

SHORT NOTE

A. Martins · A. Casimiro · M.S. Pais

Influence of mycorrhization on physiological parameters of micropropagated *Castanea sativa* Mill. plants

Accepted: 21 May 1997

Abstract Mycorrhizal micropropagated *Castanea sativa* plants were studied in terms of growth and physiological parameters following *in vitro* mycorrhization with *Pisolithus tinctorius*. Mycorrhization enhanced growth of micropropagated chestnut plants, increased their protein content and photosynthetic rates, decreased the respiratory rates and CO₂ compensation point. RuBisCO activity was not significantly different in mycorrhizal and control plants, although there was an increase in the amount of RuBisCO in the former. Mycorrhization increased plant biomass and improved plants physiological status, thus enhancing the acclimatization process.

Key words Micropropagation · *Pisolithus tinctorius* · RuBisCo · Acclimatization

Introduction

Several authors have reported on physiological parameters in studies of the increased growth and carbon metabolism of ectomycorrhizal plants. Increased phosphorus uptake and increase in nitrogen uptake by incorporation into carbon compounds to form amino acids are considered to be mainly responsible for the higher productivity of ectomycorrhizal plants (Bougher et al. 1990; Jones et al. 1990, 1991; Finlay 1992; Finlay et al. 1996). By enhancing access to the growth-limiting nutrients, mycorrhizas can significantly increase carbon fixation (Smith and Read 1997). This gain occurs primarily via increased photosynthetic rates, as has been reported for both endo- and ectomycorrhizas (Allen et

al. 1981; Reid et al. 1983). According to Allen (1991), mycorrhizal plants can also take up more carbon in drought periods than nonmycorrhizal ones, since they can maintain stomata open at lower soil water potentials. Survival and growth rates of micropropagated *Castanea sativa* were increased in plants mycorrhizal with *Pisolithus tinctorius*, allowing plants to overcome acclimatization problems in a difficult step of the micropropagation process (Martins et al. 1996). In this paper, we report the effect of mycorrhization on micropropagated *C. sativa* plants in terms of photosynthetic and respiratory rates, CO₂ compensation point, RuBisCO activity and protein content.

Materials and methods

Adult clones of the European chestnut tree *C. sativa* Mill. were micropropagated from buds of old trees (>50 years) following the method of Feijó and Pais (1992). Five weeks after root induction, plants were transferred to 250-ml flasks containing peat:perlite (3:1) substrate previously inoculated with *P. tinctorius* (Pers.) Coker and Couch (isolate 289/Marx) to induce mycorrhization. Plants were maintained in a growth chamber under a 16-h light period of ~100 mE m⁻² s⁻¹ quantum flux density (Sylvania Gro-lux fluorescent lamps) at 25 °C and in a dark period at 19 °C. After 14 weeks, plants were acclimatized *ex vitro* as described by Martins et al. (1996). Photosynthetic and respiratory rates, CO₂ compensation point, RuBisCO activity and protein content were measured at intervals after inoculation. Leaf area, dry weight and chlorophyll content were also determined.

For photosynthetic and respiratory rates, CO₂ evolution of mycorrhizal and control plants was quantified 8, 10, 12 and 14 weeks after inoculation using an infrared gas analyzer (IRGA-ADC-225-MK3) in a closed circuit at 22–25 °C and under a photosynthetic active radiation of 1000 mmol m⁻² s⁻¹, until the saturation point was reached. CO₂ evolution was then recorded in complete darkness. During the experiments, plants were maintained in the inoculation flasks with a cover to which the IRGA tubes were adapted.

RuBisCO activity of mycorrhizal and control plants was measured 8, 14 and 25 weeks after inoculation, according to Machler and Nösberger (1980). The enzyme extract was activated in the presence of NaH¹⁴CO₃ for 2 min. The reaction was started by the addition of ribulose biphosphate and stopped after 1.5 min by the addition of 0.1 ml of 2 M HCl.

