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Effect of ectomycorrhizal fungi on survival and growth of micropropagated plants and seedlings of *Castanea sativa* mill.

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Abstract Four ectomycorrhizal fungi (*Amanita muscaria, Laccaria laccata, Piloderma croceum* and *Pisolithus tinctorius*) were used to produce mycorrhiza on seedlings and micropropagated plants of *Castanea sativa* in vitro. *Pisolithus tinctorius* was most effective in colonizing roots of both micropropagated plants and seedlings. *A. muscaria* and *L. laccata* only colonized a few feeder roots of some plants and *Piloderma croceum* did not form mycorrhizas. Mycorrhization of micropropagated plants increased survival and growth during weaning.

Key words Micropropagation · Ectomycorrhizas · In vitro synthesis · *Castanea sativa*

Introduction

European chestnut (*Castanea sativa* Mill.) is a forest tree of great economic interest for wood and fruit production. This species is difficult to propagate by cuttings and shows high heterosis of seeds. Recently, adult clones of *C. sativa* have been successfully micropropagated (Feijó and Pais 1992). However, micropropagated plants require a long and difficult period of transition to become adapted 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).

Ectomycorrhizas offer several advantages to plants, including an increased root absorbing area (Bowen 1973, Harley and Smith 1983), enhanced nutrient uptake (Harley and Smith 1983), increased host resistance to plant pathogens (Marx 1969) and to drought (Duddridge et al. 1980; Boyd et al. 1986; Meyer 1987; Feil et al. 1988; Marx and Cordell 1989; Guehl et al. 1992). Ectomycorrhizas can also cause an increase in 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 acclimation of micropropagated plants. The use of compatible mycorrhizal fungi in the substrate during the weaning process may not only improve the nutritional state of the plants, but also increase their resistance to water stress in ex vitro conditions. We report here on the in vitro synthesis of ectomycorrhizas using seedlings and micropropagated plants of *C. sativa* and on their effect on survival and growth during the acclimation process.

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Materials and methods

Castanea sativa seedlings and micropropagated plants, were obtained from buds of old trees (>50 years) following the method described by Feijó (1989).

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) and *Pisolithus tinctorius* (Pers.) Coker and Couch (isolate 289/Marx) were maintained on a MMN agar medium (Marx 1969).

Preliminary assays were carried out with the four fungal species, micropropagated plants and seedlings in order to find the most efficient fungus for mycorrhization. Subsequent studies only used *Pisolithus tinctorius* and micropropagated plants.

Experiment S1

Seeds were surface sterilized with sodium hypochloride (5%) for 30 min. After sterilization, the tegument was removed and part of the cotyledons excised. Germination was carried out in sterile, hydrated perlite in a growth chamber under a 16 h-light period, ~100 μE m^{-2} s^{-1} quantum flux density (Sylvania Gro-lux fluorescent lamps), 25 °C in the light period, 19 °C in the dark period. The same conditions were used for micropropagation. In vitro mycorrhization was induced in seedlings 4 weeks after sowing and in micropropagated plants 5 weeks after root induction. Plants were transferred to 1000-ml flasks containing 250 ml sterile sub-

strate of sphagnum peat: perlite (1:3), containing MMN (Melin Norkrans modified medium with 5 g/l glucose, but without malt extract and casamino acids). Inoculation of the substrate with mycorrhizal fungi was done at the time of plant transfer. Five inocula plugs (0.5 cm^2 mycelium grown on MMN agar medium), obtained from 3-week-old fungal cultures, were introduced into the substrates equidistant from the four plants in the flask, one of which was in a central position.

Plants were maintained in the growth conditions described above. Thirty weeks after mycorrhizal inoculation, the plants were transferred to 200-ml pots containing a mixture of sphagnum peat: perlite (1:1) as substrate. Weaning occurred during 10 weeks in a chamber starting with saturated humidity and decreasing in humidity 10% every 2 weeks until environmental relative humidity was reached.

