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Review paper

The state of art of mycorrhizas and micropropagation

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Abstract: Plant micropropagation has an outstanding place in biotechnology industry. Plant production through this technique can benefit from the utilisation of mycorrhiza, the mutualistic association between plant roots and fungi. Mycorrhizas can act as bioregulators, biofertilisers and bioprotectors, making possible the production of healthy high-quality plants with low chemical inputs. Research data in this field is presented, management procedures are suggested and potentialities for the joint use of micropropagation and mycorrhizal biotechnologies are discussed.

1. Introduction

Plant micropropagation has an outstanding place in the biotechnology industry. The total number of plants presently produced by tissue culture in Europe is around 200 million units a year, of which over 180 million are produced in commercial laboratories. Assuming a unit price of 0.3 European Currency Units (ECU), the commercial production can be evaluated, for Europe alone, at more than 54 million ECUs per year (O'Riordain, 1992). An equivalent amount of plants is produced in Eastern Asia and in North America (M. Rancilla, personal communication). Micropropagated plants vary from flowers, like gerbera, lilies, roses and begonias, to rootstock for fruit trees like apple and cherry, or forest trees like wild cherry and ash. Ornamental plants have a leading position along with small fruits like strawberry, but fruit trees like citrus and pear are also of importance (Table 1). Micropropagation is being extended to many tropical plants. Banana (*Musa spp.*) is the most important species multiplied by this technique, with a production of more than 40 million microplants a year throughout the world (J. Marchal, personal communication). The majority of these plant species naturally form arbuscular mycorrhizas, some of them form ericoid (*Rhododendron, Kalmia*) or orchidoid (*Orchidaceae*) endomycorrhizas and a very limited number form either ectomycorrhizas (*Betula*, as well as *Picea* and *Juglans* which are not indicated on the table) or no mycorrhizal association at all (*Beta vulgaris*).

Table 1 - Major plant species (x1000) produced by micropropagation in Western Europe (O'Riordain, 1992)

Gerbera	18383	Vriesea	2805
Nephrolepis	14517	*Orchidaceae*	2162
Prunus	10725	*Philodendron*	2138
Spatiphyllum	9827	*Citrus*	2061
Lilium	7112	*Pyrus*	1805
Fragaria	7040	Begonia	1726
Ficus	7002	Cordyline	1175
Saintpaulia	5985	Actinidia	1112
Cynara	5727	*Beta vulgaris*	1070
Rosa	5696	Rubus	1009
Syngonium	4797	*Kalmia*	1001
Anthurium	4408	*Betula*	842
Triticum	4016	*Nicotidiana*	704
Rhododendron	3882	*Platycerium*	700
Solanum tuberosum	2817	Dieffenbachia	682
Total			132926

In nature, mycorrhizal fungi are an integral part of the plant, assuring satisfactory growth and development in microbially-rich and nutrient-poor environments. However, micropropagation technology obviously eliminates all microorganisms from plant tissues, and consequently

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mycorrhizal fungi. The absence of mycorrhiza, therefore, requires the use of nutrient-rich and microbially-poor environments to guarantee growth at outplanting. This is presently ensured by the use of artificial substrata and high chemical inputs (fertilizers and pesticides). However, economic factors, as well as the growing awareness about environmental problems, make it necessary to reduce the use of these chemical inputs and to develop technologies compatible with what is termed sustainable agricultural production.

Mycorrhizas, through their role in increasing the natural resistance of plants to abiotic and biotic stresses, and in rendering their underground organs more efficient in exploiting soil resources, have opened interesting perspectives for the production of micropropagated plants of high quality in low input systems (Gianinazzi *et al.*, 1990). The aim of this paper is to present the beneficial effects of arbuscular mycorrhiza for micro-plant production and to illustrate the most recent developments in efforts to combine these new technologies.

2. Mycorrhizas as bioregulators

Root infection by mycorrhizal fungi can affect plant growth and development. It has been demonstrated that the presence of the association avoids blocking of shoot apical growth at transplanting (Berta *et al.*, 1994), a feature which is of importance for reducing production time. Other physiological traits are affected, like branching and flowering, as has been demonstrated for micropropagated roses, for which the number of branches and flowers is increased by inoculation with arbuscular mycorrhizal fungi (Table 2). Such effects, leading to a reduction in the time needed for flowering are at the origin of the first commercial application of mycorrhizal inoculation to pot cultures of chrysanthemums in Japan (Cargeeg, 1991; Arias and Cargeeg, 1992).

