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Micropropagation coupled with mycorrhization used as modern biotechnologies for the production of mycorrhizal tissue-cultured plants for economic and medicinal uses

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Abstract

Using the tripartite system for endomycorrhizal inoculation of micropropagated plantlet *in vitro*, we showed that vesicular-arbuscular mycorrhizae (VAM) performed *in vitro* was also maintained in acclimatization stage while non-inoculated micropropagated plantlets did not display any root infection during this time. *ln vitro* infected strawberry were colonized by more than 45 % by VAM after two weeks of acclimatization stage. Shoot and root length of mycorrhizal plantlets was increased about 31 % and 32 % respectively, whereas the shoot fresh weight was significantly increased about 35 % when compared to non-mycorrhizal plantlets. All plantlets survived in greenhouse. No significant difference was detected in term of growth between inoculated and non-inoculated micropropagated plantlets during the first sampling date. After this date, we could notice some beneficial effects of the symbiosis on the growth of micropropagated plantlets. This can be explained by the increase of relative water content in mycorrhizal plant by changing root cell amino-acid composition, and altering cell starch concentrations, to reduce water stress.

Key words: Biotechnology, micropropagation, mycorrhization, *In vitro* regeneration, strawberry.

Introduction

Biotechnological tools are important for multiplication of crop and medicinal plants by adopting techniques such as *in vitro* regeneration. *In vitro* propagation is used routinely to generate a large number of high-quality clonal agricultural plant including ornamental, and vegetables species, and also holds tremendous potential for the production of high-quality plant-based medicines (Murch *et al*., 2000). Micropropagation has many advantages over conventional methods of vegetative propagation techniques. These include the potential of combining rapid large-scale propagation of new genotypes, the use of small amounts of original germaplasm, and the generation of pathogen-free material. This application of the principles of *in vitro* culture and regeneration of plant propagation (organogenesis and somatic embryogenesis) is the result of continuous studies in hundreds of laboratories worldwide in forestry and agricultural sectors, many of them in developing countries, on the standardisation of explants sources, media composition and physical state, environmental conditions (El Meskaoui *et al*., 2006) acclimatisation stage (El Meskaoui *et al*., 1995) and exogenous hormonal factors (El Meskaoui 2000, Victor 2005) .

Plant production by micropropagation technology is limited by the acclimatization stage, one of the most critical stages of this process. A high percentage of micropropagated plantlets can be lost or damaged during transfer from test tube conditions to *in vivo* environment. This transition is often very difficult because the *in vitro* produced plants are not well

adapted to the *in vivo* uncontrolled conditions (Preece & Sutter, 1991). ln practice, procedures leading to *in vitro* hardening during the last micropropagation phase generally facilitate plantlet acclimatization. It would be useful to acclimatize plantlets during the *in vitro* period to reduce the stress during transfer *ex vitro*.

In the development of sustainable plant production practices, the use of symbiotic inoculants as replacement for chemical fertilizes and pesticides is receiving attention (Rai, 2001). So, the mycorrhizal technology (Fortin *et al.,* 2002) can be applied for the conservation of rare and engendered plants in general, and aromatic and medicinal plants in particularly, by *in vitro* propagation and inoculation of growth-promoting fungi or bacteria.

ln order to salve the acclimatization problems, various aspects of *in vitro* and *in vivo* acclimatization have been studied (in El Meskaoui *et al*., 1995). ln the present experiment, our aim was to inoculate *in vitro* plants by tripartite culture system during the last step of micropropagation, i.e. before their transfer to *in vivo* conditions (El Meskaoui *et al*., 1995) and to verify if *in vitro* VAM colonization as maintained under *in vivo* conditions and to determine their influence on the growth of micropropagated plantlets during acclimatization stage.

