to use a balanced and proper composition. For example, potassium nitrate may be used to supplement both potassium and nitrogen, while nitrogen may be supplemented by foliar feeding. Potassium is also used for the maintenance of *p*H which is generally maintained between 6.0 - 6.8 for several mycorrhizal fungi. As *p*H is drastically lowered because of the K^+ ion uptake by plants in nitrogen-free medium, a proper balance of K^+ and NH_4^{++} ions has to be maintained. The equipment used in the NFT system is presented in figure 1.

We have multiplied AM inoculum dominated by *Glomus* and *Gigaspora* sp. on tomato (*Lycopersicum esculentum*) and african merigold (*Tagetes erecta*) in NFT system. The experience of NFT system suggests that a thin layer of nutrient liquid (<1cm) is appropriate for AM proliferation. The progress of AM infection was monitored employing super oxide dismutase (SOD) as a marker. The SOD activity remains

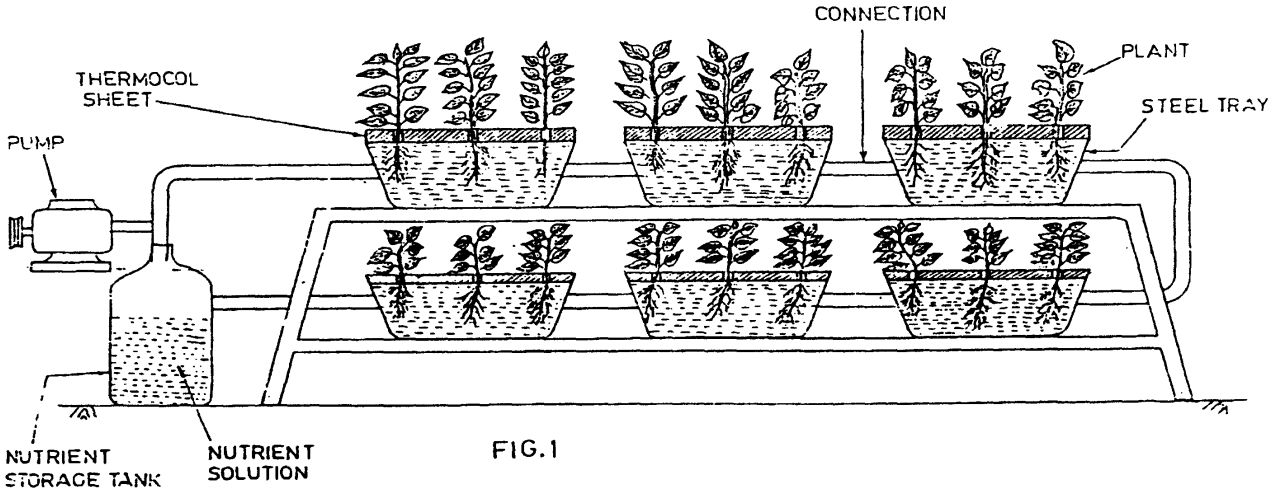

FIG.1

greater in roots at the air water interface because of higher infection level; it decreased in the root portion completely dipped in liquid which also carried a low infection level (unpublished). The composition used in our laboratory is as follows:

Contents	*Concentration (g/l)*
$Ca(NO_3)_2.4H_2O$	1.0
$MgSO_4.7H_2O$	0.512
KNO_3	0.584
KH_2PO_4	0.001
$C_6H_5O_7Fe.5H_2O$	0.027
$MnSO_4.H_2O$	0.006
H_3BO_3	0.0018
$ZnSO_4.7H_2O$	0.002
$CuSO_4.5H_2O$	0.0004
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0002

Merits

Since, a large reservoir is used in NFT system, the maintenance is low and there is no need to continuously monitor *p*H. It is easy to isolate the infected roots and spores.

Demerits

Higher sporulation compared to the soil based system is not expected and because of common nutrient solution, problem of undesirable organisms (rotifers, other protozons and eelworms) is expected from this system.

Aeroponic Systems

A culture system employing a fine mist of defined nutrient solution to the roots of the host plant is termed as aeroponic culture. The mycorrhizal culture has been successfully established on maize, sweet potato, leek and bahia grass using this system (Weathers & Zobel 1992, Mohammad et al. 2000). There are three basic methods for producing atomised nutrient solution: (a) an impeller system making use of an atomising disc (Zobel et al. 1976), (b) pressurised spray through nozzles, or (c) ultrasonically generated fog (Weathers & Zobel 1992). A diagramtic representation of these systems is provided in figure 2 where all these systems mentioned. The basic requirement in aeroponic culture is to make the fine mist of the nutrient solution but the precaution is to standardise the droplet size so that it could stick to the root system for some time. Normally, 45mm droplet size is optimum for this purpose. A dilute, modified

