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SHORT NOTE

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

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**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 $CO_2$ 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, $CO_2$ compensation point, RuBisCO activity and protein content.

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## 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 $\sim 100$ mE $m^{-2}$ $s^{-1}$ quantum flux density (Sylvania Grolux 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, $CO_2$ 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, $CO_2$ 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. $CO_2$ 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 $NaH^{14}CO_3$ for 2 min. The reaction was started by the addition of ribulose bisphosphate and stopped after 1.5 min by the addition of 0.1 ml of 2 M HCl.

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 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 nonmycorrhizal 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 2** Chlorophyll content (mg/cm$^2$) 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 |

**Table 1** Mean leaf area (cm$^2$) 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 mycorrhizal 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 |
| Myc | 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 |
| Cont | 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] $\mu l\ CO_2/cm^2$ leaf area
[2] 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 endproducts 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, 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 bisphosphate, 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.

**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 |

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] μmol $CO_2$/g/h in terms of fresh (*FW*) or dry (*DW*) weight
[2] μmol $CO_2$/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] μl $CO_2/cm^2$ leaf area
[2] ml $CO_2$/g leaf fresh wt.
[3] ml $CO_2$/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 $CO_2$ compensation point. As a consequence, plant biomass increases and the physiological status of plants was improved, thus facilitating the acclimatization process.

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