Material and methods

Biological material

Strawberry (*Fragaria x ananassa* Duch. cv Kent) cultures were established, multiplied and subcultured as previously described by El Meskaoui (1994). Axillary buds located at the base of cultures were excised and transferred on Sorbarod cellulose plug (Baumgartner Papier SA, Lausanne, Switzerland) containing rooting liquid medium as described by Kartha *et al*. (1980). The cultures were kept in a growth room at a temperature of 23 °C under a photosynthetic photon flux of 60 µmol. m^{-2} s^{-1} for a photoperiod of 16 h provided by cool-white fluorescent lamps.

The VA mycorrhizal fungus used in this study was *Glomus intraradix* Schenk and Smith (DAOM 197198, Biosystematic Reasearch Center, Ottawa, Canada). RiT-DNA transformed roots of Carrot (*Daucus carota* L.) served as partner for fungal inoculums used to inoculate micropropagated plantlets.

Primary mycorrhizal colonization

Primary mycorrhizal colonization was achieved by placing a single root explants with two groups of 20 *Glomus intraradix* non-germinated spores in the middle of a Magenta vessel (Magenta crop. Chicago, IL.) on a minimal medium (Figure 1A, El Meskaoui *et al*., 1995). The M medium contained the following ingredients per litre of distilled water: 731 mg $Mg SO_4. 7H_2O$; 80 mg KNO_3; 65 mg KCl; 4.8 mg Kh_2PO_4; 288 mg $Ca(NO_3)_2. 4H_2O$; 8 mg Na-FeEDTA; 0.75 mg KI; 6 mg $MnCI_2. 4H_2O$; 2.658 mg $ZnSO_4. 7H_2O$; 1.5 mg H_3BO_3; 0.13 mg $CuSO_4. 5H_2O$; 0.0024 mg $Na_2MoO_4. 2H_2O$; 3 mg glycine; 0.1 mg thiamine; 0.1 mg pyridoxine; 0.5 mg nicotinic acid; 50 mg myo-Inositol and 10 g sucrose. The pH of the media 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 (Gelgro, ICN Biochemicals, Cleveland, Ohio) added at a concentration of 0.3 % for the maintenance of root cultures and for the establishment of mycorrhizal associations. Afterwards, the vessels were sealed and incubated at 27 °C in the dark for five weeks. Since transformed carrot roots, display a negative geotropism, vessels were inverted to keep root apices growing into the medium (Figure 1A).

***In vitro* tripartite culture system (Figures 1A-D)**

Briefly, after primary mycorrhizal establishment and at two weeks of rooting stage, the rooting medium was removed by suction from cellulose plugs supporting rooted micropropagated plantlets and rinsed three times in sterile distilled water under sterile conditions. After washing, the minimal medium was added aseptically in the cellulose plugs (Sorbarod) supporting micropropagated plantlets. Then, the cellulose plugs were placed in contact with the primary mycorrhizae into culture vessel under aseptic conditions and transferred at 25 °C under Cool-White fluorescent lights supplying a PPF of 60 µmol. s^{-1} m^{-2} for a 16 h-photoperiod in small growth chambers enriched with 5,000 ppm CO_2 during 20 days as described in El Meskaoui *et al*. (1995). Culture vessel containing the same elements except for *Glomus intraradix* spores served as controls.