A. Martins (✉)

Escola Superior Agrária de Bragança, Qta. Sta. Apolónia,
P-5300 Bragança, Portugal
Fax: +351-73-25405; e-mail: amartins@ipb.pt

A. Casimiro · M.S. Pais

Faculdade de Ciências de Lisboa, Dept. de Biologia Vegetal,
Bloco C2, P-1700 Lisbon, Portugal

Soluble proteins from leaves of mycorrhizal and control plants were quantified 8, 10, 12, 14 and 25 weeks after inoculation. Protein extraction, precipitation and concentration were performed according to Holloway and Armdel (1988). Protein content was determined by the Coomassie Fast Blue method (Bradford 1976) using a Shimadzu UV 160 A spectrophotometer, taking an average of five observations for each sample.

Leaf area of control and mycorrhizal plants was calculated 8, 10, 12 and 14 weeks after inoculation and during the ex vitro acclimatization process at 20, 25 and 30 weeks (35, 40 and 45 weeks after inoculation). Leaf areas were measured with an area meter LI-COR, LI 3000 and LI 3000 A and total and unit leaf area were calculated. Chlorophyll contents of leaves were determined after extraction in methanol following the method of Ozerol and Titus (1965) using a Shimadzu UV 160 A spectrophotometer.

Data were analysed by conventional variance analysis and significance of the calculated F values was established for probabilities lower than 5%, 1% and 0.1%.

Results and discussion

Leaf area expressed as mean leaf area/plant was higher in mycorrhizal than in control plants (Table 1) and differences increased with time and during acclimatization. Twelve weeks after inoculation, the differences were already statistically significant (Table 1). Chlorophyll content was similar in mycorrhizal and control plants up to 25 weeks (Table 2).

Mycorrhizal plants had significantly higher photosynthetic rates in terms of leaf area, fresh weight and dry weight at 12 and 14 weeks after inoculation (Table 3). These results are in agreement with previous reports that the presence of mycorrhizas on plant root systems is correlated with higher net photosynthetic rates (Paul and Kucey 1981; Reid et al. 1983; Nylund and Wallander 1989). Two main reasons have been offered to explain the increased photosynthetic rates: (1) a nutritional effect due to increased N, P and K absorption (Brix 1971, 1981; Nátr 1972; Osman et al. 1977; Longstreth and Nobel 1980; Swada et al. 1982; Reid et al. 1983), and (2) a non-nutritional effect, suggesting that photosynthetic rate is related to demand for carbohydrates and constitutes the source-sink concept basis for carbon metabolism (Sweet and Wareing 1966; Herold 1980; Dosskey et al. 1990; Vodnik and Gogala 1996). Improved nutrient status of mycorrhizal plants, especially P and N nutrition, is a well-known phenomenon (Bougher et al. 1990; Jones et al. 1990, 1991; Finlay 1992; Botton 1996; Finlay et al. 1996), and increase in net photosynthesis following increases in N and P has

Table 2 Chlorophyll content (mg/cm²) of leaves of mycorrhizal and control plants. Mean values in columns within a sampling followed by different letters are significantly different at $P < 0.05$

	Time (weeks)				
	8	10	12	14	25
Mycorrhizal	23.9 a	25.0 a	26.0 a	29.2 a	35.0 a
Control	23.8 a	23.5 a	21.6 a	25.2 a	40.2 b

been reported for different species (Osman et al. 1977; Longstreth and Nobel 1980; Swada et al. 1982; Reid et al. 1983). According to Wallander and Nylund (1992), P-deficiency increases the carbohydrate pool in plants, which in nonmycorrhizal plants should increase chloroplast starch and thus downregulate photosynthesis. Consequently, in mycorrhizal plants, where P increases and there is a drain of carbon compounds to the fungus, photosynthesis should increase since there is no carbohydrate accumulation to inhibit photosynthesis. Rousseau and Reid (1990) compared relationships between foliar phosphorus and net photosynthetic rate in non-mycorrhizal and ectomycorrhizal pine seedlings and found that at low and medium levels of mycorrhizal development the net photosynthetic response to mycorrhizal formation appeared to be due to enhanced P nutrition. However, at high levels of mycorrhizal development, photosynthetic rates of mycorrhizal seedlings were significantly higher than nonmycorrhizal seedlings of similar foliar P concentrations, suggesting that mycorrhizas can stimulate net photosynthesis through mechanisms other than enhanced phosphorus nutrition (Rousseau and Reid 1990).

