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A tripartite culture system for endomycorrhizal inoculation of micropropagated strawberry plantlets in vitro

Abstract The objective of the current investigation was to develop a reliable method to obtain vesicular-arbuscular mycorrhizae (VAM) in micropropagated plantlets and to determine their influence on growth. An in vitro system for culturing the VA mycorrhizal fungus *Glomus intraradices* with Ri T-DNA-transformed carrot roots or nontransformed tomato roots was used in this study as a potential active source of inoculum for the colonization of micropropagated plantlets. 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 days of tripartite culture, all plantlets placed in contact with the primary symbiosis were colonized by the VAM fungus. As inoculum source, 30-day-old VA mycorrhizal transformed carrot roots had a substantially higher infection potential than 5-, 10- or 20-day-old VAM. Colonized plantlets had more extensive root systems and better shoot growth than control plants. The VAM symbiosis reduced the plantlet osmotic potential. This response may be a useful pre-adaptation for plantlets during transfer to the acclimatization stage.

Key words Micropropagation · *Glomus intraradices* · Strawberry · Vesicular-arbuscular mycorrhizae (VAM)

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Introduction

For the last decade, micropropagation has been a most powerful tool for cloning several plant species, especially the ornamentals (Ammirato et al. 1989). Despite many successful applications of this technique, there are still several problems limiting its widespread use. The transfer of in vitro plantlets to ex vitro conditions is one of the most critical factors of the micropropagation process and a cause of higher production costs. High mortality is often observed upon transfer to ex vitro conditions as the cultured plants have a poorly developed cuticle (Wetzstein and Sommer 1982), nonfunctional stomata (Lee and Wetzstein 1988) and a weak root system (Pierik 1987).

In order to increase growth rate and reduce mortality of plantlets at the stage of acclimatization, recent research has focused on control of the environmental conditions. One approach has been to modify the environmental conditions during acclimatization by increasing light intensity (Hasegawa et al. 1973; Donnelly et al. 1985; Desjardins et al. 1988; Kozai and Iwanami 1988) or both increasing the light levels and altering the CO_2 concentration (Kozai and Iwanami 1988; Laforge et al. 1988). Another approach has been to change the environment during the multiplication and rooting stages, including increasing light intensity and CO_2 concentration in culture tubes, and decreasing the sugar concentration (Hasegawa et al. 1973; Donnelly et al. 1985; Kozai et al. 1986; Desjardins et al. 1990; Laforge et al. 1990; Hdider and Desjardins 1994). All were found to be beneficial for plantlet growth in the later stages of micropropagation.

A biological approach to reducing the stress of acclimatization and providing faster growth of micropropagated plantlets, is the establishment of vesicular-arbuscular mycorrhizae (VAM) on micropropagated plantlets during acclimatization. Micropropagated plantlets are grown in a completely sterile environment and, therefore, inoculation with microorganisms may be

subsequently necessary in vivo for plants living symbiotically (Pierick 1988). Indeed, VAM colonization of horticultural plant roots can improve growth by increased uptake of phosphorus, zinc and other minerals (Menge et al. 1978; Ames et al. 1983) and may reduce the incidence of disease (Dehne 1982). Moreover, colonization with VAM fungi may increase transplant uniformity and reduce both transplant mortality (Biermann and Linderman 1983) and injury (Menge et al. 1978). Recent work has also shown improvement in water relations of the host plant using VAM. These fungi may also improve drought tolerance by decreasing leaf water potential (Koide 1985; Augé et al. 1986), by reducing stomatal and root hydraulic resistances (Allen and Boosalis 1983; Nelsen 1987), and by increasing transpiration rates (Koide 1985).

For these reasons, several studies have focused on VA mycorrhizal formation during acclimatization of cultured plantlets (Chavez and Ferrara-Cerato 1990; Ponton et al. 1990; Shubert et al 1992; Vestberg 1992; Williams et al. 1992; Wang et al. 1993). It has been shown that VAM colonization of micropropagated plantlets can improve rooting, enhance root function and increase survival rates (Strullu 1985). The benefits of VAM have also been demonstrated by higher survival rates and better overall growth (Wang et al. 1993). In most cases, the beneficial effects on growth have been observed after in vitro plantlets were fully acclimatized (Wang et al. 1993), and has been suggested that growth and survival would improve during acclimatization if VA mycorrhizal fungi were introduced during the in vitro rooting stage. However, the culture of VAM fungi in the absence of living host roots is currently not possible.