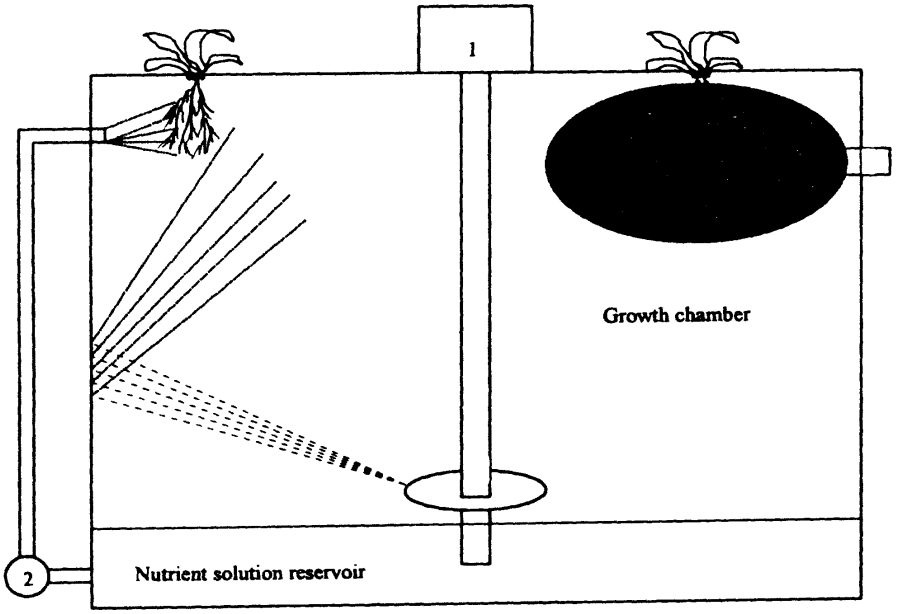

Figure 2 Three ways of producing atomised nutrient solution for the growth of mycorrhizal plants in a flat-bed aeroponic chamber (1) Impeller system for distributing nutrient spray, (2) system with nozzels for producing a pressurised spray, (3) system using ultrasonically generated nutrient for (after Jarstfer & Sylvia 1994)

Hoagland's nutrient solution (Epstein 1972) has been successfully used for the aeroponic cultivation of bahia grass (Sylvia & Hubbell 1986, Wu et al. 1995) and sweet potato (Hung & Sylvia 1988) with various *Glomus* spp. and *Entrospora kentinensis* (Wu et al. 1995). The nutrient solution described by Sylvia and Hubbell (1986) contains the following

Contents	Amount
Macronutrients	
$Ca(NO_3)_2$ .4 H_2O	1.4 μM
KNO_3	1.4 μM
$MgSO_4$.7 H_2O	0.30 μM
$Na_2FeEDTA$	0.03 μM
NaCl	0.05 μM
KH_2PO_4	0.003 μM
Micronutrients	
H_3BO_3	13 μM
$MnCl_2$ $4H_2O$	3 μM
$ZnSO_4$.$7H_2O$	0.2 μM
$CuSO_4$.$5H_2O$	0.08 μM
H_2MoO_4.H_2O	0.04 μM

pH 6.5 adjusted with KOH

Merits

Larger population of spores has been found to be produced in aeroponics than in comparable soil-based pot cultures. Since the colonized root inoculum produced in this system is free of any substrate, it can also be sheared, resulting in very high propagule number (Sylvia & Jarstfer 1992). The population of deleterious organisms can be kept low by frequent nutrient change. Since there is no involvement of soil, extraction of roots and spores is easier in this system.

Demerits

An adaptation period of few days to month for settling down the roots against stress is often necessary since the artificial environment created forcefully, is a disadvantage of this system. Souza *et al* (1996) showed that 28 days adaptation period caused a decrease in root colonization; however they were able to achieve 5082 spores^{-1} g dry wt root of *Entrophospora colombiana* after 70 days. This system is maintenance intensive and the initial cost of equipment is higher than for nutrient flow and hydroponic culture. Although long term use of the equipment could make this system more economic but initial estimated cost is about $500 for a small chamber (Jarstfer & Sylvia 1994).

Axenic Culture of Arbuscular Mycorrhizae

Being biotrophic in nature, AM fungi have only been grown sporadically in a synthetic medium. The principal approach used till date involves systematic screening of defined and complex media for support of hyphal growth and differentiation. Dramatic levels of germ tube growth have been achieved from surface sterilized spores. Extensive fungal differentiation, sporulation and hyphal subculture is still not a routine although considerable progress with abiotic media has been achieved. Further improvements were sought using exudates and extracts from the roots of AM host plants during the earlier phase of axenic culturing (Hepper 1984).