In ectomycorrhizas, photosynthates move from the autotroph to the fungal mantle, where they are rapidly converted to metabolic intermediates, of which trehalose and mannitol are dominant (Söderström et al. 1988). Since ectomycorrhizal fungi are totally dependent on their host for carbon, they undoubtedly increase the sink strength of the root system. In this manner, mycorrhizal symbiosis may be able to stimulate photosynthesis in host plants (Rousseau and Reid 1990). The translocation of carbon compounds from the host to the fungus cannot be considered only as a sink of carbon compounds from the plant, but also as a way of exchange between plant and fungus (Finlay 1992). These carbon compounds are used for sustaining the fungal

Table 1 Mean leaf area (cm²) of mycorrhizal and control plants at 8, 10, 12 and 14 weeks after mycorrhizal induction and 20, 25 and 30 weeks after acclimatization (35, 40 and 45 weeks after my-

corrhizal induction). Mean values in columns within a sampling followed by different letters are significantly different at $P < 0.05$

	Time (weeks)						
	8	10	12	14	20/35	25/40	30/45
Mycorrhizal	1.7 a	2.2 a	1.7 a	2.2 a	76.0 a	115.7 a	167.6 a
Control	1.5 a	2.0 a	1.5 a	2.0 a	37.9 b	73.2 b	91.4 b

Table 3 Photosynthetic rates of leaves of mycorrhizal (*Myc*) and control (*Cont*) plants. Mean values in columns within a sampling followed by different letters are significantly different at $P < 0.05$

	8 weeks			10 weeks			12 weeks			14 weeks		
	Area ¹	FW ²	DW ³	Area	FW	DW	Area	FW	DW	Area	FW	DW
<i>Myc</i>	28.9 a	2.0 a	12.2 a	37.8 a	2.7 a	14.8 a	36.1 a	2.6 a	15.8 a	41.6 a	2.9 a	17.8 a
<i>Cont</i>	23.3 a	1.8 a	11.1 a	34.0 a	2.5 a	16.0 a	19.4 b	1.4 b	8.3 b	26.7 b	1.8 b	10.8 b

¹ $\mu\text{l CO}_2/\text{cm}^2$ leaf area² ml CO_2/g leaf fresh wt.³ ml CO_2/g leaf dry wt.**Table 4** Protein content (mg/g) per fresh weight (*FW*) and per dry weight (*DW*) of leaves of mycorrhizal and control plants. Mean values in a column within a sampling followed by different letters are significantly different at $P < 0.05$

	8 weeks		10 weeks		12 weeks		14 weeks		25 weeks	
	FW	DW	FW	DW	FW	DW	FW	DW	FW	DW
Mycorrhizal	16.8 a	98.8 a	17.9 a	99.4 a	14.7 a	88.9 a	13.4 a	83.3 a	15.6 a	93.6 a
Control	14.4 b	84.7 b	11.5 b	71.9 b	12.2 b	71.5 b	11.6 b	72.5 b	10.0 b	62.5 b

biomass existing in the mycorrhizal root tips and in the soil mycelia network, and also for producing new fungal biomass (Söderström 1992). As the photosynthetic rate is primarily limited by the accumulation of its end-products in the leaf cells, the drain of carbohydrates by the mycobiont may offset endproduct limitation facilitating an increased photosynthetic rate (Jakobsen 1991, Goldschmidt and Huber 1992; Vodnik and Gogala 1996). The protein content of leaves of mycorrhizal plants, expressed in terms of fresh weight or of dry weight was significantly higher than in control plants (Table 4). According to Finlay (1992), carbon compounds released by the host in ectomycorrhiza are not exclusively used by the fungus. A reverse translocation to the host plant of carbon compounds as amino acids, resulting from the incorporation of ammonium into carbon skeletons derived from fungal trehalose and mannitol has previously been suggested by France and Reid (1983). Such a mechanism for increased nitrogen assimilation may explain the higher protein content found in leaves of mycorrhizal chestnut plants.

Mycorrhizal plants showed lower CO_2 compensation points than control plants, the differences being highly significant (Table 5). The activity of RuBisCO was higher in mycorrhizal than control plants for all time periods (8, 14 and 25 weeks), when expressed in terms of fresh weight, dry weight and chlorophyll content. However, when the values were expressed in terms of protein content, the activity was similar in mycorrhizal and control plants for all time periods (Table 6).

