Chapter 14

IN VITRO MYCORRHIZATION OF MICROPROPAGATED PLANTS: STUDIES ON *CASTANEA SATIVA* MILL.

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Abstract: *In vitro* mycorrhization can be made by several axenic and nonaxenic techniques but criticism exists about their artificiality and inability to reproduce under natural conditions. However, artificial mycorrhization under controlled conditions can provide important information about the physiology of symbiosis. Micropropagated *Castanea sativa* plants were inoculated with the mycorrhizal fungus *Pisolithus tinctorius* after *in vitro* rooting. The mycorrhizal process was monitored at regular intervals in order to evaluate the mantle and hartig net formation, and the growth rates of mycorrhizal and nonmycorrhizal plants. Plant roots show fungal hyphae adhesion at the surface after 24 hours of mycorrhizal induction. After 20 days a mantle can be observed and a hartig net is forming although the morphology of the epidermal cells remains unaltered. At 30 days of root–fungus contact the hartig net is well developed and the epidermal cells are already enlarged. After 50 days of mycorrhizal induction, growth was higher for mycorrhizal plants than for nonmycorrhizal ones. The length of the major roots was lower in mycorrhizal plants after 40 days. Fresh and dry weights were higher in mycorrhizal plants after 30 days. The growth rates of chestnut mycorrhizal plants are in agreement with the morphological development of the mycorrhizal structures observed at each mycorrhizal time. The assessment of symbiotic establishment takes into account the formation of a mantle and a hartig net that were already developed at 30 days, when differences between fresh and dry weights of mycorrhizal and nonmycorrhizal plants can be quantified. *In vitro* conditions, mycorrhization influences plant physiology after 20 days of root–fungus contact, namely in terms of growth rates. Fresh and dry weights, heights, stem diameter and growth rates increased while major root growth rate decreased in mycorrhizal plants.

K**ey words**: *Castanea sativa;* micropropagation**;** mycorrhization.

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1 INTRODUCTION

European chestnut (*Castanea sativa* Mill.) has great economic interest for wood and fruit production but is difficult to propagate by cuttings and show high heterosis of seeds. *C. sativa* has been successfully micropropagated demonstrating that micropropagation of adult clones can provide an effective way to overcome propagation difficulties. However, micropropagated plants require a long and difficult adaptation period to *ex vitro* conditions. During the first step of weaning, roots obtained *in vitro* usually have a very low efficiency of absorption of water and nutrients (Bonga, 1977; Flick *et al.,* 1983).

Ectomycorrhizal (ECM) fungi bring several advantages to plants, including increased root area for absorption (Bowen, 1973; Harley and Smith, 1983), enhanced uptake of nutrients (Harley and Smith, 1983), resistance to plant pathogens (Marx, 1969), and drought (Duddridge *et al.*, 1980; Boyd *et al.,* 1986; Meyer, 1987; Feil *et al.,* 1988; Marx and Cordell, 1989). ECM can also increase growth and nutrient content of plants growing in low nutrient soils (Jones *et al.,* 1991). Water stress appears to be one of the major causes for the failure of micropropagated plants during acclimation. The compatible mycorrhizal fungi in the substrates during the weaning process not only improve the nutritional state of the plants, but also increase their resistance to the water stress of *ex vitro* conditions, increasing their weaning rates.

The first practical work to evaluate the role of mycorrhizae in plant growth was performed by Frank (1894) with seedlings of the *Pinus* sown in sterile and non-sterile soils. The results showed that plants from non-sterile soils could develop mycorrhizas and grew better than plants from sterilized soils (Smith and Read, 1997). Sterilization by heat was responsible for the production of toxic compounds that could be harmful for plant development. Other sterilizing methods and new methods of mycorrhizal synthesis were used along time, confirming the results originally shown by Frank (Hacskaylo, 1953; Marx and Zak, 1965; Trappe, 1962; 1967; Pachlewska, 1968; Skinner and Bowen, 1974; Mason, 1975; 1980; Mullette, 1976; Fortin *et al.,* 1980; 1983; Sohn, 1981; Biggs and Alexander, 1981; Nylund, 1981; Rancillac, 1983; Duddridge and Read, 1984a; b; Branzanti and Zambonelli, 1986; Kahr and Arveby, 1986; Kottke *et al.,* 1987; Wong and Fortin, 1988; Bougher *et al.*, 1990, Jones *et al.*, 1990). Mycorrhizae formed in non-sterile soils are responsible for the increased performances of the plants.