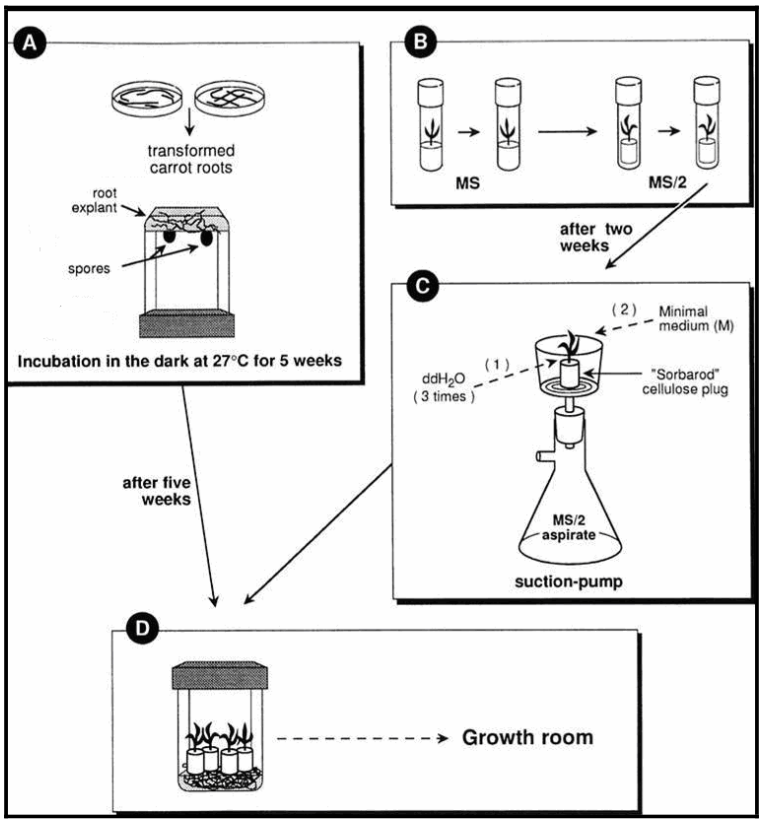

Figure 1. A-D. *In vitro* tripartite culture system used for establishment of mycorrhizae on micropropagated strawberry plantlets. **A.** Inoculation of tissue cultured roots (primary establishment). **B.** Micropropagation of strawberry plantlets. **C.** Transfer to rooting medium. **D.** Inoculation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

Plant acclimatization and greenhouse conditions

The main experiment was performed in a greenhouse of the Horticulture Research Centre at Laval University. ln vitro plantlets were first transferred from test tubes to multicell containers; cell volume was 100 cm^3. They were then grown in a small plastic tunnel and misted twice daily during the first two weeks to maintain a saturating air humidity for acclimatization. Temperature inside the tunnel was maintained at 23/20 °C ± 2 °C days/nights, respectively.

Plantlets were acclimatized for two weeks and then transferred to greenhouse conditions. Plants were watered as needed and fertilized weekly with 100 ml commercial solution which contained 480 mg $MgSO_4$. $7H_2O$, 750 mg Ca $(NO_3)_2$. $4H_2O$, 31.4 mg 10-52-10 N-P205-K20 (Plant Products Co Ltd., Bramalea, Ontario, Canada) and 550 mg 12-0-44 N-P205-K20 (Plant Products Co. Ltd) per litre. This nutrient solution was based upon the nutrient solution that of Long Ashton (Hewitt, 1966) and is particularly weak in phosphorus (P). The pH of solution was adjusted at 5.5.

Mycorrhizal infection and growth

Mycorrhizal infection ratio of roots was determined microscopically by counting the relative frequency of colonization in 1 cm root pieces (75 pieces per treatment). Every week for four weeks, roots of micropropagated plantlets were cleared in 10 % KOH at 90 °C for 10 min, rinsed with deionised water, immersed in 1 % HCl at 25 °C for 2 min and stained by chlorazol black at 90 °C for about 4.5 min. At weekly intervals, shoots and roots length and fresh weight were measured.

Experimental design

A randomized complete block design was used to evaluate the impact of *in vitro* VA mycorrhizal infection on acclimatization and growth of micropropagated strawberry plantlets. There were a total of 36 plants per treatment randomly distributed to 3 blocks.

Data analysis

An ANOVA was performed on data with the Statistical Analysis System (SAS institute Inc. 1982).

Results

Colonization rate

At all sampling dates root examination showed that VA mycorrhizal symbiosis performed *in vitro* was also maintained in acclimatization stage while non-inoculated micropropagated plantlets did not display any root infection during this time. Microscopic observations of mycorrhizal micropropagated plantlets roots clearly revealed typical vesicles, arbuscules and intraradical mycelia (Figures 2G & H). *In vitro* infected strawberry plants used for this work, were colonized by more than 45 % by VAM after two weeks of acclimatization stage. This value increased to about 68 % at the end of the experiment (Figure 3).