Quantitative assessment of mycorrhiza was made by visually according to Grand and Harvey (1982). Roots were observed under a Wild-M8 stereomicroscope. Hand-sections from material fixed in acetic acid:ethanol (1:3) were stained with safranin and observed under a Wild-Leitz-Dialux microscope. Semi-thin sections from material fixed with glutaraldehyde (2% in 0.1% cacodylate buffer, pH 7.0)/osmium tetroxide (1%), dehydrated in a graded acetone series, embedded in Spurr resin and double-stained with safranin-methylene blue (Warmke and Sheu-Ling 1976), were observed by light microscopy.

Roots for scanning electron microscopy were fixed in a glutaraldehyde solution (2% in 0.1% cacodylate buffer, pH 7.0), dehydrated in a graded acetone series and critical point dried. Material was mounted on scanning electron microscope stubs, gold coated and observed in a Jeol SM T220 scanning electron microscope.

Plant survival was calculated at the moment of plant transfer to ex vitro conditions, 30 weeks after mycorrhizal inoculation. Height was measured during and after the acclimation process of seedlings.

Experiment S2

The growth substrate described above was inoculated in pots with *Pisolithus tinctorius* 3 weeks before plant transfer by introducing five inocula plugs (0.5 cm^2) of mycelium grown on MMN agar medium. Plants were transferred to the substrate 4 weeks after root induction and placed between the inocula. Weaning in pots occurred for 10 weeks as described above. As in experiment S1, roots were monitored and assessed for mycorrhizas according to Grand and Harvey (1982).

Plant survival was calculated at time of plant transfer to ex vitro conditions and 10 weeks after transfer. Heights of micropropagated plants were measured during and after the acclimation process. Leaf area and fresh weight of whole plants, roots, shoots, leaves and stems were measured as well as the ratio of fresh and dry weights of roots, leaves and stems at the time of plant transfer to ex vitro conditions. Total leaf area and unit leaf area were calculated in control and mycorrhizal plants 8, 10, 12 and 14 weeks after inoculation and during the weaning process (18, 25 and 30 weeks of ex vitro acclimation). Leaf areas were measured with an area meter LI-COR, LI 3000 and LI 3000 A.

Significance of differences was analyzed by $\chi 2$ test for plant survival and using variance analysis for height and fresh weight. Significance of calculated F values was established for $P<5\%$, 1% and 0.1%.

Results

The fungi tested in experiment S1 differed in their capacity to form mycorrhizas with *C. sativa* plants (Table 1). *Pisolithus tinctorius* showed the highest capacity to colonize chestnut roots either from seedlings or from micropropagated plants. Thirty weeks after inoculation, 50% of the inoculated plants were mycorrhizal and 75% of lateral roots showed a true mantle and a well-developed Hartig net (Fig. 1f).

Table 1 Percentage of mycorrhization of *Castanea sativa* seedlings and micropropagated plants 30 weeks after mycorrhiza inoculation. Mean values within a sampling followed by different letters are significantly different at $P<0.05$

Fungi	Seedlings	Micropropagated plants
Amanita muscaria	35.3 a	30.4 a
Laccaria laccata	25.5 a	22.5 a
Pisolithus tinctorius	54.8 a	47.5 b
Piloderma croceum	0.0 b	0.0 c

Amanita muscaria and *L. laccata* colonized only 10% and 20% of the lateral roots, respectively (Fig. 1a) and only 20% and 30% of the inoculated plants were mycorrhizal. *Amanita muscaria* formed a true mantle, but in most cases it did not form a Hartig net. All roots, even nonmycorrhizal, showed an external mycorrhizal morphology, with short lateral branching. When the Hartig net was formed, elongation of epidermal cells was observed.

Laccaria laccata formed a thin mantle and penetrated the epidermal cells forming a true Hartig net in 20% of lateral roots. Most of the nonmycorrhizal roots showed short lateral branches. The mycorrhizas obtained with this fungus seemed to be unstable, since root growth of the apex was not accompanied by fungal growth (Fig. 1b).

Piloderma croceum induced lateral branches but did not colonize *C. sativa* roots.

