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Effects of CO_2 concentration on acclimatization and physiological responses of two cultivars of carob tree

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Abstract

This study reports survival and physiological responses of micropropagated *Ceratonia siliqua* L. cvs. Galhosa and Mulata plants during *ex vitro* acclimatization under ambient (AC; 330 μmol mol^{-1}) or elevated (EC; 810 μmol mol^{-1}) CO_2 concentration and a photosynthetic photon flux density of 125 μmol m^{-2} s^{-1}. CO_2 enrichment during acclimatization did not improve survival rate that was around 80 % for both treatments. Eight weeks after *ex vitro* transplantation, photosynthetic capacity and apparent quantum yield in acclimatized leaves were higher in comparison with those in *in vitro*-grown leaves, without any significant difference between CO_2 treatments. Chlorophyll content increased after acclimatization. However, EC led to a decrease in the total amount of chlorophyll in new leaves of both cultivars, compared to those grown at AC. Soluble sugars and starch contents were not markedly affected by growth EC, although starch had significantly increased after transfer to *ex vitro* conditions. EC induced an increase in the stem elongation and in the effective life of leaves, and a decrease in the number of new leaves.

Additional key words: *Ceratonia siliqua* L., chlorophylls, photosynthesis, sugars, survival rate, tissue culture.

Introduction

Carob (*Ceratonia siliqua* L.) is a slow growing evergreen tree of the *Fabaceae*. It has been commonly cultivated in the Mediterranean area since historic times. This species is of substantial importance for the locust bean gum (E410, also called carubin) obtained from its pods for use in the food industry. Although the methods for multiplication by plant tissue culture are well known and the procedures for micropropagation of these cultivars have been described (Romano *et al*. 2002), there are no reports on environmental control during acclimatization to *ex vitro* conditions of this species.

The concentration of CO_2 in the atmosphere during acclimatization or throughout *ex vitro* photoautotrophic conditions has been manipulated in order to promote survival (Desjardins *et al*. 1987, Solárová and Pospíšilová 1997, Pospíšilová *et al*. 1999, Carvalho and Amâncio 2002, Sha Valli Khan *et al*. 2003, Morini and Melai 2004). Plants in growth cabinets or greenhouses generally respond to atmospheric CO_2 enrichment by increasing net carbon gain and water use efficiency (Chaves 1994). Growth responses to elevated CO_2 reflect changes in dry matter production, whereas carbon allocation responses reflect changes in net assimilation (Atkinson *et al*. 1997). The initial growth stimulation often tends to decline after weeks, days or even hours and some species exhibit similar or even lower net photosynthetic and growth rates than plants growing at ambient CO_2 (Epron *et al*. 1996, Atkinson *et al*. 1997). The photosynthesis inhibition to long-term exposure is species-dependent and it seems that young, fully unfolded leaves are more responsive to CO_2 enrichment than very young or old leaves (Kelly *et al*. 1991). Increasing CO_2 concentration is described as having a positive effect on the acquisition of autotrophic behaviour as well as on growth and photosynthetic rates of plantlets during acclimatization *ex vitro* (Carvalho and Amâncio 2002).

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Abbreviations: AC - ambient CO_2 concentration (330 μmol mol^{-1}); BA - 6-benzyladenine; Chl - chlorophyll; c_i - substomatal CO_2 concentration; EC - elevated CO_2 concentration (810 μmol mol^{-1}); ϕ - apparent quantum yield; g_s - stomatal conductance; IBA - indole 3-butyric acid; MS - Murashige and Skoog medium; NL - new leaves; PL - persistent leaves; P_N - net photosynthetic rate; P_{Nmax} - photosynthetic capacity; PPFD - photosynthetic photon flux density.

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The present research was undertaken in order to investigate how CO_2 concentration during acclimatization affects survival, stomatal density, photosynthetic competence and carbon allocation, either in persistent or in new leaves of two Portuguese carob cultivars Galhosa and Mulata.