Rate-setting of the plant growth

All plantlets survived in greenhouse. No significant difference was detected in term of growth between inoculated and non-inoculated micropropagated plantlets during the first sampling date. After this date, we could notice some beneficial effects of the symbiosis on the growth of micropropagated plantlets. Shoot and root growth increased significantly for VAM inoculated treatments as compared to the control plants (Figures 4 and 5). Indeed, shoot and root length of mycorrhizal plantlets was increased about 31 % and 32 % respectively, whereas the shoot fresh weight was significantly increased about 35 % when compared to non-mycorrhizal plantlets (Figures 6 and 7).

Discussion

Recently, a novel and reliable technique to establish arbuscular mycorrhizal symbiosis in micropropagated plantlets and to evaluate their effect on the plant growth has been developed (El Meskaoui *et al*. 1995, Declerck *et al*. 1998). An *in vitro* tripartite culture system, consisting of co-culture of *in vitro* plantlets, VAM fungi, and a carrot-organ culture, has been proved useful in highlighting the capacity of VAM fungi to establish a Mutualistic association with *in vitro* plantlets (El Meskaoui *et al*., 1995). The tripartite culture system with CO_2

enrichment favors proliferation of the inoculums (Figure 2), growth of plantlets and establishment of the mycorrhizal association. Interestingly, it allows direct *in vitro* VAM inoculation in an axenic environment.

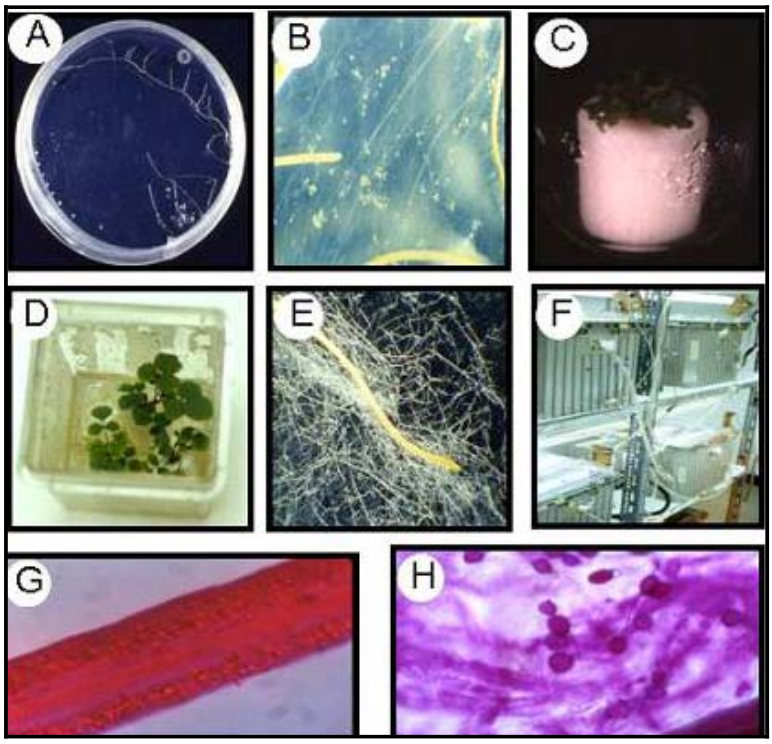

Figure 2. A-H. **A.** Transformed carrot roots. **B.** Mycorrization of tissue cultured root. **C.** Micropropagation of strawberry plantlet on cellulose plugs. **D.** Tripartite culture. **E.** Development of the mycorrizal colonization. **F.** Small growth chambers for carbon dioxide enrichment. **G** & **H.** Typical VAM on the root of micropropagated plantlets during in vitro and acclimatization stages.