Mycorrhization of micropropagated plants only occurred on ex vitro-formed roots. Roots obtained in vitro never formed mycorrhizas. Root morphology and mycorrhiza extension were not significantly different between seedlings and micropropagated plants. Roots from uninoculated micropropagated controls showed very poor or no branching when compared with those from seedlings or from mycorrhizal micropropagated plants.

At the moment of ex vitro transfer in experiment S1, plants mycorrhizal with *A. muscaria*, *L. laccata* and *Pisolithus tinctorius* had significantly better survival than other treatments (Table 2). Plants mycorrhizal with *Pisolithus tinctorius* had the highest survival, followed by those inoculated with *A. muscaria*, *L. laccata* and *Piloderma croceum* (Fig. 2). The differences in survival between plants mycorrhizal with *Pisolithus tinctorius*, *L. laccata*, *Piloderma croceum* and control plants are significant for both seedlings and micropropagated plants but not significant comparing *Pisolithus tinctorius* and *A. muscaria* ($P>0.05$).

Plants mycorrhizal with *Pisolithus tinctorius* were higher than all other plants (Figs. 2, 3). Height was highly influenced by fungal presence in the substrate, even without mycorrhiza formation (Fig. 2). At 0, 180 and 290 days of weaning plants mycorrhizal with *Pisoli-*