2 EFFECT OF MYCORRHIZA INOCULATION ON PLANT GROWTH

The beneficial effect of mycorrhizal associations is the enhanced uptake of mineral nutrients, namely phosphorus (Reid *et al.,* 1983; Jones *et al.,* 1990; Tam and Griffiths, 1993; Eltrop and Marschner, 1996; Smith and Read, 1997). Mycorrhizal symbiosis is frequently associated with increased photosynthetic rates of mycorrhizal plants (Harley and Smith, 1983; Reid *et al.*, 1983; Bougher *et al.,* 1990; Dosskey *et al.,* 1990; Rousseau and Reid, 1990; Guehl and Garbaye, 1990; Jones *et al.,* 1990; Martins, 1992; Martins *et al.,* 1997; Smith and Read, 1997). ECM may influence the assimilation capacity for CO_2 in two distinct forms: increased absorption of P and N in mycorrhizal plants influence the photosynthetic rates, as observed for forestry species when amended with P; the other resulting from enhanced flux of carbon compounds to the roots, promoted by mycorrhizal associations (Martins *et al.,* 1997; 1999). This hypothesis considers that the increased photosynthetic rates are related with the fungus necessity of carbon compounds and is named source-sink concept (Dosskey *et al.,* 1990; 1991) although this seems to be just one of mechanism involved in photosynthetic increment in mycorrhizal plants (Martins *et al.,* 1997; 1999).

The effect of mycorrhization on plant growth is well documented (Garbaye *et al.,* 1988, Bougher *et al.,* 1990, Grove and Le Tacon, 1993; Le Tacon *et al.,* 1997; Généré, 1995; Martins *et al.,* 1996; Généré *et al.,* 1997; Parladé *et al.,* 1997; Dell and Malajczuk, 1997). Bougher *et al.* (1990) made several trials in controlled inoculation of *Eucalyptus diversicolor* seedlings with different fungal species and different P supplementations. Plants with higher P and N availability (culture media or soil) showed increased metabolism of proteins and phosphorus compounds (nucleic acids and inositol phosphates). The synthesis of these compounds implies an increase in energy use (carbon compounds) and a lower translocation of carbon compounds to the root. The amount of root soluble carbon compounds condition the nutrition of the associated fungus interferes with the mycorrhizal infection rates (Le Tacon *et al.,* 1997). High levels of mineral nutrients, generally, decrease mycorrhizal efficiency or even infection rates. Under nutrient deficiency, growth rates of mycorrhizal plants increase. Bougher *et al.* (1990) also evidenced that the response of plants to mycorrhization does not depend only upon nutrient availability but also on the fungus species or even strains of a same species.

The abilities of mycorrhizal species and strains to promote plant growth opened new perspectives for the use of these fungi inoculations in nurseries and forestry. Inoculations of forestry species were performed with different species of hosts and fungi, under different conditions and inoculum

types. The influence of mycorrhization on growth rates reveal that plants grow better (Grove and Le Tacon, 1993; Tam and Griffiths, 1994; Eltrop and Marschner, 1996; Le Tacon *et al.,* 1997; Généré 1995; Généré *et al.,* 1997; Parladé *et al.,* 1997; Dell and Malajczuk, 1997), have more extended root systems and both roots and shoots have increased dry weights, although the ratio between the dry weights of roots and shoots were lower for mycorrhizal plants. The similar results were observed in young plants growing with high nutrient levels, behaving like mycorrhizal plants in comparison with plants growing with limited nutrient levels, exhibit nonmycorrhizal like growth (Smith and Read, 1997).