Materials and methods

Plants and culture conditions: Shoots (3 cm long) were obtained from stock cultures of *Ceratonia siliqua* L. cultivars Galhosa and Mulata established from adult female trees growing in the field, maintained through monthly subcultures on MS medium (Murashige and Skoog 1962) supplemented with 2.2 μM 6-benzyladenine (BA) (Romano *et al*. 2002). For root induction, the basal ends of the shoots were dipped in 4.9 mM indole-3-butyric acid (IBA) for 3 min, followed by culture on half-strength MS auxin-free medium supplemented with 58 mM sucrose and 0.7 % (m/v) agar (*Iberagar*). The pH was adjusted to 5.8 before autoclaving at 121 ºC and 1.1 kg cm^{-2} for 20 min. Shoots were grown individually in test tubes (32 × 200 mm) containing 20 cm^3 culture medium and covered with aluminium foil. Shoots were grown in the dark for one week and then placed for four weeks at 25 ± 2 ºC under a 16-h photoperiod (photosynthetic photon flux density, PPFD, of 60 μmol m^{-2} s^{-1}), provided by cool-white fluorescent lamps.

Acclimatization: Each rooted plantlet was transplanted into 350 cm^3 plastic pots with a mixture of peat and *Perlite* 3:1 (v/v). The acclimatization phase took place in a plant growth chamber (*500E*, *Aralab*, Lisboa, Portugal), with control of relative humidity and CO_2 concentration. Light was provided by 21 *Gro-Lux F18W/GRO* lamps placed at the top of the chamber. At the plant level PPFD was 125 μmol m^{-2} s^{-1} during the 16-h photoperiod. Temperature inside the growth chamber was kept at 25 ± 2 ºC during the day and 22 ± 1 ºC at the night. Relative humidity (RH) was programmed through an ultrasonic fog system controlled by a hygrometer initially at 98 %, but was decreased during the third, fourth and the fifth week until 70 % RH was attained. An infrared gas analyser measured the CO_2 concentration in the chamber and CO_2 was automatically added from a cylinder with compressed CO_2. The CO_2 concentration was 330 μmol mol^{-1} for the ambient CO_2 treatment (AC) and 810 μmol mol^{-1} for the elevated CO_2 treatment (EC). Plantlets were watered with a 10 % diluted solution of MS macronutrients twice a week. All determinations were done at the end of cultivation *in vitro* (day zero of acclimatization) and at the end of acclimatization *ex vitro* under AC or EC treatment (eight weeks). At the end of acclimatization, both persistent leaves (PL) formed *in vitro* and remained attached to the plantlet and the first new leaf (NL) totally expanded during acclimatization, were analysed.

Growth parameters: Percentage of surviving plants, number of new and dead leaves and stem height were determined weekly.

Stomatal density and stomatal index: Epidermal peels were taken from the abaxial surface of the leaves *in vitro* on day zero of acclimatization, and of new leaves (NL) fully expanded during the acclimatization period. Stomatal density (number of stomata per mm^2) and stomatal index (percentage of stomata per total number of epidermic cells) were determined using an optical microscopy (*Olympus*, Tokyo, Japan).

Photosynthesis measurements: Photosynthetic rates at saturating CO_2 (P_{Nmax}) and apparent quantum yield (ϕ) were determined from O_2 evolution rate measured in a leaf disc oxygen electrode Clark type (*Hansatech*, Kings Lynn, UK), at 25 ºC and saturating CO_2 concentration (5 %), immediately after detaching the leaves. The different PPFD was obtained with a Björkman lamp (*LS2 Hansatech*) equipped with neutral filters. The determinations of ϕ were done increasing PPFD from 18 to 100 μmol m^{-2} s^{-1} and photosynthetic capacity (P_{Nmax}) was measured at saturating PPFD (950 μmol m^{-2} s^{-1}).