Table 2 - Number of branchese (B) and flowers (F) obtained in *post vitro* inoculated or uninoculated 7, 9, and 11 week-old micropropagated roses (var. Ruth Levrik) (Gianinazzi *et al.*, 1990)

	Number of weeks					
	7		9		11	
Treatment	B	F	B	F	B	F
Uninocultated	1.35 a	0.00 a	1.55 a	0.75 a	1.78 a	1.20 a
Inoculted with						
- *Glomus fasciculatum* (LPA.7)	2.55 b	0.90 b	3.15 b	1.45 b	3.80 b	1.60 b
- *Glomus sp.* (LPA 21)	2.25 b	0.50 b	2.75 b	1.30 b	3.45 b	1.85 b

Values in each column followed by the same letter are not significantly different (P=0.05).

The pattern of root morphogenesis and development is also modified in mycorrhizal plants. Plants forming mycorrhizas tend to have a lower root/shoot (R/S) ratio (Fig. 1), which means a greater biomass efficiency, since

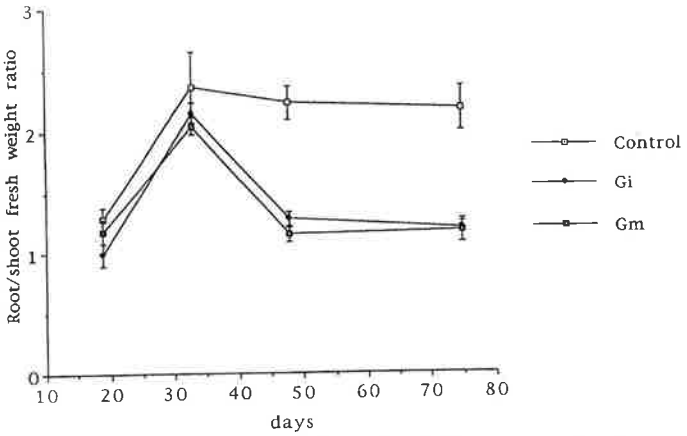
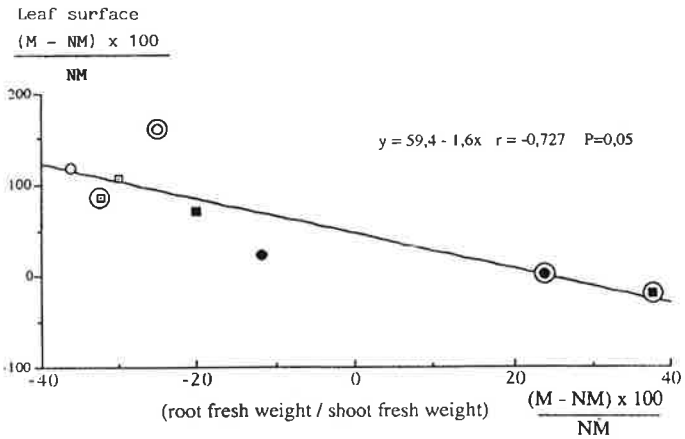


Fig. 1 - Root/shoot fresh weight ratio of micropropagated *Prunus cerasifera* uninoculated (Control), or inoculated with *Glomus mosseae* (GM) or *Glomus intraradices* (GI) (Berta *et al.*, 1994).

less energy is directed to root formation. Using micropropagated pineapple, Guillemin *et al.* (1991) were even able to demonstrate the existence of a negative correlation between the mycorrhizal effect on root and shoot development: the higher the R/S ratio, the less efficient the system for shoot production (Fig. 2). This mycorrhizal effect on root development is partly due to architectural changes in the root system, as has been demonstrated in particular for micropropagated woody species such as grapevine (Schellenbaum *et al.*, 1991) and *Prunus* (Berta *et al.*, 1994). These studies showed that, following fungal effects on meristem activity, mycorrhiza formation changes root topology from a herring-bone pattern to a more dichotomous pattern, the latter being more efficient for the scavenging and uptake of the soil nutrients.



	complete solution		solution without P	
	acid soil	alkaline soil	acid soil	alkaline soil
clone CY0	■	◉	▫	◎
clone CY5	●	◉	○	◎

Fig. 2 - Negative correlation between mycorrhizal effect on leaf surface and root/shoot fresh weight of two clones of micropropagated pineapple (cv. Queen Tahiti) (Guillemin *et al.*, 1991).