The difference in ratio between dry weights of roots and shoots is more related to plant dimension than to the colonization rate (Bougher *et al.,* 1990). The total number of short roots of mycorrhizal plants is higher than for nonmycorrhizal ones, exhibiting completely altered root morphology by the association with the mycorrhizal fungi. The number of roots per unit length and per unit weight was higher for mycorrhizal root systems (Brundrett *et al.,* 1996). Root colonization by mycorrhizal fungi can result in lower plant growth rates if fungus compatibility, nutrient availability, light intensity or temperature is not suitable for plant development (Marx and Bryan, 1971; Marx, 1979; Nylund and Wallander, 1989; Dosskey *et al.,* 1990; Colpaert *et al.,* 1992; Conjeaud *et al.,* 1996; Smith and Read, 1997). Decrease of growth rates is expected when a symbiont depends on the others to obtain the carbon compounds for survival, and the other depends on the essential mineral nutrients provided by the former for its growth and photosynthesis. Decrease in growth is also expected under light conditions limiting photosynthesis (Conjeaud *et al.,* 1996), nutrient availability in soil, conditioning plant growth but not colonization intensity (Colpaert *et al.,* 1992; Smith and Read, 1997). Son and Smith (1988) observed an increase in plant growth after colonization of plants under high PAR (photosynthetic active radiation) and a decrease in growth of plants colonized under low PAR, independently of the levels of P availability. When nutrient availability allows fungal growth and there is no light or temperature limitation, fungal growth can require large amounts of carbon compounds conditioning plant growth (Colpaert *et al.,* 1992).

3 *IN VITRO* MICORRHIZATION

Large numbers of in vitro studies have been carried out to evaluate the factors that influence mycorrhization. Under natural conditions, interactions of biotic and abiotic factors make the interpretation of the results difficult. The methods of axenic synthesis are object of criticism because working under conditions where (1) interacting factors are eliminated, (2)

carbon sources are provided to allow fungal growth before the infection sets in, and (3) substrates are sterilized, may change the efficiency and type of infection (Piché and Peterson, 1988).

In parallel with *in vitro* studies, non axenic studies have been made (Fortin *et al.*, 1980; Piché *et al.*, 1982). It was possible to demonstrate that there are no significant differences between mycorrhizae synthesized under axenic and non axenic conditions (Piché and Peterson, 1988) other than the time of infection (Duddridge and Read, 1984a). The axenic system studied had a time of infection starting at 3 weeks and completed by weeks 6 to 8, while in natural soils, the association was retarded until 11 to 19 weeks. Morphological differences between axenic and non axenic synthesized mycorrhizae exist only when high sucrose levels are used (Duddridge and Read, 1984b). Under these conditions the host-fungus interface is changed and there is callose deposition at the cells walls in response to host infection.

Non axenic systems allow detailed studies of the root colonization by the fungus (Fortin *et al.*, 1983). Fungus connection to the root epidermis is due to the root polysaccharides secretion (Nylund, 1980). The translocation of photosynthetic products to the root increases the concentration of carbon compounds in root exudates. These are mainly amino acids, proteins, carbon compounds, organic acids and plant growth regulators. Mineral balance and plant growth regulators concentrations, directly control cell permeability and the mechanism of fungus adhesion to the roots when mycorrhization takes place (Barea, 1986).

Axenic and non axenic mycorrhizal syntheses mainly differ in the time and degree of infection (Duddridge and Read, 1984a). These findings validated the use of *in vitro* mycorrhization techniques. Mycorrhizas obtained by different methods of *in vitro* synthesis had mantles and hartig nets with similar structures (Brunner, 1991). Mantle thickness and number of hyphae penetrating between cortical cells may vary with substrate and the synthesis method used.

4 *IN VITRO* MYCORRHIZATION OF MICROPROPAGETED PLANTS

Micropropagated plants are adversely affected by water stress, either due to low absorption capacity of their roots or due to stomata deficient regulation of water loss (Bonga, 1977; Flick *et al.*, 1983). Acclimation of micropropagated plants corresponds to a transition period when roots become adapted to a substrate with less available nutrients, and to an autotrophic condition. At this stage, the presence of mycorrhizae could increase the availability of limiting nutrients such as phosphorus (P) and

nitrogen (N), facilitating the absorption. Water stress can be responsible for the low survival of many micropropagated woody plant species during the acclimation process and *C. sativa* is one of these species.