The measurements of net photosynthesis rate (P_N) and stomatal conductance (g_s) were performed using a portable *Minicuvette System HCM 1000* (*Walz*, Effeltrich, Germany). Measurements were taken in the youngest fully expanded leaf during acclimatization, at the middle of the light period, in ambient CO_2 (approximately 330 μmol mol^{-1}) for both sets of plants. Plants exposed to 810 μmol CO_2 mol^{-1} were maintained at ambient CO_2 10 min prior to taking measurements.

Chlorophyll determination: Leaf samples (0.85 cm^2) were ground to powder in liquid nitrogen and the pigments extracted in 10 cm^3 acetone (100 %). The homogenate was centrifuged for 3 min at 14 000 g. The supernatant absorbances at 661.6 nm, 644.8 nm and 470 nm were measured in a spectrophotometer *Shimadzu UV-160* (Kyoto, Japan). The contents of chlorophyll *a* and *b* and total carotenoids were calculated according to Lichtenthaler (1987).

Sugar analysis: Glucose, fructose, sucrose and starch were quantified in an alcoholic extract from the leaf discs used in the oxygen electrode (2.12 cm^2), using the enzymatic methods described by Jones *et al*. (1977) for soluble sugars and by Stitt (1978) for starch.

Statistical analysis: Each of the acclimatization treatments was repeated twice. All the determinations were obtained with randomly chosen plants. Data were subjected to one or two-way analysis of variance (*ANOVA*) using the *SPSS* statistical package for Windows (release *11.0*, *SPSS Inc.*). All pairwise comparisons of individual means were done by the Bonferroni test. Differences were considered significant at $P \leq 0.05$.

Results

Growth and survival: After 8 weeks of acclimatization AC and EC plants showed almost the same frequency of survival, 86 % for cv. Mulata and 76 - 82 % for cv. Galhosa. The number of new leaves developed during acclimatization period (NL) was greater on AC plants than on EC plants. The number of dead leaves at the end of acclimatization on AC plants, that corresponds to leaf senescence and abscission, was nearly three times that of EC plants. So, the total number of functional leaves was similar under both CO_2 concentrations. In contrast, stem height of EC plants was significantly greater and nearly double compared to AC plants (Fig. 1). Stem height of Mulata was significantly greater than stem height of Galhosa regardless of CO_2 concentration.

Stomatal density and stomatal index: The acclimatization had a marked effect on the stomatal density of both cultivars (Table 1). The stomatal density in NL of acclimatized plants decreased to less than one half of that observed in *in vitro* grown leaves, despite of CO_2 concentration during acclimatization. Stomatal density in *in vitro* leaves was significantly higher in Galhosa than in Mulata, whereas the stomatal index was similar in all leaf types and cultivars.