Morandi et al. (1992) showed a differential reponse of isoflavonoids and flavanoids on the spore germination and hyphal growth of *Gigaspora margarita*. The authors suggested that, while working on axenic culture of AM fungi it was important to maintain the concentration of solvent, such as ethanol low, which is used to dissolve these phenolics. If root extracts are to be used then it is better to record the concentration of phenolics in inoculated and uninoculated AM roots. It was suggested that since lower concentrations of isoflavonoids and flavonoids were mostly stimulatory towards spore germination and hyphal growth, use of uninoculated roots could be more promising compared with inoculated ones; high concentration of these phenolics are likely to be more in the latter due to triggering of the resistance mechanism of plants by AM infection.

Relatively little progress has been made during the last three decades using axenic approach which has led to various speculations concerning non-nutritional foundations for the obligate symbiosis. Major hypothesis include the followings, (i) Permeability barriers in AM germ tubes and hyphae which prevent nutrient uptake

except across arbuscular membranes (Millner 1988); (ii) AM fungi lack genomic instructions for critical enzymes or biochemical pathways, and thus depend on host cells for specific metabolites or metabolic processes; (iii) AM fungi are genetically competent but genes critical for growth and differentiation have to be activated through association with the host plant. The activation may involve chemical triggers. Hence, dual culture technique employing root organ culture was developed using roots of the host plant and surface sterilized AM spores. Ri-plasmid transformed root cultures offer an efficient method for growing colonized roots as no plant growth regulators are required for sustained growth (Mugnier & Mosse 1987). Presence of specific polypeptides in mycorrhizal roots established a clear cut feasibility of this method for adoption since these polypeptides have not been traced in the uninoculated roots (Simoneau et al. 1994). Nitrogen and sucrose supply (Mugnier & Mosse 1987), P concentration (Becard & Fortin 1988) and *p*H (Mosse & Hepper 1975) have all been shown to influence the spread of AM fungi in cultured roots. Looking at the available information, one can conclude that a compromise between adequate nutrients for rapid root growth and nutrient concentrations that did not inhibit fungal spread to the new roots, is a necessary prerequisite of root organ culture.

With continued exercise in the sphere of root organ culture, several systems have been designed of which some have been patented (Menge 1984, Dehne & Backhaus 1986, Mugnier & Mosse 1987). However, inspite of these developments, this activity with AM fungi is yet to reach a stage where real commercial benefits in agriculture and forestry could be capitalized. Present success employing tools of modern biology appears to indicate a bright future.

The failure of the presently available inoculants to attain widespread commercial use is also coupled to the issue of high costs, limited efficacy and limited grower needs. Therefore, in order to make mycorrhizal technology a reality biotechnological approaches will have to provide answers not only for these limitations/but will have to play a vital role. The possible strategies include, (i) development of improved culture system for AM fungi to reduce inoculum production cost, (ii) development of multiagent inoculants to expand breadth of efficacy, and (iii) development of genetic transformation system to introduce new traits of significant commercial interest to AM fungal strains.

Improved Culture System

At present, biotechnology does not provide any immediate solution for culturing AM fungi, but it does provide a precise tool to facilitate study of their biology. For example, monoclonal antibodies (MCA) can be developed against the specific membrane proteins of AM spores which would help in understanding the permeability factor. Proteins can be extracted from the saprophytic fungi that are closely related to the AM fungi. MCA's could be developed against it, tagged and used to probe the membranes of germ tube, arbuscules and hyphae of AM fungi. A significant number of proteins can help in identifying the role of a particular protein in AM system. Proteins of interest could also be sequenced and compared with sequences in data banks to search for a particular protein and their specific function in AM fungi.

In a study of pea mutants, it was observed that the growth and appressorium formation of AM fungus was not inhibited on the root surface of even myc pea mutants while it stoped on non-host plant (Gollote et al. 1993). This indicated that the effect was due to genetic response from the host plant rather that the fungus. This study suggested probable involvement of a number of genes. It was envisaged that plant sensor genes determine a specific binding site for the fungal elicitor at the root surface. Binding product could lead to activation of integrators/ symbiosis genes, the products of which interact or interfere with the expression of receptor/ defence genes. Understanding of this model could be an important aspect to break barriers in completing cycle of sporulation of AM fungus *in vitro*.

Complementary DNA (cDNA) could be produced from the messenger RNA extracted from the germinated fungal spores/hyphae and

then cloned for amplification. Hybridization techniques could then be used to identify RNAs that are present and hence genes that are active in the symbiotic state as opposed to the asymbiotic state. The cDNA specific to those RNAs could be sequenced and compared with sequences in gene data banks in an attempt to identify gene product and their functions. This study can provide the clues as to which gene is being activated as a result of mycorrhizal formation and which cell function is lacking in the symbiotic state.