Micropropagated plants develop under high moisture and low lighting conditions, often with low lignifications levels and decreased functionality of the root systems that cause low survival rates to weaning. Mycorrhization of micropropagated plants before acclimation increases survival, enhancing the functionality of the root system and the mineral plant nutrition (Rancillac, 1982; Grellier *et al.*, 1984; Heslin and Douglas, 1986; Poissonier, 1986; Tonkin *et al.*, 1989; Martins, 1992; 2004; Martins *et al.*, 1996; Herrmann *et al.*, 1998; Díez *et al.*, 2000). Similarly, *in vitro* mycorrhization of micropropagated plants can be used to increase survival and growth during *ex vitro* weaning (Nowak, 1998).

Mycorrhization trials have been made with different micropropagated plant species: pine (Rancillac, 1982; Normand *et al.*, 1996), birch (Grellier *et al.*, 1984), poplar (Heslin and Douglas, 1986), eucalyptus (Poissonier, 1986; Tonkin *et al.*, 1989), oak (Herrmann *et al.*, 1998), chestnut (Strullu *et al.*, 1986; Martins, 1992; 2004; Martins *et al.*, 1996; Martins and Pais, 2005), cork oak (Díez *et al.*, 2000). These trials were performed as an effort to make micropropagation a sustainable propagation method for plant species recalcitrant to conventional propagation, increasing *in vitro* plant performances.

Herrmann *et al.* (1998) used an *in vitro* mycorrhizal system of *Quercus robur* micropropagated plants, intending to develop a method to analyze the mycorrhization of forest species without the constraints of the methods using seedlings. Genetic heterogeneity of seedlings (reflected in different germination times), seedling vigour and asynchronous development are only some of these constraints. These trials were made to work with (1) genetically uniform plants deprived of cotyledons, to function as older plants, (2) with selected material, to warranty the uniformity of repetitions, and (3) with a mycorrhizal system that allows following the development along the trials, in order to characterize mycorrhizal effects on plant morphology.

Castanea sativa micropropagated plants were studied along 90 days of plant-fungus association *in vitro*, after preliminary studies on plant-fungus compatibility with four fungi species (Martins *et al.*, 1996). The studies included: (1) development of mycorrhizal morphological structures (mantle and Hartig net) along 90 days; and (2) mycorrhizal influence on growth rates (heights, stem diameter, length of major root, total plant length, fresh weights and dry weights).

5 *CASTANEA SATIVA* MYCORRHIZATION *IN VITRO*

Plants were first inoculated with 4 different mycorrhizal fungi species to test their mycorrhizal capacities *in vitro*. *Amanita muscaria* Hooker isolate from Schönbuch/Tübingen, *Laccaria laccata* (Scop. ex Fr.) Berk and Br., isolate from Molina, *Piloderma croceum* Erikss and Hjortst, isolate from Unestam and Nylund 01.01.1976, Sweden and *Pisolithus tinctorius* (Pers.) Coker and Couch, isolate 289/Marx, were used (Martins *et al.,* 1996).The fungi tested differed in their capacity to form mycorrhizas with *C. sativa* plants *in vitro*. *P. tinctorius* showed the best capacity to colonize chestnut roots either from seedlings or from micropropagated plants(Martins *et al.*, 1996, 1997).

Mycorrhizal (M) and nonmycorrhizal plants (NM) were followed for 90 days since inoculation with the mycorrhizal fungus *P. tinctorius*. Mycorrhizal synthesis was performed in Petri dishes 13 cm in diameter, as can be seen in fig. 1. Agarized MS modified medium plated in slant was used (Murashige and Skoog, 1962). The plants were placed with the root system adhering to the medium in Petri dishes inoculated with the fungus (three weeks before). Control plants were placed in non-inoculated Petri dishes. The root system was covered with aluminium foil to prevent photo-oxidation (Martins and Pais, 2005).