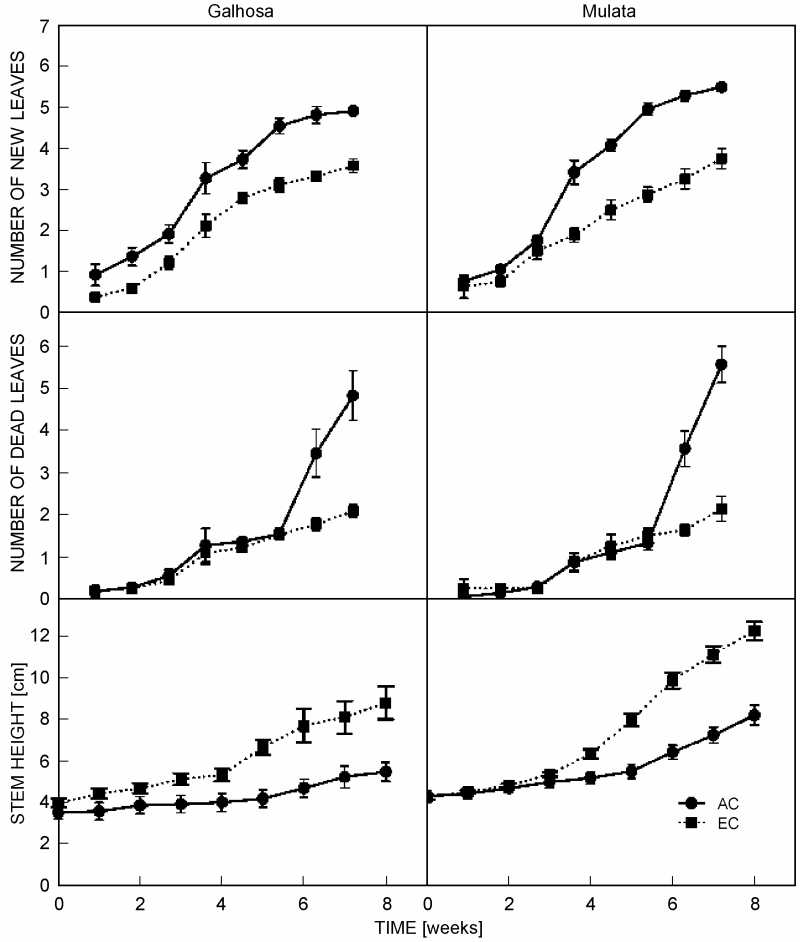

Fig. 1. Number of new and dead leaves and stem height of *ex vitro*-grown cvs. Galhosa and Mulata during 8 weeks of acclimatization to AC [330 μmol(CO_2) mol^{-1}] or EC [810 μmol(CO_2) mol^{-1}]. Values are means ± SE, $n = 30$.

Table 1. Stomatal density and stomatal index on cvs. Galhosa and Mulata leaves before (*in vitro*) or 8 weeks after acclimatization in AC [330 μmol(CO_2) mol^{-1}] or EC [810 μmol(CO_2) mol^{-1}]. Values are means ± SE, $n = 5$. For each cultivar means followed by different letters are significantly different at $P < 0.05$ (Bonferroni test); NL - new leaves.

Cultivar		Stomatal density [mm^2]	Stomatal index [%]
Galhosa	*in vitro*	1378 ± 364 a	11.1 ± 0.4 a
	AC (NL)	671 ± 73 b	11.1 ± 0.8 a
	EC (NL)	576 ± 78 b	10.6 ± 1.6 a
Mulata	*in vitro*	1016 ± 145 a	11.1 ± 0.6 a
	AC (NL)	581 ± 33 b	9.1 ± 0.9 a
	EC (NL)	456 ± 75 b	8.8 ± 1.7 a

Photosynthesis: Eight weeks after transplantation, photosynthetic capacity at saturating levels of irradiance and carbon dioxide (P_{Nmax}), was higher in acclimatized leaves (PL or NL) than in *in vitro*-grown ones; however, no significant differences were observed between the two CO_2 treatments (Fig. 2). Differences were more visible in Galhosa than in Mulata. Apparent quantum yield (ϕ) increased in acclimatized leaves as compared with *in vitro* leaves, regardless of the treatment and the cultivar (Table 2). CO_2 enrichment did not affect significantly stomatal conductance (g_s) measured under growth irradiance and ambient CO_2 (Table 2). In contrast, it led to a significant decrease in net photosynthetic rate (P_N) measured in AC, in NL of Mulata, although. substomatal CO_2 concentration (c_i) was maintained high (Table 2).