The values obtained for the CO_2 compensation point suggest that mycorrhizal plants are able to photosynthesise at lower levels of CO_2 than control plants. According to Coombs (1985), the affinity of RuBisCO for CO_2 is very low, and if there is no mechanism to concentrate CO_2 on photosynthetic cells, RuBisCO will be used at half capacity. In mycorrhizal chestnut plants,

Table 5 CO_2 compensation point (ppm CO_2) of mycorrhizal and control plants. Mean values in columns within a sampling followed by different letters are significantly different at $P < 0.05$

	Time (weeks)			
	8	10	12	14
Mycorrhizal	173.0 a	110.0 a	121.1 a	130.0 a
Control	250.0 b	222.5 b	288.0 b	187.5 b

the similar specific activity of RuBisCO compared to nonmycorrhizal plants, with a higher activity in terms of fresh and dry weights, suggests a higher content of the enzyme in the mycorrhizal plants (Table 6). According to Allen (1991), under water-stress conditions mycorrhizal plants can take up more CO_2 by maintaining their stomata open. The water-stress conditions to which chestnut plants were submitted during photosynthetic and respiration measurements could induce stomata to close earlier in nonmycorrhizal plants than in mycorrhizal ones, so that these can continue to photosynthesize at lower CO_2 levels. Stomata seem to respond to the capability of the cells to fix CO_2 by maintaining the intercellular concentrations of CO_2 at a constant level, by feedback involving ribulose biphosphate, ATP or NAD(P)H (Chandler and Dale 1993). Considering that mycorrhizal plants have increased photosynthetic rates, to maintain the intercellular concentrations of CO_2 at a constant level, stomata of these plants must be maintained open for longer, allowing plants to photosynthesise at lower concentrations of CO_2 (lower CO_2 compensation points). Increased water-stress resistance and increased photosynthesis could be responsible for the lower values of CO_2 compensation points obtained for mycorrhizal chestnut plants.

Respiratory rates were higher in leaves of control than of mycorrhizal plants (Table 7). Differences were

Table 6 RuBisCO activity in leaves of mycorrhizal and control plants. Mean values in columns within a sampling followed by different letters are significantly different at $P < 0.05$

	8 weeks				14 weeks				25 weeks			
	FW ¹	DW ¹	Protein ²	Chlor ²	FW	DW	Protein	Chlor	FW	DW	Protein	Chlor
Myc	92.8 a	545.7 a	5.6 a	41.0 a	107.4 a	667.0 a	9.7 a	25.3 a	333.0 a	2497.5 a	22.4 a	72.5 a
Cont	76.4 b	449.2 b	5.3 a	34.1 a	95.7 a	598.1 b	9.7 a	18.6 b	267.0 b	1949.1 b	20.5 a	68.5 a

¹ $\mu\text{mol CO}_2/\text{g/h}$ in terms of fresh (FW) or dry (DW) weight

² $\mu\text{mol CO}_2/\text{mg/h}$ in terms of protein or chlorophyll (Chlor)

Table 7 Respiratory rates of mycorrhizal (Myc) and control (Cont) plants. Mean values in columns within a sampling followed by different letters are significantly different at $P < 0.05$

	8 weeks			10 weeks			12 weeks			14 weeks		
	Area ¹	FW ²	DW ³	Area	FW	DW	Area	FW	DW	Area	FW	DW
Myc	16.1 a	1.0 a	6.1 a	10.1 a	0.7 a	4.0 a	13.1 a	0.9 a	5.4 a	13.9 a	0.6 a	3.5 a
Cont	16.8 a	1.3 b	7.6 b	23.5 b	1.7 b	10.9 b	27.1 b	2.0 b	11.6 b	17.0 a	1.2 b	7.2 b

¹ $\mu\text{l CO}_2/\text{cm}^2$ leaf area

² $\text{ml CO}_2/\text{g}$ leaf fresh wt.