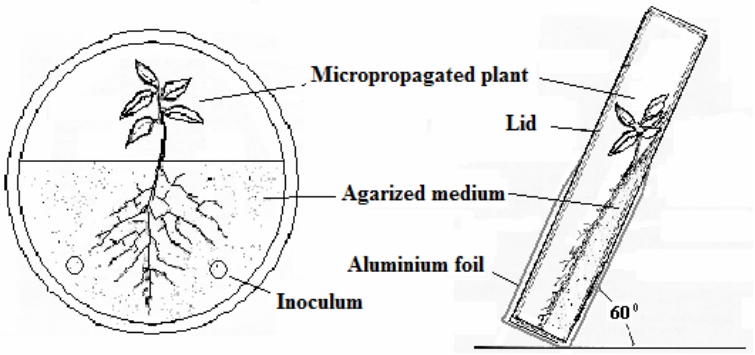

Fig. 1 – Axenic synthesis of micropropagated *Castanea sativa* mycorrhizas with *P. tinctorius.*

Mycorrhizal and nonmycorrhizal plants were maintained in a plant tissue culture chamber with a photoperiod of 16 h, light intensity of ~100 $\mu E \cdot m^{-2} \cdot s^{-1}$ and temperatures of 25 °C and 19 °C respectively during light and dark periods for 90 days after plant transference to the pre-inoculated media (Martins, 2004; Martins and Pais, 2005). Plant development was monitored along 90 days of *in vitro* mycorrhization. Root mycorrhizal status was observed at regular intervals and mycorrhizal evolution compared with

growth parameters for the same time of mycorrhization (Martins, 2004; Martins and Pais, 2005).