Variability in plant growth can be diminished by the presence of a mycorrhizal association, as shown by Branzanti *et al.* (1992), who observed that the coefficient of variation for several growth parameters of micropropagated apple was lower in mycorrhizal plants than in uninoculated ones. Another example of the role of mycorrhizas in regulating plant growth comes from experiments with micropropagated oil palms by Blal and Gianinazzi-Pearson (1989). Variations in the response to applications of phosphate fertilizers of different clones of this plant species were completely eliminated by the presence of mycorrhizal fungi. Consequently, plant homogeneity in growth and in response to inputs is a goal which can be attained more easily by coupling micropropagation with mycorrhization.

3. Mycorrhizas as biofertilizers

The best known effect of mycorrhizal infection in the improvement of growth due to increased uptake of several nutrients (Gianinazzi-Pearson and Gianinazzi, 1983; Harley and Smith, 1983). This results from the increased soil volume explored by the mycorrhizal fungal mycelium, to which should be added the modifications in root morphology discussed above. The association of these factors creates a highly efficient underground organ, which improves uptake of nutrients with low diffusion coefficients in the soil, like phosphorus, potassium, copper and zinc.

Phosphorus has been the most extensively studied nutrient because of its importance in plant nutrition and the reliability of methods to study it. Blal *et al.* (1990) showed that mycorrhizal oil palm microplants obtain P from the same pool in the soil as non-mycorrhizal plants, but that the coefficient of fertiliser utilisation is multiplied by four, in particular with rock phosphate (Table 3). Similar results have been obtained with several other

Table 3 - Yield and utilisation of triple superphosphate (TSP) or non calcinated rock phosphate (RP) by oil palms in a ^{32}p-labelled tropical acid sandy clay soil (Blal *et al.*, 1990)

Treatment	Fertiliser	Dry weight g/shoot	Utilisation coeff. %	% P derived from fertiliser	$^{32}p/^{31}p$
Non mycorrhizal	0	0.6	-	-	-
	TSP	1.6	5.0	75	1.9
	RP	1.5	4.3	65	1.7
Mycorrhizal	0	2.4	-	-	-
	TSP	3.1	13.8	74	2.0
	RP	3.3	16.8	76	1.9

NM = non mycorrhizal; M = mycorrhizal; D.wt = dry shoot weight; coeff. = coefficient of fertiliser utilisation.

plant species. For example, Williams *et al.* (1992) investigated the interest of mycorrhizas to reduce the use of chemical fertilizers in the production of commercially micropropagated strawberry in the United Kingdom and in Finland. Their data show that mycorrhizal plants receiving 25% of the minimum recommended commercial rate of slow-release fertilizer have a similar growth to non-mycorrhizal plants which receive the full recommended rate. These studies confirm that fertilizer inputs can be reduced with mycorrhizal inoculation of micropropagated plants, whilst maintaining high production levels. Besides increasing plant growth through improved nutrient uptake, the presence of a functional mycorrhizal infection will help the plant tolerate or overcome several abiotic stresses, like drought or nutrient depletion, without yield losses (for a discussion on the subject see Sylvia and Williams, 1992).

4. Mycorrhizas as biocontrols

Mycorrhizas can therefore be considered as an "insurance" for plant production (Gianinazzi and Gianinazzi-Pearson, 1988), and their role in the alleviation of stresses can be extended to those of biotic origin. Although mycorrhizal infection can favour the colonization of roots by other symbiotic microorganisms (Barea *et al.*, 1987), it often reduces susceptibility, or increases tolerance, of roots to soil-borne pathogens like fungi or nematodes. For example, mycorrhizal micropropagated oil palms growing in a substratum infested with the pathogen *Fusarium oxysporum* did not show necrotic symptoms, whilst these developed in non-mycorrhizal controls which had been fertilised to have the same growth as the mycorrhizal plants (Blal, 1989). The contribution of arbuscular mycorrhizal infection to the resistance of micropropagated plants to soil-borne pathogens has also been successfully shown by Guillemin *et al.* (1994a) in micropropagated pineapple plants where the presence of arbuscular mycorrhizas prevented growth depressions caused by heavy applications of *Phytophthora cinnamomi*.