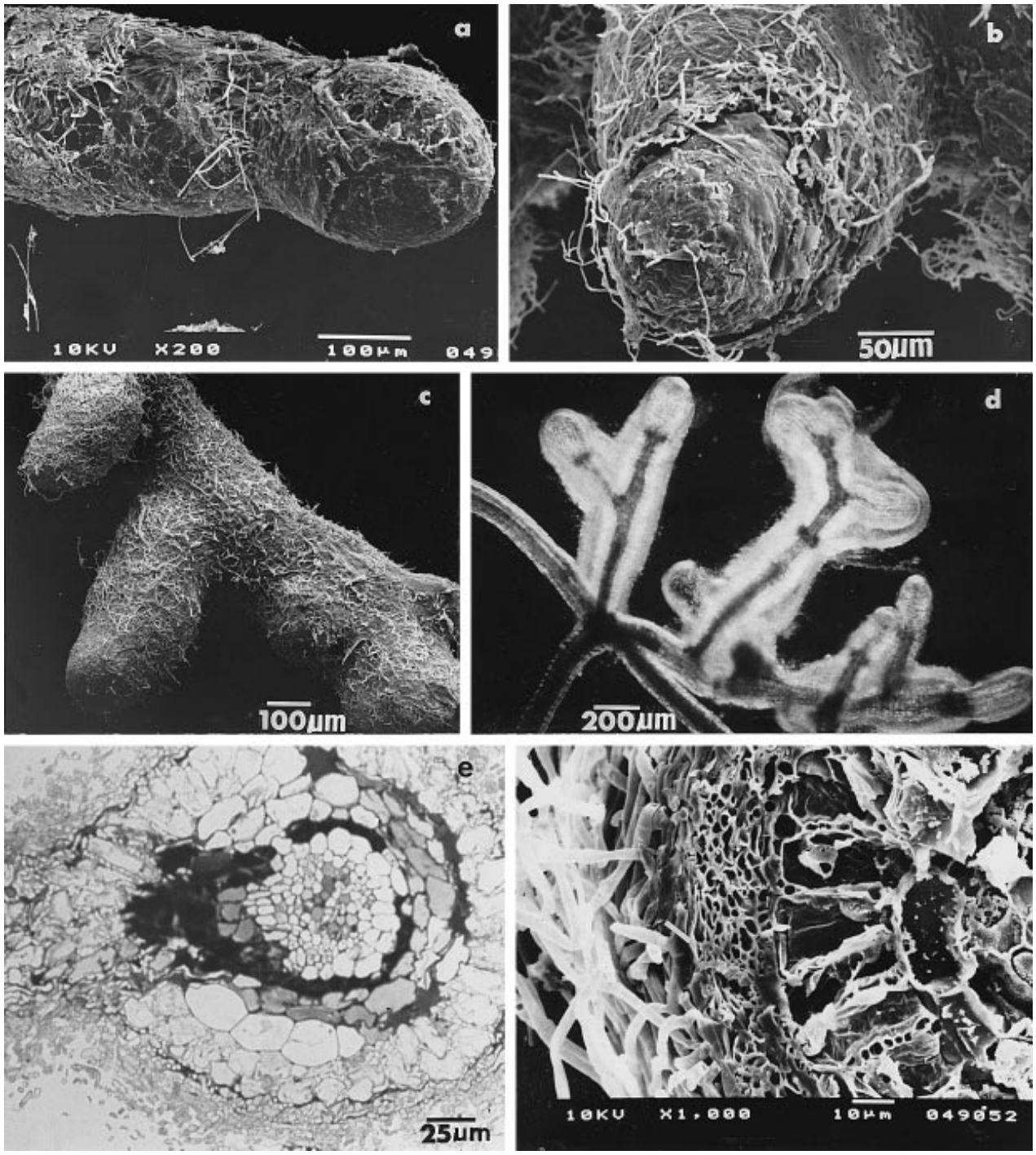

Fig. 1 **a** Scanning electron microscopy (SEM) of *Amanita muscaria* mycorrhiza. **b** SEM of *Laccaria laccata* mycorrhiza. Notice that the root apex growth is not enveloped by the hyphal mantle. **c** SEM of *Pisolithus tinctorius* mycorrhiza 5 weeks after inoculation. **d** Light micrograph of *P. tinctorius* mycorrhiza 5 weeks after inoculation. **e** Light micrograph of a cross-section from a *P. tinctorius* mycorrhiza 5 weeks after inoculation. Elongation of epidermal cells and the Hartig net are apparent. **f** SEM of a cross section of a *P. tinctorius* mycorrhiza 30 weeks after inoculation. The Hartig net between the elongated epidermal cells is apparent

thus tinctorius showed the best growth compared with all other treatments. Differences between plants mycorrhizal with *Pisolithus tinctorius* and the treatments increased with weaning time (except for plants mycorrhizal with *A. muscaria*).

In experiment S2, *Pisolithus tinctorius* formed mycorrhizas 5 weeks after plant inoculation. Ca. 80% of the inoculated plants formed mycorrhizas and more than 50% of the roots were colonized and had a well-developed Hartig net, elongated epidermal cells and typical root branching (Fig. 1c, d, e).

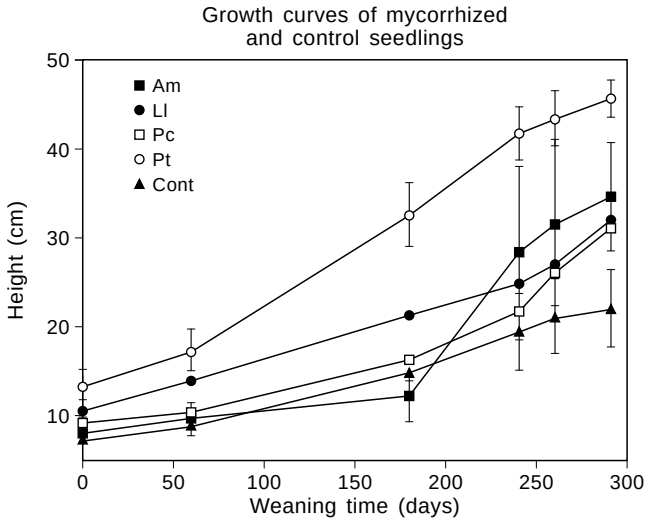

Fig. 2 Average growth curves of seedlings mycorrhizal with *Amanita muscaria* (Am), *Laccaria laccata* (Ll), *Pisolithus tinctorius* (Pt), *Piloderma croceum* (Pc) and control (Cont) after ex vitro transfer (day 0) until 290 days of weaning ($P<0.05$ for all measured times); *bars* SEM