The culture system for mycorrhizal establishment in vitro on Ri T-DNA-transformed carrot roots (Bécard and Fortin 1988) could be used as a source of inoculum to initiate mycorrhizae rapidly on micropropagated plantlets in vitro. It has been proposed that VA mycelium associated with root organ culture could provide vigorous and uniform fungal hyphal growth (Bécard and Piché 1992); thus this system may have a higher potential for mycorrhizal colonization than axenically germinated spores or surface-sterilized VAM colonized root fragment. In light of these observations, the objectives of the present investigation were: (1) to develop a culture system for the establishment of VAM fungi on micropropagated plantlets; (2) to determine the effects of VA mycorrhizae on the growth of in vitro plantlets inoculated by this novel method; (3) to determine the efficiency of transformed and nontransformed roots inoculated by *Glomus intraradices* as a primary source of inoculum; (4) to determine the age of primary mycorrhizae at which the most rapid and intense colonization occurs.

Materials and methods

Fungal inoculum

The VA mycorrhizal fungus used in this study was *G. intraradices* Schenck et Smith (DAOM 197198, Biosystematic Research Center, Ottawa, Canada). Spores of this fungus were obtained from a VAM tomato root culture routinely produced on minimal medium (M) according to the procedure described by Chabot et al. (1992a). The M medium contained the following ingredients in 1 l of distilled water: $MgSO_4 \cdot 7H_2O$, 731 mg; KNO_3, 80 mg; KCl, 65 mg; KH_2PO_4, 4.8 mg; $Ca(NO_3)_2 \cdot 4H_2O$, 288 mg; Na-FeEDTA, 8 mg; KI, 0.75 mg; $MnCl_2 \cdot 4H_2O$, 6 mg; $ZnSO_4 \cdot 7H_2O$, 2.658 mg; H_3BO_3, 1.5 mg; $CuSO_4 \cdot 5H_2O$, 0.13 mg; $Na_2\ MoO_4 \cdot 2H_2O$, 0.0024 mg; glycine, 3 mg; thiamine-HCl, 0.1 mg; pyridoxine·HCl, 0.1 mg; nicotinic acid, 0.5 mg; myoinositol, 50 mg; sucrose, 10 g. The pH was adjusted to 5.5 after addition of all components. The medium was autoclaved at 121° C for 15 min. Bacto agar was replaced by Gellan gum (Gel-Gro, ICN Biochimicals, Cleveland, Ohio) and added at a concentration of 0.3% for the maintenance of root cultures and for the establishment of mycorrhizal associations.

Micropropagation of strawberry

Strawberry (*Fragaria* × *ananassa* Duch. cv. Kent) cultures were established, multiplied and subcultured as previously described by Desjardins et al. (1987). Axillary buds located at the base of cultures were excised and transferred to sorbarod cellulose plugs (Baumgartner Papier SA, Lausanne, Switzerland), containing rooting liquid medium as described by Kartha et al. (1980) (Fig. 1B). The liquid rooting medium contained one-half strength MS salts (Murashige and Skoog 1962) with 0.12 μM thiamine·HCl, 0.56 μM myo-inositol, 0.40 μM nicotinic acid, 2.40 μM pyridoxine-HCl, 3% sucrose, and without growth regulators. The pH was adjusted to 5.5 before the liquid medium was dispensed in 5-ml aliquots to 25×150-mm culture tubes. These were then autoclaved at 1.4 kg cm^{-2} and 121° C for 15 min and left at room temperature for 24 h before use. The cultures were kept for 2 weeks in a growth room at a temperature of 23±1° C under a photosynthetic photon flux density (PPFD) of 60 μmol m^{-2} s^{-1} for a photoperiod of 16 h provided by cool-white fluorescent lamps until inoculation.