³ $\text{ml CO}_2/\text{g}$ leaf dry wt.

significant at all times when expressed in terms of fresh weight and dry weight, but were only significantly different in terms of leaf area for plants of 10 and 12 weeks after inoculation (Table 7). That mycorrhizal plants present lower respiratory rates than control ones is an unexpected observation. However, respiration can be highly variable in ectomycorrhizal plants (Nylund and Wallander 1989). Rousseau and Reid (1990) found that the effect of ectomycorrhizas on seedling dark respiration, net photosynthesis, and foliar P concentration was qualitatively very similar to the effect of phosphorus fertilization on these same parameters. While increased ectomycorrhizal infection was related to increased photosynthetic rate and foliar P, it did not significantly alter dark respiration. Since mycorrhizal plants exhibited higher photosynthetic rates and lower respiratory rates, they present higher productivity, which is in agreement with results previously reported (Martins et al. 1996). The respiratory quotient of the plants (the amount of CO₂ lost in respiration as a percentage of the amount of CO₂ gained in photosynthesis) show that mycorrhizal plants, even with a sink of carbon to the fungus, may have a gain of carbon that can balance the loss.

The results reported here show that mycorrhization enhanced growth of micropropagated chestnut plants, increased their protein content and photosynthetic rates and decreased the respiratory rates and CO₂ compensation point. As a consequence, plant biomass increases and the physiological status of plants was improved, thus facilitating the acclimatization process.

References

- Allen MF (1985) Phytohormone action: an integrative approach to understanding diverse mycorrhizal responses. In: Molina R (ed) Proceedings of the 6th North American Conference on Mycorrhizae. Forest Science Laboratory, Corvallis Ore, pp 158–160
- Allen MF (1991) The ecology of mycorrhizae. Cambridge University Press, Cambridge
- Allen MF, Smith WK, Moore TS, Christensen M (1981) Comparative water relations and photosynthesis of mycorrhizal and nonmycorrhizal *Bouteloua gracilis* (J. B. K.) Lag ex Steud. New Phytol 88:683–693
- Botton B (1996) Regulation of the nitrogen-assimilating enzymes in ectomycorrhizas. In: Azcon-Aguilar C, Barea JM (eds) Proceedings of the 4th European Symposium on Mycorrhizas. Granada European Commission. Mycorrhizas in integrated systems from genes to plant development. pp 294–300
- Bougher NL, Grove TS, Malajczuk N (1990) Growth and phosphorus acquisition of karri (*Eucalyptus diversicolor* F. Muell.) seedlings inoculated with ectomycorrhizal fungi in relation to phosphorus supply. New Phytol 114:77–85
- Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
- Brix H (1971) Effects of nitrogen fertilization on photosynthesis and respiration in Douglas-fir. For Sci 17:407–414
- Brix H (1981) Effects of nitrogen fertilizer source and application rates on foliar nitrogen concentration, photosynthesis and growth of Douglas-fir. Can J For Res 11:775–780
- Chandler JW, Dale JE (1993) Photosynthesis and nutrient supply in needles of Sitka spruce (*Picea sitchensis* (Bong.) Carr.). New Phytol 125:101–111
- Coombs J (1985) Carbon metabolism. In: Coombs J, Hall DO, Long SP, Scurlock JMO (eds) Techniques in bioproductivity and photosynthesis. Pergamon Oxford New York, pp 139–157
- Dosskey MG, Linderman RG, Boersma WG (1990) Carbon-sink stimulation of photosynthesis in Douglas-fir seedlings by some ectomycorrhizas. New Phytol 115:269–274
- Feijó JA, Pais MS (1992) Patent n° 94663/Processo para multiplicação e enraizamento in vitro de plantas adultas (40 a 160 anos) de castanheiro