Other groups of organisms may be damaging to micropropagated plants when they are taken out of axenic conditions. Nematodes, for example, cause reductions in the growth of non-mycorrhizal pineapple microplants but do not affect the development of mycorrhizal plants, even if the pathogen is inoculated at the same time as the symbiotic fungus (Guillemin *et al.*, 1994b). This protective effect was not due simply to the nutritional effects of the mycorrhiza, since the presence of the symbiosis reduced the number of nematodes colonising the root system. These data suggest that the presence of mycorrhizal fungi in roots induces some kind of resistance mechanism. The processes involved in this increased resistance of mycorrhizal plants to root pathogens are not clear, but one possibility is a weak and permanent activation of plant defences by the mycorrhizal fungus (the subject is discussed elsewhere, see Gianinazzi, 1991).

5. Procedures for mycorrhization of microplants

As well established mycorrhizal infection is a prerequisite to obtaining maximum benefits from the role of mycorrhizas as bioregulators, biofertilisers and bioprotectors. Consequently, plant and soil management should take the symbiosis into consideration. Three main factors are of importance in the production of mycorrhizal micropropagated plants: time and form of mycorrhizal inoculation, substrate to be used and choice of the mycorrhizal inoculum.

Time and form of inoculation

Three periods during micropropagated plant production can be identified for introducing mycorrhizal fungi: during the *in vitro* phase, during the weaning phase, and

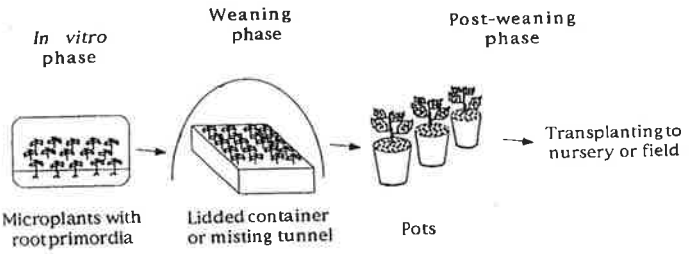


Fig. 3 - Schematic representation of the different phases of microplant production from *in vitro* techniques to field growth.

after the weaning phase (Fig. 3). Micropropagated plants can be inoculated with arbuscular mycorrhizal fungi *in vitro*, but this requires an extension of the *in vitro* phase, so the economic impact of the procedure is questionable. In fact, Ravolanirina *et al.* (1989b) showed that growth increases in grapevine rootstocks due to the presence of mycorrhiza was greater if inoculation was introduced in the *post vitro* phase, even if the levels of infection obtained were comparable to those of plants inoculated during the *in vitro* phase. Further work (Ravolanirina *et al.*, 1989a; Branzanti *et al.*, 1992) has demonstrated that the best results for plant growth are obtained if microplants are inoculated at the beginning of the weaning phase when they show only two root primordia (Table 4). This is compatible with the present trend to couple the weaning or acclimatization period with a rooting phase, following induction or root formation during the *in vitro* phase. The validity of mycorrhizal inoculation at the beginning of the rooting phase has been confirmed in our laboratory for an increasing number of plant species produced by micropropagation (Table 5).

Table 4 - Effect of time of arbuscular mycorrhizal fungus inoculation and fertilisation procedures on the growth (shoot height, in cm) at 8 weeks, of micropropagated grepevine rootstock S04102 non-mycorrhizal or inoculated with *Glomus fasciculatum* (Ravolanirina *et al.*, 1989a)

		Time of inoculation	
Inoculation	Fertilization	After *in vitro* rooting	During *post-vitro* rooting
Non-mycorrhizal	once a week	22.0 f	13,0 g
G. fasciculatum	"	66.6 d	77.0 bc
Non-mycorrhizal	twice a week	39.0 e	45.0 e
G. fasciculatum	"	70.0 cd	79.8 b
Non-mycorrhizal	once a day	-	80.5 b
G. fasciculatum	"	-	95.0 a

Table 5 - Micropropagated plant species inoculated with arbuscular mycorrhizal fungi

Plant	Reference
Pear	Gianinazzi *et al.*, 1985
Grapevine	Ravolanirina *et al.*, 1989a, 1989b
Oil palm	Ravolanirina *et al.*, 1989b
Rose	Gianinazzi *et al.*, 1990
Pineapple	Guillemin *et al.*, 1991
Apple	Branzanti *et al.*, 1992
Platanus	Tisserant and Gianinazzi-Pearson, 1992
Rhododendron	Lemoine *et al.*, 1992
Citrus	Blal *et al.*, unpublished
Banana	Guillemin, unpublished
Asparagus	Delaitre and Gianinazzi, unpublished
Wild cherry	Lovato *et al.*, 1994
Common ash	Lovato *et al.*, 1994