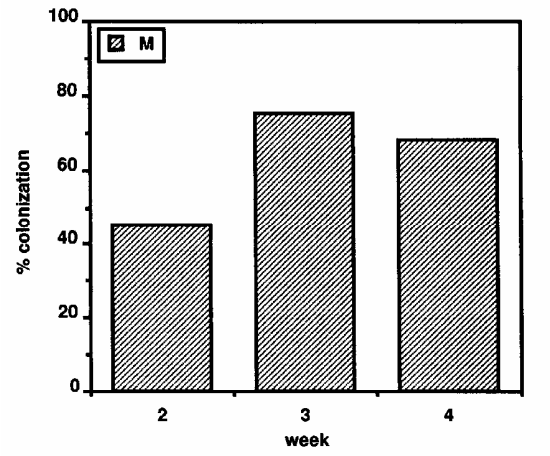

Figure 3. Percentage of roof length colonization of *in vitro* mycorrhizal micropropagated plantlets during acclimatization stage. Mean separation by Duncan multiple range test ($p < 0.05$).

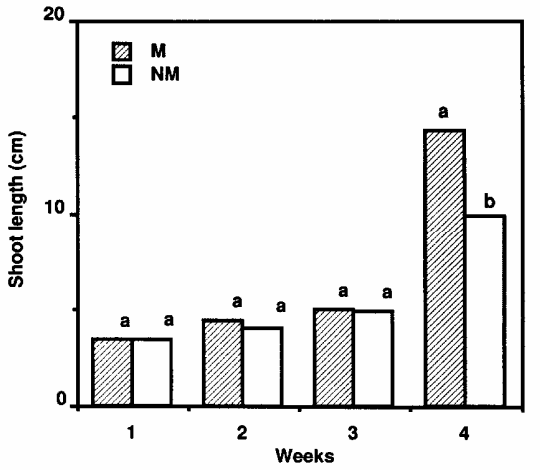

Figure 4. Shoot length of *in vitro* mycorrhizal strawberry plantlets during acclimatization stage. Mean separation by Duncan multiple range test ($p < 0.05$).

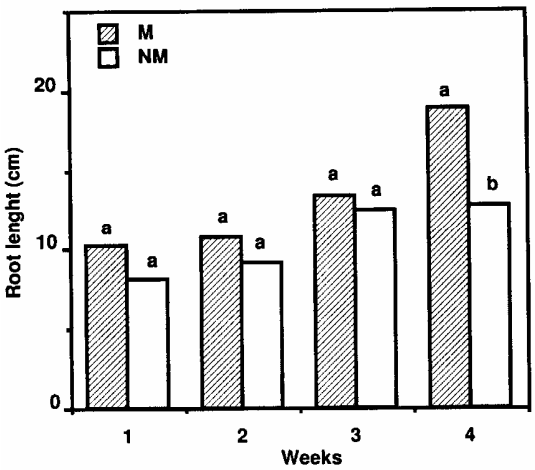

Figure 5. Root length of *in vitro* mycorrhizal strawberry plantlets during acclimatization stage. Mean separation by Duncan multiple range test ($p < 0.05$).

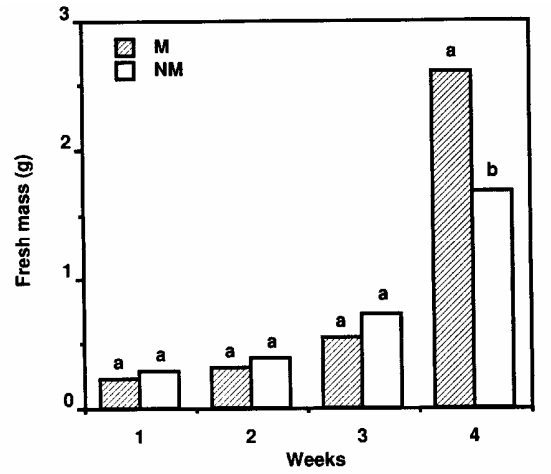

Figure 6. Shoot fresh mass of *in vitro* mycorrhizal strawberry plantlets during acclimatization stage. Mean separation by Duncan multiple range test ($p < 0.05$).