Ri T-DNA-transformed roots of carrot (*Daucus carota* L.) and nontransformed roots of tomato (*Lycopersicum esculentum* Mill. Var. Vendor) served as plant partners for fungal inoculum used to colonize micropropagated plantlets. Primary mycorrhizal colonization of these hosts was achieved by placing a single root explant of either carrot or tomato (7 cm long) with two groups of 20 *G. intraradices* nongerminated spores in the middle of a Magenta vessel (Magenta Corp., Chicago, Ill.) on a minimal M medium (Fig. 1A). Spores which appeared to be viable (light colored, globule filled) were obtained from VAM tomato root cultures. The Magenta vessels were sealed and incubated at 27° C in the dark for 5 weeks. Since transformed carrot roots display negative geotropism, vessels were inverted to maintain root apices in the medium.

In vitro tripartite culture system

After root induction on micropropagated plantlets and primary mycorrhizae establishment, secondary symbiosis was achieved by removing the rooting medium from cellulose plugs by suction and rinsing with sterile distilled water three times under sterile conditions (Fig. 1C). After washing, M medium was added aseptically to sorbarod plugs supporting micropropagated plantlets. The plugs were then placed in contact with the primary mycorrhizae in the culture vessel under aseptic conditions (Fig. 1D). Tripartite cultures were grown at 25° C in small growth chambers with a PPFD of 60 μmol s^{-1} m^{-2} provided by cool-white fluorescent lights for a 16-h photoperiod (Laforge et al. 1990) with or without