Several other laboratories have demonstrated that the acclimatization period of micropropagated plants can be shortened by application of mycorrhizal technology. Barea and co-workers, who also have a wide experience in mycorrhizas and micropropagation, succeeded in shortening the acclimatization process for a micropropagated woody legume from 18 to 10 weeks by introducing mycorrhizas (Salamanca *et al.*, 1992). This reflects the potential gain in time and cost that is made possible through the use of mycorrhizal tecnology.

This procedure for inoculation of micropropagated plants consists in grouping them together in trays containing the mycorrhizal inoculum mixed into the weaning substratum, since survival of plants is higher than in individual pots. Furthermore a reduced amount of inoculum is required because there is a greater probability of developing roots rapidly encountering mycorrhizal fungal propagules. Five grams of freshly chopped mycorrhizal fragments (i.e. roots well-infected by mycorrhizal fungi) are enough to guarantee a high level of infection for 25 to 50 plants after two weeks in trays, before transplanting to pots. For other types of inoculant, like soil-based inocula or commercial products, a 1% dose has proved to be sufficient for improving the growth of microplants like grapevine and pineapple (Lovato *et al.*, 1992). Other conditions for the weaning phase do not have to be modified: plants are outplanted in misting tunnels or tall lid containers to control humidity, and after two to four weeks, they are transplanted to individual pots without adding extra inoculum.

Substratum composition

Substratum composition is very important to obtain optimal mycorrhizal effects, and in particular the presence of soil in the rooting mix is advisable. For example, comparing three types of substratum for producing mycorrhizal propagated avocado, Azcón-Aguilar *et al.*

(1992) concluded that 50% disinfected soil gives a better mycorrhizal effect. However, this may vary with the plant species used. Schubert *et al.* (1992) obtained good growth improvement of micropropagated kiwi following mycorrhizal infection using only 5% sandy soil in a peat-perlite mix, whilst Lovato *et al.* (1992) observed growth enhancement of mycorrhizal wild cherry and common ash using a potting mixture containing 20% clay loam soil. The latter proportion is recommended in British nurseries (N. Hammatt, personal communication), which means that it is representative of management practices used at the production level.

Choice of the inoculant

Inoculants should be chosen according to the target plant since, for arbuscular mycorrhizas, there are differences among fungal strains in promoting growth of diverse plant species or varieties (Azcón-Aguilar *et al.*, 1992; Fortuna *et al.*, 1992; Guillemin *et al.*, 1992; Lovato *et al.*, 1994). The most frequent solution is to use a mixture ("cocktail") of isolates, each of which is adapted to a specific set of environmental factors, so that a wide range of hosts and environmental/management conditions can be covered. However, it is possible that in a given situation a less efficient fungal strain present in the inoculant may be more competitive for infection of host roots. This could introduce limitation in the use of commercial inoculants now available to plant producers. These products are aimed at a wide range of host plants and soil conditions, but this does not guarantee their maximum efficiency in all situations. This has been demonstrated for one such inoculant which infected less in an acid than in an alkaline soil (Table 6). Optimal plant improvement can, therefore, only be obtained through mycorrhizal fungal inoculation of micropropagated plants if, as in any production system, soil characteristics and other environmental features are carefully considered.

6. Transfer of mycorrhizal microplants to the nursery or field

The beneficial effects of mycorrhiza observed in pots have been confirmed at the field or nursery level. Six months after outplanting, oil palm plants grown in non-disinfected soil under nursery conditions grew better when they were mycorrhizal, even in a phosphorus-amended soil (Fig. 4). The persistence of the mycorrhizal effect after one year in the field has been demonstrated for several micropropagated plant species, both in disinfected and non-disinfected plots (Table 7). The results