Our results provide evidences that VA mycorrhizal symbiosis, obtained during *in vitro* stage (by tripartite culture system), have been maintained during *in vivo* environment and influenced positively the behaviour plants. *ln vitro* VAM inoculation had a similar positive effect on growth of micropropagated plantlets as was observed when micropropagated strawberry plants were inoculated at the start of the acclimatization stage (Robertson *et al.* 1988, Vestberg 1992). ln our case, the beneficial effect were observed generally at the 4th week of acclimatization stage, independently of the intensity of roof fungal colonization. These results indicate that there is not always a strict relation between colonization rate and growth response of host plants. It has been reported that the most infective endophytes are not necessarily the most effective (Mosse *et al.*, 1973). At the week 4 of acclimatization stage, *in vitro* VAM inoculation improved root and shoot growth. Such responses were also noted by another investigator (Gerdemann, 1964). The absence of significant differences in growth rates of the mycorrhizal plantlets until the last week may be attributed to the fungi growth which might be under stress during the first two weeks after transfer to *in vivo* conditions.

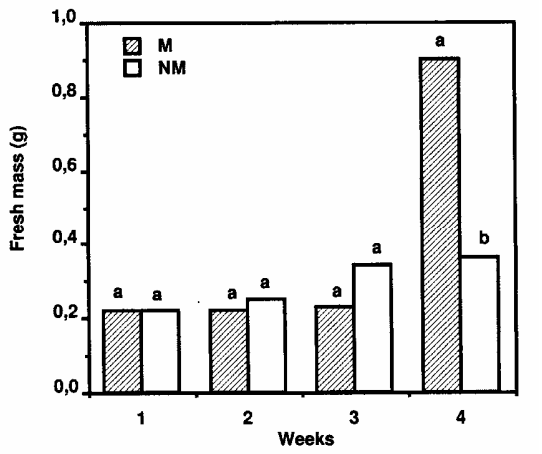

Figure 7. Root fresh mass of *in vitro* mycorrhizal strawberry plantlets during acclimatization stage. Mean separation by Duncan multiple range test ($p < 0.05$).

Even if the fungus was present tram *in vitro* stage its growth may have been checked by the stressful conditions found during the acclimatization stage. Another explanation for the absence of significant differences in growth between mycorrhizal and non-mycorrhizal plantlets may be the competition between symbionts for available photosynthetic products. Indeed, in our experiment the mycorrhizal strawberry plantlets were very young and were not capable to meet the requirements for development of the two symbiotic partners. The demand of partners may be

satisfied by the development at good photosynthetic ability of the host plant (Brown and Bethlenfalvay, 1988). ln this work, the absence of saturated humidity during the last two weeks of acclimatization, was coupled with the increase of growth of *in vitro* micropropagated plantlets compared with non-mycorrhizal plantlets.

The improved growth of the *in vitro* mycorrhizal strawberry plantlets grown under the absence of saturated humidity may be attributed to the growth of extraradical hyphae developed on mycorrhizal plants, which could have facilitated water and nutrient uptake VA mycorrhizal fungi (Hardie 1985, Davies *et al*. 1992). However, during the first two weeks no significant differences were detected between the mycorrhizal plantlets and non-mycorrhizal plantlets. Indeed, during this period the micropropagated plantlets were grown under saturated air humidity; therefore, the plantlets did not have to use any water loss control mechanism. Using this tripartite systeme, Hernández-Sebastià *et al*. (1999) showed that colonization of *in vitro* cultivated plantlets by *Glomus intraradices* increased relative plant water content. This effect was related to enhanced water content of mycorrhizal roots. However, root osmotic potential and dry weight did not significantly differ from that of non-mycorrhizal controls. In an attempt to explain this phenomenon, Hernández-Sebastià *et al*. (2000) proposed that mycorrhizal strawberry plantlets were able to change root cell amino-acid composition, and alter cell starch concentrations, to reduce water stress. This interestingly biological approach combining the micropropagation and mycorrhizae will be developed for propagation of some Moroccan aromatic and medicinal plants used in food and traditional medicine under investigation (Ennabili *et al*., 2006).

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