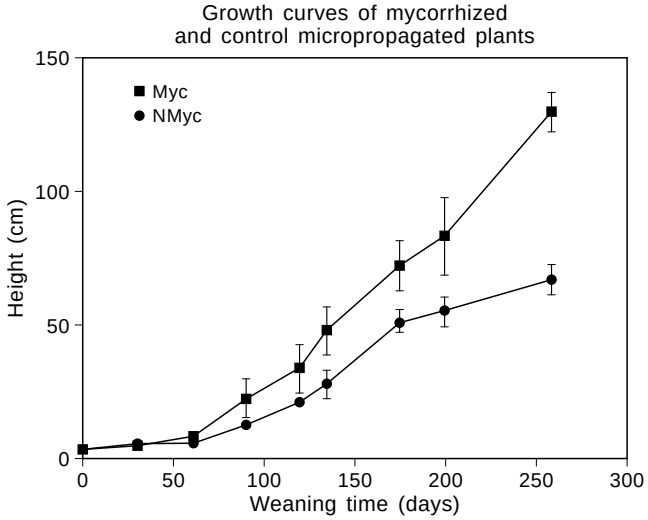

Fig. 3 Average growth curves of micropropagated plants mycorrhizal with *Pisolithus tinctorius* (Myc) and control plants (NMyc) ($P<0.05$ from day 175 of weaning); *bars* SEM

Plant survival in experiment 2 increased significantly after mycorrhization (Table 3). Inoculation of the substrates before plant transfer allowed a faster association of the fungus with the plant and consequently increased the number of surviving plants (47.5% S1 vs 83.0% S2 for *Pisolithus tinctorius* mycorrhizal plants) (Tables 2, 3). Survival of mycorrhizal plants at ex vitro transfer was significantly better when compared with the control plants (83% vs 62.9%) and they continued to be significant (72.2% vs 49.4%) 10 weeks after starting the weaning process.

Growth in height during experiment S2 showed a pattern similar to that in experiment S1 (Fig. 3). Differences in growth of mycorrhizal and control plants were not significant until 135 days of weaning.

Also in experiment S2, fresh weights of mycorrhizal plants at the time of transfer to ex vitro conditions were higher compared with control plants (Table 4). Major differences were found for root fresh weight. The shoot/root ratios were significantly lower for mycorrhizal plants (2.0) than for control plants (3.4). Differences in the leaves/stems ratio between mycorrhizal plants and the controls were not significant. The ratio of dry and fresh weights of leaves, stems and roots from mycorrhizal and control plants were not significantly different.

Leaf area of micropropagated mycorrhizal plants 8–13 weeks after inoculation and control plants were not statistically significant. After 18 weeks of ex vitro acclimation, leaf area of mycorrhizal plants was significantly larger than control plants either for total leaf area or for area per leaf (Table 5).

Table 2 Percentage of survival of *C. sativa* seedlings and micropropagated plants 30 weeks after mycorrhiza inoculation with four different fungi. Mean values within a sampling followed by different letters are significantly different at $P<0.05$

Fungi	Seedlings	Micropropagated plants
Amanita muscaria	35.3 a	30.4 a
Laccaria laccata	25.5 a	22.5 a
Pisolithus tinctorius	54.8 a	47.5 b
Piloderma croceum	19.5 b	13.3 c
Control	19.4 b	9.1 c

Table 3 Percentage of survival of micropropagated mycorrhizal and control plants, before and after weaning. Mean values within a sampling followed by different letters are significantly different at $P<0.05$

Material	Before weaning	After weaning
Mycorrhizal	83.0 a	72.2 a
Control	62.9 b	49.4 b

Discussion

Only *Pisolithus tinctorius* was able to form complete mycorrhizas in both seedlings and micropropagated *C. sativa* plants. *A. muscaria* and *L. laccata* stimulated roots, inducing changes in root morphology, plant growth and survival without forming extensive mycorrhizas.