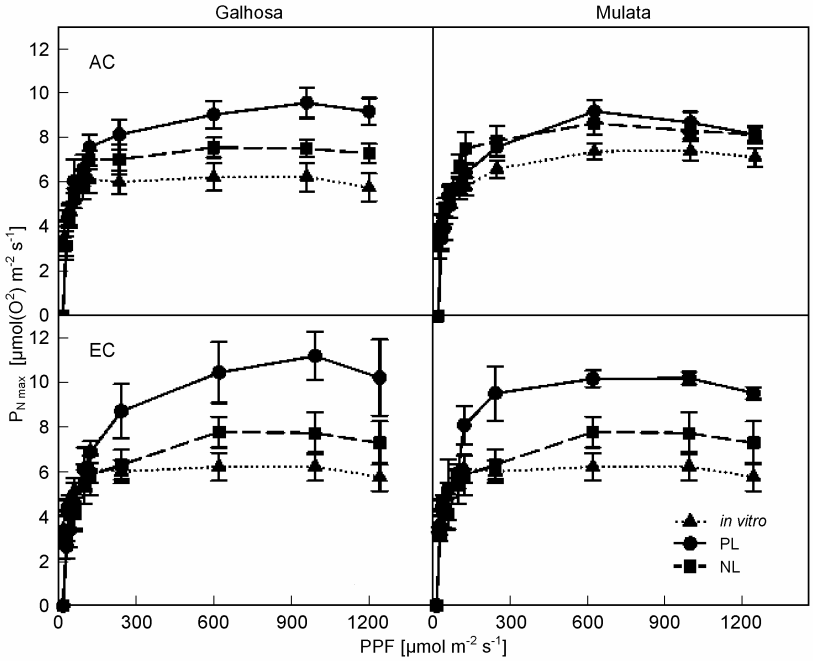

Fig. 2. Response of photosynthetic capacity ($P_{N\,max}$) to PPFD, measured as O_2 evolution in oxygen electrode at CO_2 saturation, of cvs. Galhosa and Mulata grown *in vitro* or 8 weeks after acclimatization (new leaves: NL, persistent leaves: PL) in AC [330 μmol(CO_2) mol^{-1}] or EC [810 μmol(CO_2) mol^{-1}]. Values are means ± SE, $n = 5$.

Table 2. Net photosynthetic rate (P_N), stomatal conductance (g_s), sub-stomatal CO_2 concentration (c_i) and apparent quantum yield (ϕ) on cvs. Galhosa and Mulata leaves before (*in vitro*) or 8 weeks after acclimatization in AC [330 μmol(CO_2) mol^{-1}] or EC [810 μmol(CO_2) mol^{-1}]. Values are means ± SE, $n = 5$. For each cultivar and variable, a single factor *ANOVA* was performed between treatment means. For each cultivar means followed by different letters are significantly different at $P < 0.05$.

Cultivar		P_N [μmol m^{-2} s^{-1}]	g_s [mmol m^{-2} s^{-1}]	c_i [μmol mol^{-1}]	ϕ [μmol(O_2)/μmol(photon)]
Galhosa	*in vitro*	-	-	-	0.03 ± 0.01 b
	AC	1.76 ± 0.29 a	49.52 ± 4.95 a	238.8 ± 9.9 b	0.05 ± 0.01 a
	EC	1.08 ± 0.33 a	82.23 ± 15.80 a	291.4 ± 8.6 a	0.06 ± 0.00 a
Mulata	*in vitro*	-	-	-	0.02 ± 0.00 b
	AC	2.15 ± 0.63 a	59.98 ± 16.11a	241.8 ± 7.6 a	0.06 ± 0.01 a
	EC	0.49 ± 0.13 b	52.24 ± 5.98 a	303.7 ± 4.4 a	0.06 ± 0.01 a

Table 3. Contents of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and Chl *a*/*b* ratio on cvs. Galhosa and Mulata leaves before (*in vitro*) or 8 weeks after acclimatization (persistent leaves: PL, new leaves: NL) in AC [330 μmol(CO_2) mol^{-1}] or EC [810 μmol(CO_2) mol^{-1}]. Values are means ± SE, $n = 5$. For each cultivar different letters indicate significant effects ($P < 0.05$; Bonferroni test).