Development of Multiagent Inoculum

Despite the potential of AM fungi in enhancing nutrient uptake, stress tolerance etc. the monoculture of these organisms is not often enough to achieve maximum benefit for the plant. It is, therefore, necessary to build up a multiagent inoculum consisting of growth promotory microbial consortia.

Phosphate solubilizing bacteria and fungi including species in the genera *Agrobacterium, Bacillus, Pseudomonas, Aspergillus* and *Penicillum* are one class of organisms that have been included in multiagent inocula. Tarafdar and Marschner (1995) reported increased biomass of *Triticum aestivum* and better solubility and uptake of organic phosphorus when *Aspergillus* and *Glomus mosseae* were used together. Barea et al. (1975) and Raj et al. (1981), both showed that phosphate solubilizing bacteria maintained higher population for longer periods in the rhizosphere of mycorrhizal as opposed to non-mycorrhizal plants. In addition, some phosphate solubilizing bacteria and fungi have been shown to produce hormones, vitamins and other growth factors that stimulate mycorrhizal development and plant growth independently of the phosphorus effect (Bagyaraj 1984).

The legume-*Rhizobium*-mycorrhiza interaction is a well documented case of growth promotory microbial interaction (Barea & Azcon-Aguilar 1983, Bagyaraj 1984, Sharma 1990). Increased biomass through enhanced phosphorus uptake and increased activity of rhizobia through AM fungi has also been reported in *Leucaena leucocephala* by Aguirre-Medina and Velazco-Zevadua (1994). Jha et al. (1993) reported an increased activity of nitrogenase, nodule weight and nodule nitrogen content in *Alnus nepalensis* when inoculated with *Frankia* and *Glomus mosseae*. Bagyaraj and Menge (1978) inoculated tomato with *Glomus fasciculatum* and *Azotobacter chroococcum* and found beneficial influence; mycorrhizal colonization increased and stabilized the *A. chroococcum* population in the rhizosphere, whereas the bacteria, in turn enhanced AM colonization and spore production.

Selected strains of plant growth promoting rhizobacteria (PGPR), *Pseudomonas fluorescens, P. putida* and *P. syringe* isolated from root surfaces and rhizosphere soils, have been used as soil and seed inoculants to promote growth and yield of canola, potato, radish, rice, sugarbeet and wheat (Schroth & Weinhold 1986, Kloepper *et al* 1988). The mode of action is thought to involve suppression of pathogens via the action of siderophores (Leong 1986) as well as production of plant growth regulators (Kloepper et al. 1988). Contrary to this, an additive effect on fresh and dry weight, nitrogen and phosphorus content and alkaline and acidic phosphatases was noticed in maize plants when *Glomus mosseae* was used in combination with *Pseudomonas* in our laboratory. Therefore, it is necessary to find out the most suitable microorganism which would not only influence the plant growth but would also have a better adaptability with the mycorrhizal fungus.

Efficacy Through Genetic Transformations

Different approaches for manipulating AM fungi are possible for developing their: (i) ability to suppress root disease (ii) tolerance to specific fungicides, (iii) production of *Bacillus thuringensis* (Bt) endotoxin to suppress root damage caused by insect larvae, and (iv) production of antinematode agents and others.

Hyphal Anastomosis

AM fungal spores are known to possess multinuclear status but whether these nuclei have multiple copies of the same genome or not is still uncertain. Looking at the diversity of AM efficacy for similar species at different places, an indication of phenomenon of some kind of sexual reproduction looks probable but no specific details are yet available.

The hyphae arising from the spores of a particular strain when germinated on filter paper or 2% agar fuse or anastomose. It is however, not known whether any exchange of cytoplasm or nuclei occurs during anastomosis. However, possibility of genetic recombination through this process can not be ruled out.

Based on the above hypothesis it is possible to surmise, that two strains of AM fungi can be germinated on filter paper and checked for anastomosis. If anastomosis is confirmed, then these strains could be cultured together. Since PCR techniques are successful in amplification of small amount of DNA (Clapp & Simon 1995), the parent and progeny could be analyzed for genetic recombination. The next would be to search for inter or intra specific crosses, in order to develop a superior strain of AM fungi.

Conclusion

Till the time, we are not able to culture the AM fungi on synthetic media, we are bound to use the normal cultural practices but efforts should be made to propogatge the fungi in the field itself and try to explore the possibility of using indigenous consortium if it is suitable for supporting the better growth of desired crop. For horticulture and forest crops where the AM fungi is needed at nursey stage, the extraneous inoculum can be propogated by means of soil or soilless techniques but with the precaution so that there is no contamination of any pathogenic microbes. Use of molecular tools can further strengthen our knowledge to make the inoculum of these fungi on mass scale.

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