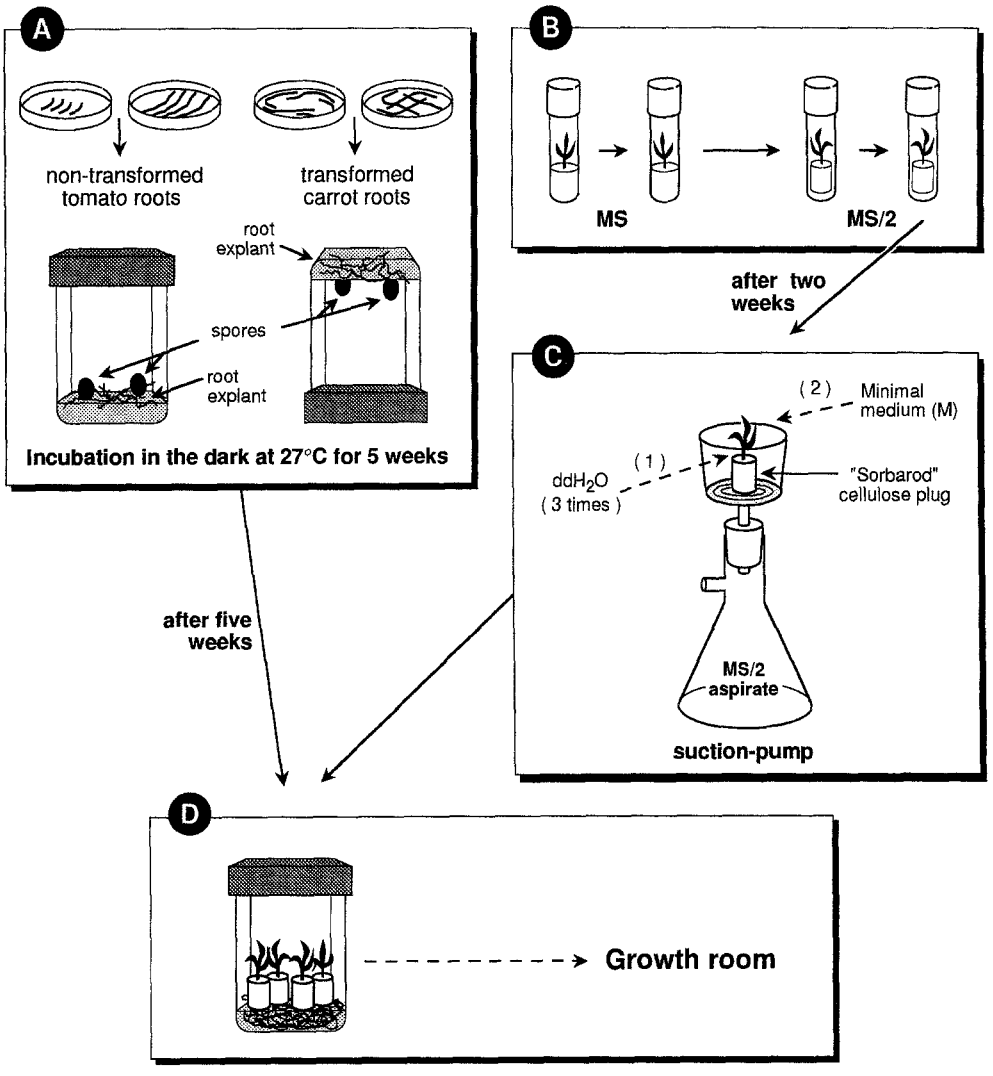

Fig. 1A–D In vitro tripartite culture system used for establishment of mycorrhizae on micropropagated strawberry plantlets. **A** Inoculation of tissue cultured roots (primary establishment). **B** Micropropagation of strawberry plantlets. **C** Transfer to rooting medium. **D** Inoculation of strawberry plantlets by tripartite culture (secondary establishment)

5000 ppm CO_2. Vessels without spores of *G. intraradices* served as controls. A total of 72 micropropagated plantlets per treatment was assigned randomly to the two CO_2 treatments in a split-plot experimental design where the main plots received CO_2 enrichment treatments and the subplots the fungal inoculum treatments. The main plots were duplicated in each of two growth chambers.

Assessment of mycorrhizae influence on micropropagated plantlets

Destructive growth analyses were conducted at 5, 10, 20 and 30 days after micropropagated plantlets were inoculated. Two mycorrhizal inoculated root treatments (carrot and tomato) and a non-inoculated control were carried out. A total of 72 micropropagated plantlets per treatment was randomly distributed in four replicate growth chambers. An analysis of variance was performed using a general linear model (SAS Institute Inc. 1982), and for all parameters studied, a Duncan's multiple range test ($P<0.05$) was used to separate means.

The root length and shoot height were determined at each sampling date. The osmotic potential of each plant was measured with a vapor pressure osmometer (Wescor 5500) calibrated with a series of NaCl solutions (Chapman and Augé 1994). In order to obtain tissue sap, leaves were pressed on 6-mm filter paper disks using a leaf press (Wescor) and the soaked filters were immediately placed in the osmometer chamber for measurement (Markhart and Lin 1985). At each date, roots of micropropagated plantlets were cleared in 10% KOH at 90° C for 10 min, rinsed with deionized water, immersed in 1% HCl at 25° C for 2 min and stained in acid fuchsin at 90° C for 4.5 min. Stained roots were carefully mounted on slides and observed under a binocular microscope Olympus BHT.

Assessment of the preferential age of primary mycorrhizae

Different aged primary mycorrhizae root inocula have been used to colonize micropropagated plantlets. In this present experiment only mycorrhizal transformed roots of carrot served as a partner for the fungal inoculum. Tripartite cultures were established as above. For percentage root length colonization, destructive root analysis was conducted 20 days after micropropagated plantlets were placed in tripartite culture. On this date, 25 root segments (1 cm long) per treatment were stained as described above for quantification of VAM colonization. Stained roots were mounted on slides and observed under a binocular microscope (Olympus BHT). The percentages of root segments containing hyphae, arbuscules and vesicules were determined using the method described by Brundrett et al. (1984).

A randomized complete block design was used to investigate the effect of primary mycorrhizae age on rate of root length colonization of plantlets in vitro. A total of 32 micropropagated plantlets per treatment was randomly distributed in four replicate growth chambers, two for each CO_2 treatment.

Results

Assessment of mycorrhizae influences on micropropagated plantlets

VAM fungi establishment

Examination of micropropagated plantlet roots inoculated with mycorrhizal transformed carrot roots or with mycorrhizal nontransformed tomato roots revealed the presence of typical VAM on micropropagated plantlets during the tripartite culture stage. For either source of inoculum, exposure to 5000 ppm CO_2 resulted in a greater percentage of colonized plantlets (Fig. 2). This was evident as early as 10 days after tripartite culture establishment. The level of colonization was 100% at 30 days for plantlets placed on transformed VAM-inoculated carrot roots.