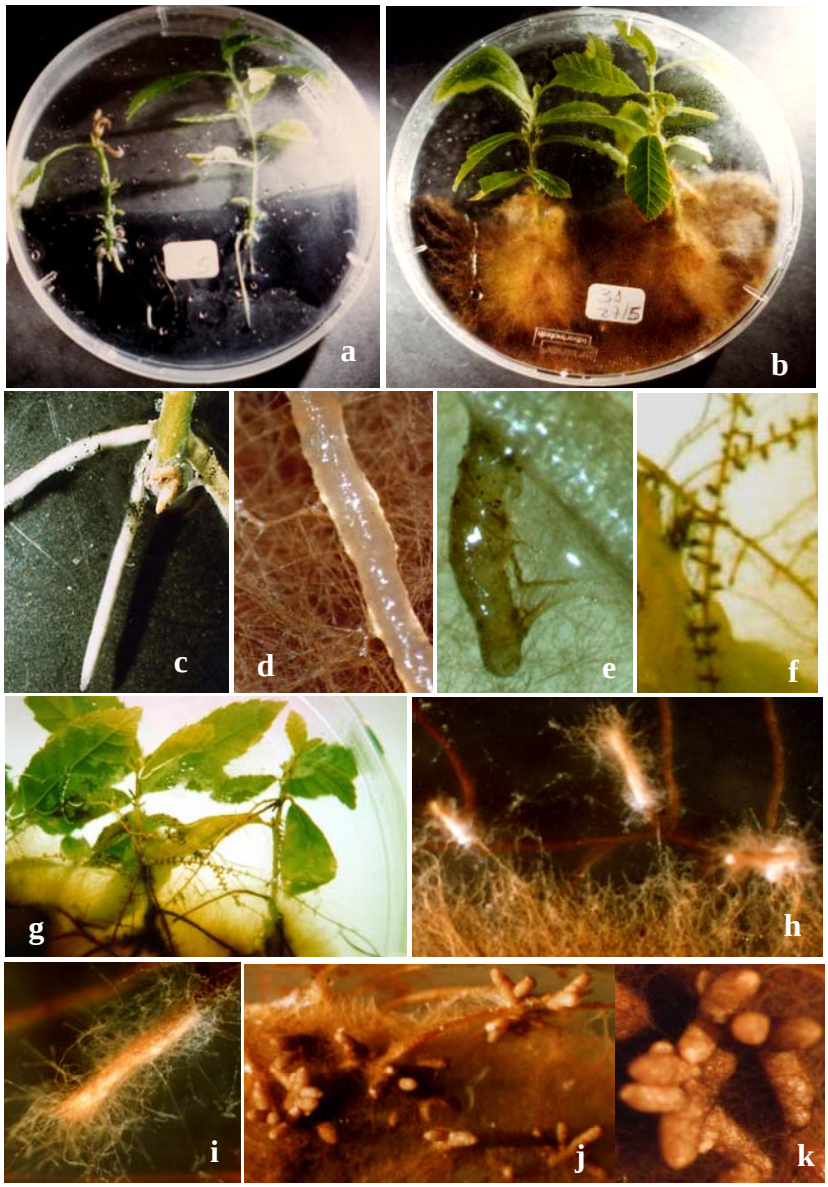

Fig. 2 – Micropropagated chestnut plants (a) 20 days after transference to Petri dishes without fungus; (b) 20 days after transference to Petri dishes with fungus (*P. tinctorius*); (c) Root system of a micropropagated plant before mycorrhization (7.5×); (d) Inoculated root 24 h after root-fungus contact (30×); (e) Inoculated root 10 days after root-fungus contact (60×); (f)

Inoculated roots 40 days after root-fungus contact (5×); (g) Mycorrhizal plants 40 days after inoculation; (h) Colonized root apices emerging from the medium (25×); (i) Details from a mycorrhizal apex (60×); (j) Inoculated root 60 days after root-fungus contact (40×); (k) Detail of colonized roots 60 days after inoculation (60×).