None of the fungi tested had been previously shown to form mycorrhizas with chestnut. *Pisolithus tinctorius* was very effective for in vitro mycorrhization of *C. sativa* micropropagated plants, and its broad host range and confirmed capacity to improve survival and growth of tree seedlings (Marx and Cordell 1989) indicate that

Table 4 Fresh weights of roots, shoots, leaves and stems of mycorrhizal and control plants. Mean values within a sampling followed by different letters are significantly different at $P<0.05$

Fresh weight per plant (mg)							
Material	Roots	Shoots	Leaves	Stems	Plant	Shoot/Root	Leaves/Stem
Mycorrhizal	114.46 a	233.70 a	96.93 a	91.56 a	348.15 a	2.01 a	1.06 a
Control	51.56 b	175.90 b	51.08 a	76.35 b	218.48 b	3.4 b	0.67 a

Table 5 Leaf area of plants mycorrhizal with *Pisolithus tinctorius* and nonmycorrhizal plants, 18, 25, and 30 weeks after weaning. Mean values within a sampling followed by different letters are significantly different at $P<0.05$

Leaf area (cm^2)						
Material	18 weeks		25 weeks		30 weeks	
	Total	Leaf area/leaf	Total	Leaf area/leaf	Total	Leaf area/leaf
Mycorrhizal	1063.5 a	76.0 a	2197.0 a	115.7 a	2680.9 a	167.6 a
Control	360.3 b	37.9 b	1005.8 b	73.2 b	1280.2 b	91.4 b

Pisolithus tinctorius could be a biological tool to improve survival and growth of *C. sativa* micropropagated plants, as previously proposed for pines by Marx and Cordell (1989).

Micropropagated plants are adversely affected by water stress, either due to a low nutrient absorption capacity of their roots or due to deficient stomatal regulation of water loss (Bonga 1977; Flick et al. 1983). Acclimation of micropropagated chestnut 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 mycorrhizas could increase the availability of limiting nutrients such as phosphorus and nitrogen by facilitating their absorption. Water stress may be responsible for the low survival of *C. sativa* plants during the acclimation process. The best results for survival of *C. sativa* were obtained for plants mycorrhizal with *Pisolithus tinctorius* although survival of both micropropagated plants or seedlings was improved by *L. laccata* and *A. muscaria.* According to Marx and Cordell (1989), *Pisolithus tinctorius* increases plant survival and also productivity under stress conditions. Oak and pine seedlings mycorrhizal with *Pisolithus tinctorius* showed differences in survival related to the extent of mycorrhization of their roots, and seedlings with abundant *Pisolithus tinctorius* ectomycorrhizas tolerated environmental stresses, such as soil water deficit and high temperatures, better than seedlings without *Pisolithus tinctorius* ectomycorrhizas (Marx and Cordell 1989). Heslin and Douglas (1986) did not find a significant improvement in the survival of micropropagated poplar plants mycorrhizal with different fungi at the moment of ex vitro transfer, and other reports with micropropagated plants do not refer to survival (Rancillac 1983; Grellier et al. 1984; Poissonier 1986; Strullu et al.1986). However, other reports on seedling survival and growth after mycorrhization with different fungal species support our conclusions (Boyd et al. 1986; Meyer 1987; Feil et al. 1988; Le Tacon et al. 1992).

In experiment S1, both mycorrhizal seedlings and mycorrhizal micropropagated plants all showed low survival rates. This may be due to the long time needed for induction of in vitro mycorrhizas, with associated water and nutrient stresses, since no water or nutrient was supplied during the 30 weeks of mycorrhization. Under these conditions, however, significant differences were found between survival of mycorrhizal and nonmycorrhizal plants.

Our results show that mycorrhization improves the general condition of micropropagated chestnut plants, increasing survival and growth. Presence of the fungi increases root branching and, consequently, the plant root absorbing area to improve the nutritional status of the plant. Mycorrhizal inoculation prior to transfer to ex vitro conditions enables plants to acclimate more readily.