In presence of CO_2, both the spread of fungal colonization and the growth of micropropagated plantlets were increased. The inoculum source significantly affected the percentage of plants colonized by VAM. Carrot roots produced a higher percentage of VAM-inoculated plants than tomato roots. After 30 days of tripartite culture, all strawberry plantlets were colonized when growing in the presence of mycorrhizal transformed carrot roots, while 40% of the strawberry plantlets growing in the presence of mycorrhizal nontransformed tomato roots were infected (Fig. 2).

Plant growth

Both inoculated and noninoculated micropropagated plantlets in enriched CO_2 conditions grew well compared with the poor growth and obvious decline by the 20th day of plants that received no additional CO_2 (results not shown). In enriched CO_2 conditions (Figs. 3, 4), no significant difference in growth was detected between inoculated and noninoculated micropropagated plantlets until day 20. No significant growth difference was detected between mycorrhizal plantlets inoculated with transformed carrot roots or nontransformed tomato roots. From 10 to 20 days of tripartite culture, mycorrhizal plantlet shoot heights and root lengths were significantly greater than those of nonmycorrhizal control plants.

Leaf osmotic potential

Leaf water potential of mycorrhizal plantlets was significantly lower than that of nonmycorrhizal plants (Fig. 5). At the third sampling (20 days following inoculation), control plants had a leaf osmotic potential 64%

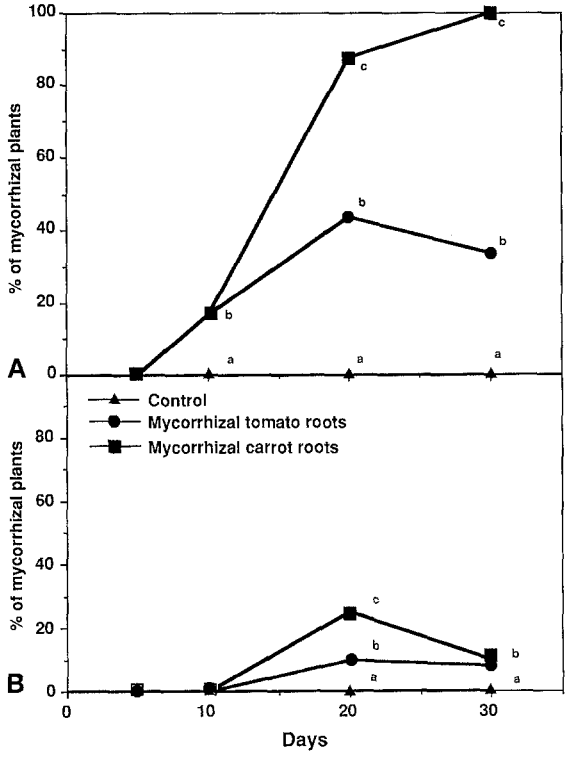

Fig. 2A, B Percentage of strawberry plants colonized with VA mycorrhizal fungi after 5, 10, 20 and 30 days following in vitro inoculation: **a** in the presence of 5000 ppm CO_2. **b** without CO_2 enrichment. Mean separation by Duncan multiple range test ($P<0.05$)

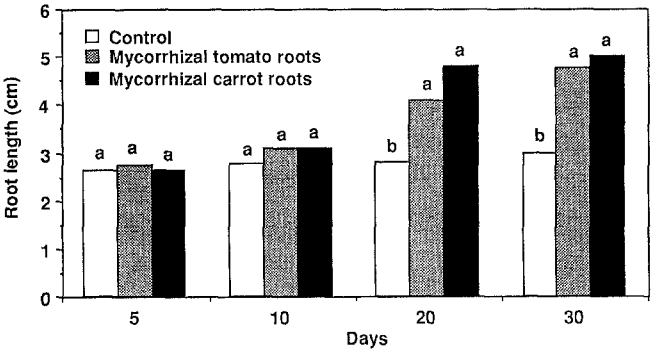

Fig. 3 Effect of inoculum source on the root length of strawberry plants after in vitro inoculation in the presence of 5000 ppm CO_2. Mean separation by Duncan multiple range test ($P<0.05$)