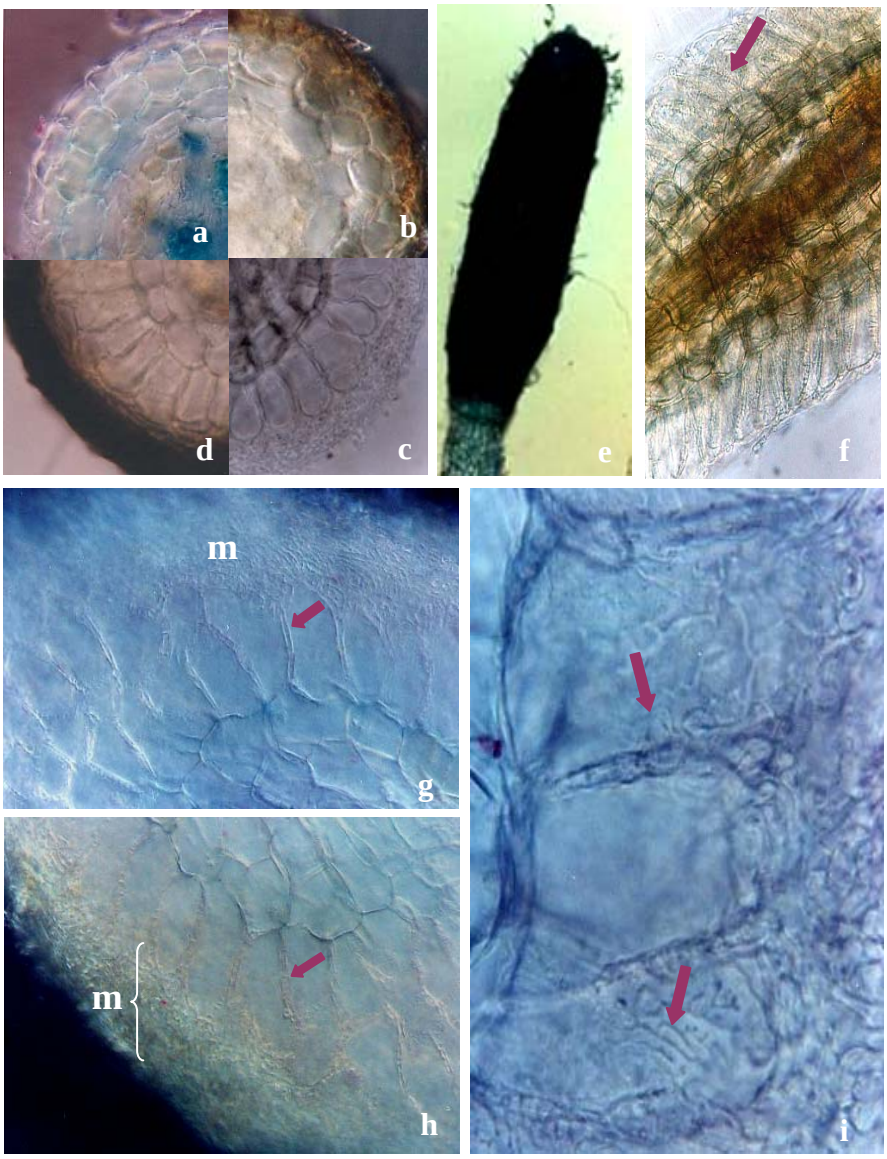

Fig. 3 –Cross sections of inoculated roots at different times after plant-fungus interaction (a) 10 days (100×); (b) 20 days (some hyphae can be seen at the surface and an Hartig net is forming,,100×); (c) 30 days (elongation of epidermal cells and a well developed Hartig net can be seen, 100×).; (d) 60 days (100×); (e) Mycorrhizal root 30 days after root-fungus contact

(40×); (f) Longitudinal section 30 days after root-fungus contact (100×); (g) - (i) Details of a cross section of a mycorrhizal root seen 30 days after root-fungus contact. The Hartig net is visible between the epidermal cells and a mantle (m) is well developed (400×/1000×).

Roots from micropropagated plants were white and without root hairs or ramifications (Fig. 2a, c) at the time of transference to co-culture with the fungus. *Pisolithus tinctorius* hyphae adhere to the root surface 24 h after root-fungus contact (Fig. 2d); after the first contact roots ramify very quickly compared with non-inoculated ones. Root ramifications became visible five days after inoculation, while control plants still have no ramifications. The fungus surrounds root ramifications forming mycorrhizae after 20 days (Fig. 2e). Establishment of mycorrhizas favours plant growth and leaf expansion (Fig. 2a, b). A successive ramification was observed after the first mycorrhizas form giving rise coralloid roots (Fig. 2j, k). The microscopical observation showed that a mantle (m) forms after 20 days but, the hartig net, with longitudinal elongation of epidermal cells, could only be observed after 30 days of root-fungus contact (Fig. 3).

6 GROWTH OF MYCORRHIZAL *CASTANEA SATIVA*

The mycorrhizal process was monitored 90 days of plant-fungus co-culture, to evaluate the growth rates of mycorrhizal and nonmycorrhizal plants, in terms of heights (h), stem diameters at the collar level (d_{collar}), lengths of the major roots (l_{root}) and total plant lengths (l). Fresh weight (FW) and dry weight (DW), as well as growth rates ($\Delta x/\Delta t$) and relative growth rates for each parameter (x) (RGR(x) = $(1/x)\times(\Delta x/\Delta t)$) were also determined for 90 days of association (Martins and Pais, 2005).

Plant heights and stem diameter at the collar level were higher in M plants after 50 and 40 days of mycorrhization respectively while the maximum root length was smaller in M plants after 40 days. The total growth at the end of 90 days (Δx) and the growth per unit time ($\Delta x/\Delta t$) were significantly higher of M plants. After 90 days, M plants had higher growth rates in heights and stem diameter at the collar level. The length of the major root had lower growth rates in M plants. The relative growth rates (RGR) also showed differences between M and NM plants with the exception of the total plant length (Table 1). The larger differences in RGR occurred in the length of the major root. RGR values obtained for the growths in height and stem diameter were higher in M plants. RGR values for total plant length were not significantly different between M and NM plants (Table 1). The ratio h/d_{collar} showed that M plants had a higher increased growth in stem diameter in comparison with growth in heights. The ratios between h/l_{root}

were also significantly different in M and NM plants after 50 days of mycorrhization, showing that in M plants the increase in heights was higher than the increase in root length (Table 1).