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References

Bonga JM (1977) Applications of tissue culture in forestry. In: Reinert J, Bajaj YPS (eds) Plant cell tissue and organ culture. Springer, New York Berlin Heidelberg, pp 93–107

Bowen GD (1973) Mineral nutrition of mycorrhizas. In: Marks GC, Kozlowski TT (eds) Ectomycorrhizas. Academic Press, New York, pp 151–201

Boyd R, Furbank RT, Read DJ (1986) Ectomycorrhiza and the water relations of trees. In: Gianinazzi-Pearson V, Gianinazzi S (eds) mycorrhizae, physiology and genetics. INRA, Paris, pp 689–693

Duddridge JA, Malibari A, Read DJ (1980) Structure and function of mycorrhizal rhizomorphs with special reference to their role in water transport. Nature 287:834–836

Feijó JA (1989) Propagação in vitro de *Castanea sativa* Mill. MSc thesis, University of Lisbon

Feijó JA, Pais MS (1992) Processo para multiplicação e enraízamento in vitro de plantas adultas (40 a 160 anos) de castanheiro. Patent no. 94663, Portugal

Feil W, Kottke I, Oberwinkler F (1988) The effect of drought on mycorrhizal production and very fine root system development of Norway spruce under natural and experimental conditions. Plant Soil 108:221–231

Flick CE, Evans DA, Sharp WR (1983) Organogenesis. In: Evans DA, Sharp WR, Ammirato PV, Yamada Y (eds) Handbook of plant cell culture, vol 1. McMillan, New York, pp 13–81

Grand LF, Harvey AE (1982) Quantitative measurement of ectomycorrhizae on plant roots. In: Schenck NC (ed) Methods and principles of mycorrhizal research. The American Phytopathological Society, St Paul, Minn, pp 157–164

Grellier B Letouzé R, Strullu DG (1984) Micropropagation of birch and mycorrhizal formation in vitro. New Phytol 94:591–599

Guehl JM, Garbaye J, Wartinger A (1992) The effects of ectomycorrhizal status on plant-water relations and sensitivity of leaf gas exchange to soil drought in Douglas Fir. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in ecosystems. C.A.B. International, Wallingford, UK, pp 323–332

Harley JL, Smith SE (1983) Mycorrhizal symbiosis. Academic Press, Toronto

Heslin MC, Douglas GC (1986) Effects of ectomycorrhizal fungi in growth and development of poplar plants derived from tissue culture. Sci Hortic 30:143–149

Jones MD, Durall DM, Tinker PB (1991) Fluxes of carbon and phosphorus between symbionts in willow ectomycorrhizas and their changes with time. New Phytol 119:99–106

Le Tacon F, Alvarez IF, Bouchard D, Henrion B, Jackson RM, Luff S, Parlade JI, Pera J, Stenström E, Villeneuve N, Walker C (1992) Variations in field response of forest trees to nursery ectomycorrhizal inoculation in Europe. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in ecosystems. C.A.B. International, Wallingford, UK, pp 119–134

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163

Marx DH, Cordell CE (1989) The use of specific ectomycorrhizas to improve artificial forestation practices. In: Whipps JM, Lumsden RD (eds) Biotechnology of fungi for improving plant growth. British Mycological Society. Cambridge University Press, Cambridge, pp 1–25

Marx DH, Bryan WC, Cordell CE (1977) Survival and growth of pine seedlings with *Pisolithus tinctorius* after two years on reforestation sites in North Carolina and Florida. For Sci 23:363–373

Meyer FH (1987) Extreme site conditions and ectomycorrhizae (especially *Cenococcum geophyllum*). Mycorrhiza and plant stress. Angew Bot 61:39–46

Poissonier M (1986) Mycorhization in vitro de clones d' Eucalyptus. Note de Laboratoire. Ann AFOCEL 1986:80–93

Rancillac M (1983) La mycorhization in vitro influence de la morphologie et des structures anatomiques de l′apareil racinaire sur l′établissement des ectomycorhizes. Bull Soc Bot 130:47–52

Strullu DG, Grellier B, Marciniak D, Letouzé R (1986) Micropropagation of chestnut and conditions of mycorrhizal synthesis in vitro. New Phytol 102:95–101

Warmke HE, Sheu-Ling JL (1976) Improved staining procedures for semithin epoxy sections of plant tissues. Stain Technol 51:179–185