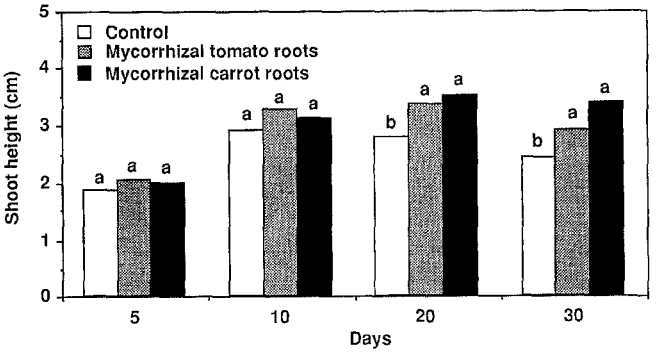

Fig. 4 Effect of inoculum source on the shoot height of strawberry plants after in vitro inoculation in the presence of 5000 ppm CO_2. Mean separation by Duncan multiple range test ($P<0.05$)

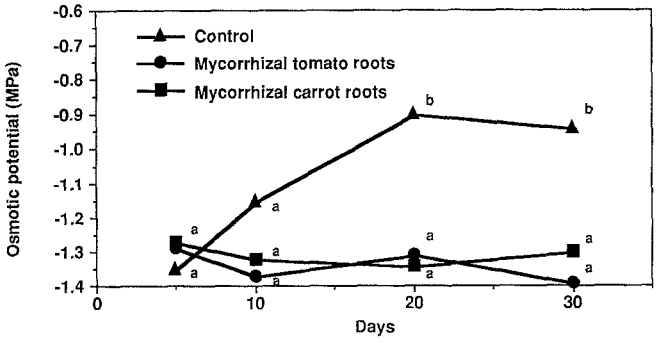

Fig. 5 Effect of inoculum source on the osmotic potential of strawberry plants after in vitro inoculation in the presence of 5000 ppm CO_2. Mean separation by Duncan multiple range test ($P<0.05$)

higher than mycorrhizal plants. Mycorrhizal plantlets inoculated by either of the primary mycorrhizal types had almost the same osmotic potential during the tripartite culture period.

Assessment of preferential age for the primary mycorrhizae inoculum

There were significant differences between the effects of different aged primary mycorrhizae on the percentage root length colonization of micropropagated plantlets. With increase in mycorrhizae age, the percentage of VA mycorrhizal colonization of micropropagated plantlets increased. After 20 days of tripartite culture, the use of a 30-day-old primary inoculum resulted in 70% colonization of plantlets, compared with 0, 0 and 15% for the 5-, 10- and 20-day-old root inocula, respectively.

Discussion

The aim of this present study was to find a reliable system to obtain VA mycorrhizal micropropagated plantlets in vitro with high colonization and growth stimulation. This is the first report on the effect of mycorrhizae on micropropagated plantlets in vitro. In previous studies, VAM inoculation of micropropagated plantlets was only reported at the end of the culture period, during the acclimatization stage, and no growth stimulation was documented (Chavez and Ferrara-Cerato 1990; Shubert et al. 1992; Vestberg 1992; Williams et al. 1992; Wang et al. 1993). To overcome the difficulties in obtaining co-culture between soil VAM fungi and whole plants in vitro, the technique presented here uses VAM-colonized roots cultivated in minimal medium (dual culture system) for inoculation of micropropagated plantlets grown on an artificial substrate (tripartite culture system). The tripartite culture system with CO_2 enrichment favors proliferation of the inoculum, growth of plantlets and the establishment of the mycorrhizal association. It is unique in that it allows direct in vitro VAM inoculation in an axenic environment.

Establishment of mycorrhizae and development of VA mycorrhizal fungi depend mainly on the concentrations of sodium sulphate, phosphorus and sucrose in the culture medium (Bécard and Fortin 1988). The MS rooting medium contains these elements at high concentrations and we suggest a change to a minimal medium before the tripartite culture stage. This change in medium should create conditions favorable for the establishment of secondary mycorrhizae on micropropagated plantlets.