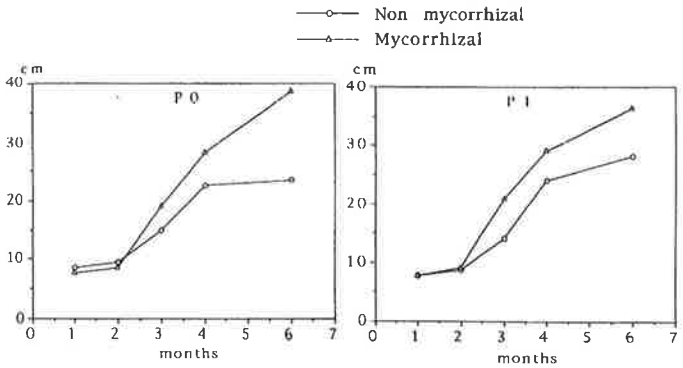


Fig. 4 - Growth in nursery (La Mé, Ivory Coast) of micropropagated oil palms, non-inoculated or inoculated with *Glomus sp.* (LPA 22), in a non-disinfected soil without addition of P (PO) or receiving 31 mg P. Kg^{-1} (P1) (B. Blal; V. Gianinazzi-Pearson; J. Renard, unpublished results).

Table 6 - Shoot dry mass and endomycorrhizal infection [intensity of infection (M%)] of micropropagated pineapple plants (Smooth Cayenne variety) uninoculated or inoculated with *Glomus spp.* or a commercial inoculant (AGC) at two application rates, in acid and alkaline soils (Lovato *et al.*, 1992)

				AGC	
	Uninoculated	*Glomus sp.*	*G. intraradices*	1%	3%
Alkaline soil					
Shoot dry weight (g)	0.43 b	-	0.97 a	1.31 a	1.64 a
Intensity of infection (M%)	0.00 b	-	64.00 a	69.00 a	74.00 a
Acid soil					
Shoot dry weight (g)	1.04 b	3.29 a	-	1.76 b	1.05 b
Intensity of infection (M%)	0.00 c	87.00 a	-	42.00 b	36.00 b

Values in each line followed by the same letter are not significantly different (P=0.05).

Table 7 - Mycorrhizal effect on growth of non inoculated (NM) and mycorrhiza inoculated (Myc) plants under field conditions, in disinfected or non disinfected soil. Non inoculated plants = 100

		Field plots				
		Disinfected		Non-disinfected		
Plant species	Shoot growth	NM	Myc	NM	Myc	Reference
Grapevine	fresh weight	100	364	-	-	Ravolanirina *et al.*, 1989 b
Apple	fresh weight	100	485	205	290	Gianinazzi *et al.*, 1989
Strawberry	dry weight	-	-	100	455	Vestberg, 1992

obtained by Vestberg (1992) are remarkable because the growth promoting effects observed in strawberry plants, outplanted to a non-disinfected field prepared according to the recommendations for commercial production, persisted after overwintering into the second year. This suggests that the highly efficient, introduced mycorrhizal fungal isolated were able to compete with indigenous strains and survive through the winter. Such data show that the beneficial effects of mycorrhizal inoculation may persist in the long term, and consequently become agronomically and economically significant.

7. Conclusions and Perspectives

A growing body of knowledge and experience is showing that it is possible to use mycorrhizas in order to market healthy and strong microplants that are able to overcome outplanting stress and assure optimal growth, even in adverse conditions. For example, in Finland, within the next few years, mycorrhizal inoculation is planned to be integrated in the commercial production of micropropagated strawberry elite plants (M. Vesterberg, personal communication). Furthermore, mycorrhizal technology is not limited to plants forming arbuscular mycorrhizas, but it can also be applied to species forming ericoid endomycorrhizas like *Rhododendron* (Lemoine *et al.*, 1992) or ectomycorrhizas, like the truffle hazelnut (Guinberteau *et al.*, 1989; J. Chevalier, personal communication). Consequently, mycorrhiza and micropropagation technologies may be used with the vast majority of plant species.

Developments in the production of mycorrhizal micropropagated plants depend on efforts being invested at several levels. A basic aspect involves the genetic plant determinants controlling mycorrhizal association. These genetic mechanisms are still poorly understood, but the use of new approaches, such as combining plants mutated for their ability to form mycorrhizas (myc^-) with molecular biology analyses, will improve knowledge about them and so pave the way to obtaining plants that are better adapted to mycorrhiza biotecnology (i.e. more responsive to the symbiotic association).

A perspective for the near future should be the development of integrated biotechnologies in which not only 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 substrata for micropropagated plant production. In the short term, it is necessary to develop management practices taking into account the establishment, functioning and benefits of the mycorrhizas. Parameters such as substrate composition, forms and rates of fertilisers or other chemical products, as well as schedules for weaning and outplanting, may be optimized by the combination of the two biotechnologies discussed in this work.

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