Table 1 - Growth parameters of the plants in terms of heights (h), stem diameter at the collar level (D_{collar}). Increases of growth (Δx), increases of growth per day ($\Delta/\Delta t$) and relative growth rates (RGR) per plant, in NM and M plants along 90 days of mycorrhization.

	Heights		**Diameter**		**Length major root**		**Maximum plant length**	
	NM	M	NM	M	NM	M	NM	M
	h (cm)		d_{collar} (cm)		l_{root} (cm)		l_{max} (cm)	
Δx (cm)	6.2a	8.2b	0.15a	0.26b	9.1b	3.8a	15.3b	12.0a
$\Delta x/\Delta t$ (mm/day)	0.69a	0.91b	0.02a	0.03b	1.01b	0.42a	1.70b	1.33a
RGR (mm/cm.day)	0.06a	0.07b	0.06a	0.07b	0.08b	0.05a	0.07a	0.06a

Fresh and dry weights of M and NM plants were larger in M plants after 30 and 20 days of root-fungus contact, respectively. Differences in FW and DW of roots between M and NM plants were earlier than other plant organs. Roots of M plants showed higher FW and DW than NM plant roots, since 20 days, while the shoots only showed differences since 50 days. Increments in DW (ΔDW, ΔDW/Δt) and RGR were significantly higher for M plants (Table 2). The leaves were the plant organs that showed larger increments in DW after 90 days (ΔDW) and per day (ΔDW/Δt) (Table 2). The ratios DW/FW of plant roots and the whole plant are higher for M plants since 75 days of mycorrhization. The differences between the ratios for M and NM plants increase along the mycorrhizal synthesis.

Table 2 - Increments in DW (ΔDW), DW increments per day (ΔDW/Δt) and relative growth rates (RGR) per plant in NM and M plant roots, stems, leaves, shoots and whole plant, along 90 days of mycorrhization.

	Dry weight/Plant organ (mg)						**Dry weight/Plant (mg)**			
	Roots		Stem		Leaves		Shoots		Plant	
	NM	M	NM	M	NM	M	NM	M	NM	M
ΔDW (mg)	39.5a	51.2b	30.5a	39.5b	43.7a	57.3b	74.2a	96.8b	114.4a	148.2b
ΔDW/**Δt** (mg/d)	0.4a	0.6b	0.3a	0.4b	0.5a	0.6b	0.8a	1.1b	1.3a	1.6b
RGR (mg/g.d)	9.6a	9.9b	7.6a	8.2b	8.7a	9.3b	8.2a	8.8b	8.7a	9.2b

7 CONCLUSIONS

In vitro mycorrhization (endo and ectomycorrhizas) of micropropagated plants can be used to increase survival and growth during *ex vitro* weaning (Martins *et al.*, 1996; Nowak, 1998). In the case of fruit trees, the inoculations of arbuscular fungi facilitate *in vitro* plants adaptation to *ex vitro* conditions (Sbrana *et al.*, 1994). However, *in vitro* ectomycorrhization can improve microcutting rooting (Normand *et al.*, 1996) and enables vitro plants to acclimate more readily (Martins *et al.*, 1996, Díez *et al.*, 2000). The *in vitro* mycorrhization of micropropagated plants like *Helianthemum* spp. (Morte *et al.*, 1994) and *Cistus* spp. (Díez and Manjón, 1996) has been obtained only in very few mediterranean species. Even somatic embryos acclimation can be improved through mycorrhization (Díez *et al.*, 2000). Increase in the root functioning and mineral nutrition of the plants through mycorrhization prior to the acclimation phase can overcome the low performance of micropropagated plants improving their survival and weaning (Martins, 1992, 2004; Martins *et al.*, 1996; Herrmann *et al.*, 1998, Díez *et al.*, 2000**).**

Under *in vitro* conditions, mycorrhization increases the growth parameters of plants and those are in consistency with the morphological development of mycorrhizal structures, for the same times of mycorrhization. Micropropagated plants improve their performances and survival capacities also increase accordingly. Micropropagation and mycorrhization can be combined as a tool to give viability to the production of difficult propagating species, increasing their survival and growth. Mycorrhization can provide a sustainable method for plant production, either by micropropagation or through traditional propagation methods.

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