The increase in plant growth observed in this experiment following the inoculation of plants with VAM fungi confirms results from ex vitro conditions (Vestberg 1992; Williams et al. 1992; Wang et al. 1993) and demonstrates that in vitro mycorrhizal formation has effects on the growth of micropropagated plantlets similar to those observed for seedlings (Hayman 1983). Results of our experiments show that in vitro VAM colonization significantly improves shoot and root growth by decreasing the osmotic potential. These in vitro improvements may be attributed to nutritional effects within the symbiotic association as previously reported (Strullu 1985). In addition, the significant increase in root/shoot length ratio during tripartite culture could represent a significant pre-adaptation of micropropagated plantlets to acclimatization. This is an interesting result because it indicates that effects other than those of nutrition, probably hormonal, are produced during a mycorrhizal symbiosis, as suggested by Allen et al. (1982).

In most cases, a higher percentage of micropropagated mycorrhizal plantlets was produced by tripartite culture with transformed mycorrhizal carrot root than with nontransformed mycorrhizal root. Volatile root products, especially CO_2, and root exudates might be responsible for the increased growth of the fungus, as was also demonstrated with transformed carrot roots and the VA mycorrhizal fungus *Gigaspora margarita* (Bécard and Piché 1989). These root exudates stimulated the growth of VAM fungi before they penetrated the root (Hepper 1981; Ferguson and Menge 1982; Azcon and Ocampo 1984). The present investigation suggests that VA mycorrhizal transformed carrot roots would be a more desirable partner a in tripartite culture system for the large-scale production of VAM fungal hyphae and to obtain a higher percentage of mycorrhizal plantlets.

The very poor growth of plantlets grown in the absence of added CO_2, is probably attributable to the composition of the culture medium. The medium developed by Bécard and Fortin (1988) is low in sugar and mineral nutrients in order to favor growth of the mycorrhizae. It is thus not suitable for heterotrophic growth, and the lack of CO_2 reduces autotrophy of micropropagated plants. Enrichment with CO_2 is important for two reasons in the tripartite culture system: it provides conditions necessary for autotrophic carbon

nutrition, and stimulates the development of the mycorrhizal colonization, as was shown by Chabot et al. (1992b).

Plant leaf osmotic potential has been most often used to indicate adaptation to water stress. Many researchers have demonstrated that the VAM formation plays a key role in resistance to drought stress (Allen and Boosalis 1983; Nelsen 1987; Augé 1989). Under our controlled growth conditions, VAM symbiosis significantly reduced the osmotic potential of micropropagated plantlets. This response could represent a significant pre-adaptation to acclimatization by maintaining a certain degree of leaf hydration under the water stress conditions of the acclimatization stage. The mechanism of this adaptation is not yet known but may include increased root hydraulic conductivity, osmotic adjustment, cell wall elasticity and stomatal conductance (Safir et al. 1972; Allen et al. 1982; Augé et al. 1986, 1987).

Primary mycorrhizae age had a significant effect on colonization rate of micropropagated plantlets. The 30-day-old VA mycorrhizal transformed carrot roots produced the highest level of colonization. An explanation for this higher infectivity may be that younger roots, 10 days after the primary colonization, had not fully established and produced infective hyphae. In addition they may not have produced the secondary infective structure described by Friese and Allen (1991) as the root-derived infection network. However, one should not dismiss the possibility that older roots simply supported more hyphae and thus had a higher inoculation potential.

Our investigation has established an efficient technique to inoculate whole tissue-cultured plants. The use of minimal M medium, synthetic substrates and mycorrhizal roots (as a source of inoculum) would certainly be very useful components of a system to produce in vitro micropropagated mycorrhizal plantlets. In this study, micropropagated plantlets forming a successful association with VAM in vitro, recovered faster and grew better than non-inoculated plantlets during tripartite culture. Thus, inoculation of micropropagated plantlets at the rooting stage by this method may have potential in the commercial production of some tissue culture plants. The tripartite culture system may be a powerful tool for the investigation of changes in the host brought about by mycorrhizal infection which are not attributable to the presence of any other microorganisms.

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