- Finlay RD (1992) Uptake and translocation of nutrients by ectomycorrhizal fungal mycelia. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) *Mycorrhizas in ecosystems*. CAB International, Wallingford, UK, pp 91–97
- Finlay RD, Brun A, Chalot M, Söderström B (1996) Interactions in the carbon and nitrogen metabolism of ectomycorrhizal associations. In: Azcon-Aguilar C, Barea JM (eds) *Proceedings of the 4th European Symposium on Mycorrhizas*. Granada European Commission. *Mycorrhizas in integrated systems from genes to plant development*. pp 279–283
- France RP, Reid CPP (1983) Interactions of nitrogen and carbon in the physiology of ectomycorrhizae. *Can J Bot* 61:964–984
- Goldschmidt EE, Huber SC (1992) Regulation of photosynthesis by end-product accumulation in leaves of plants starch, sucrose, and hexose sugars. *Plant Physiol* 99:1443–1448
- Herold A (1980) Regulation of photosynthesis by sink activity – the missing link. *New Phytol* 86:131–144
- Holloway PJ, Armdel PH (1988) High-resolution two-dimension electrophoresis of plant proteins. *Anal Biochem* 172:8–15
- Jakobsen I (1991) Carbon metabolism in mycorrhiza. In: Norris JR, Norris DJ, Varma AK (eds) *Methods in microbiology*, vol. 23. Academic, New York London, pp 149–180
- Jones MD, Durall DM, Tinker PB (1990) Phosphorus relationships and production of extramatrical hyphae by two types of willow ectomycorrhizas at different soil phosphorus levels. *New Phytol* 115:259–267
- 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
- Longstreth DJ, Nobel PS (1980) Nutrient influences on leaf photosynthesis. Effects of nitrogen, phosphorus and potassium for *Gossypium hirsutum* L. *Plant Physiol* 65:541–543
- Machler F, Nösberger J (1980) Regulation of ribulose biphosphate carboxylase activity in intact wheat leaves by high CO₂ and temperature. *J Exp Bot* 31:1485–1491
- Martins A, Barroso J, Pais MS (1996) Effect of ectomycorrhizal fungi on survival and growth of micropropagated plants and seedlings of *Castanea sativa* Mill. *Mycorrhiza* 6:265–270
- Nátr L (1972) Influence of mineral nutrients on photosynthesis of higher plants. *Photosynthetica* 6:80–99
- Nylund J-E, Wallander H (1989) Effects of ectomycorrhiza on host growth and carbon balance in a semi-hydroponic cultivation system. *New Phytol* 112:389–398
- Osman AM, Goodman PJ, Cooper JP (1977) The effects of nitrogen, phosphorus and potassium on rates of growth and photosynthesis of wheat. *Photosynthetica* 11:66–75
- Ozerol NH, Titus JF (1965) The determination of total chlorophyll in methanol extracts. *Trans Ill State Acad Sci* 58:15–19
- Paul EA, Kucey RMN (1981) Carbon flow in microbial associations. *Science* 213:473–474
- Reid CPP, Kidd FA, Ekwebelam SA (1983) Nitrogen nutrition, photosynthesis and carbon allocation in ectomycorrhizal pine. *Plant Soil* 71:415–431
- Rousseau JVD, Reid CPP (1990) Effects of phosphorus and ectomycorrhizas on the carbon balance of loblolly pine seedlings. *For Sci* 36:101–112
- Smith SE, Read DJ (1997) *Mycorrhizal symbiosis*. Academic, New York London, 2n
- Söderström B (1992) The ecological potential of the ectomycorrhizal mycelium. In: *Mycorrhizas in ecosystems*. Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) CAB International, Wallington, UK, pp 77–83
- Söderström B, Finlay RD, Read DJ (1988) The structure and function of the vegetative mycelium of ectomycorrhizal plants. IV. Qualitative analysis of carbohydrate contents of mycelium interconnecting host plants. *New Phytol* 109:163–166
- Swada S, Igarashi T, Miyachi S (1982) Effects of nutritional levels of phosphate on photosynthesis and growth studied with single, rooted leaf of dwarf bean. *Plant Cell Physiol* 23:27–33
- Sweet GB, Wareing PF (1966) Role of plant growth in stimulating photosynthesis. *Nature* 210:77–79
- Vodnik D, Gogala N (1996) Photosynthesis in mycorrhizal spruce seedlings. In: Azcon-Aguilar C, Barea JM (eds) *Proceedings of the 4th European Symposium on Mycorrhizas*. Granada European Commission. *Mycorrhizas in integrated systems from genes to plant development*. pp 388–390
- Wallander H, Nylund J-E (1992) Effects of excess nitrogen and phosphorus starvation on the extramatrical mycelium of ectomycorrhizas of *Pinus sylvestris* L. *New Phytol* 120:495–503