Cultivar		Chl *a* [g m^2]	Chl *b* [g m^2]	Chl *a*/*b*
Galhosa	*in vitro*	0.22 ± 0.03 b	0.13 ± 0.02 c	2.08 ± 0.29 ab
	AC (PL)	0.69 ± 0.08 a	0.36 ± 0.04 a	1.94 ± 0.01 b
	AC (NL)	0.41 ± 0.16 b	0.25 ± 0.06 b	1.61 ± 0.16 b
	EC (PL)	0.42 ± 0.11 b	0.21 ± 0.01 cb	2.13 ± 0.61 ab
	EC (NL)	0.35 ± 0.07 b	0.12 ± 0.04 c	3.32 ± 0.60 a
Mulata	*in vitro*	0.24 ± 0.02 b	0.12 ± 0.01 b	2.06 ± 0.19 b
	AC (PL)	0.55 ± 0.04 a	0.29 ± 0.02 a	1.89 ± 0.05 b
	AC (NL)	0.49 ± 0.09 a	0.28 ± 0.03 a	1.75 ± 0.14 b
	EC (PL)	0.52 ± 0.05 a	0.18 ± 0.03 b	2.89 ± 0.33 a
	EC (NL)	0.33 ± 0.06 b	0.12 ± 0.05 b	3.21 ± 0.48 a

Table 4. Soluble sugars and starch contents and soluble sugars/starch ratio on cvs. Galhosa and Mulata leaves before (*in vitro*) or 8 weeks after acclimatization (persistent leaves: PL, new leaves: NL) in AC [330 μmol(CO_2) mol^{-1}] or EC [810 μmol(CO_2) mol^{-1}]. Values are means ± SE, $n = 5$. For each cultivar different letters indicate significant effects ($P < 0.05$; Bonferroni test).

Cultivar		Soluble sugars [mmol m^{-2}]	Starch [mmol m^{-2}]	Soluble sugars/starch
Galhosa	*in vitro*	39.14 ± 5.04 a	7.24 ± 1.40 b	6.41 ± 1.01 a
	AC (PL)	54.45 ± 18.14 a	48.48 ± 11.24 a	1.26 ± 0.31 b
	AC (NL)	38.49 ± 10.45 a	37.75 ± 20.62 a	1.02 ± 0.31 b
	EC (PL)	74.91 ± 24.86 a	64.60 ± 15.38 a	1.50 ± 0.15 b
	EC (NL)	54.66 ± 15.18 a	51.23 ± 16.12 a	1.12 ± 0.21 b
Mulata	*in vitro*	29.26 ± 3.07 a	6.27 ± 0.97 c	5.73 ± 1.24 a
	AC (PL)	56.07 ± 10.15 a	28.85 ± 6.31 bc	2.05 ± 0.44 b
	AC (NL)	31.70 ± 8.53 a	40.03 ± 18.46 ab	2.41 ± 0.70 b
	EC (PL)	39.57 ± 11.97 a	36.15 ± 9.62 ab	1.06 ± 1.04 b
	EC (NL)	43.40 ± 13.19 a	60.01 ± 11.33 a	1.21 ± 0.68 b

Chlorophyll contents: Chl *a* and Chl *b* contents in acclimatized leaves (PL and NL) of both cultivars increased significantly during acclimatization under AC as compared to *in vitro* leaves and Chl *a*/*b* ratio was maintained (Table 3). Under EC, no significant difference was observed between photosynthetic pigments content in NL or PL *vs in vitro* leaves of Galhosa, while in PL of Mulata the effect was similar to that of AC treatment. Chl *a*/*b* ratio increased after acclimatization at elevated CO_2, although this increase was not statistically significant for PL in Galhosa. On the other hand, in NL of Mulata, enhanced CO_2 concentration led to a significant depression of all Chl contents as compared to AC, whereas in NL of Galhosa this depression was only observed in Chl *b*.

Soluble sugars and starch: For both cultivars, a significant increase in starch content, without differences between AC and EC treatments, was observed in acclimatized leaves (NL or PL), as compared with *in vitro* leaves (Table 4). The soluble sugars content in acclimatized leaves increased less than the starch content, leading to a significant reduction in soluble sugars/starch ratio, as compared with *in vitro* leaves. During acclimatization the soluble sugars content was not affected by CO_2 concentration.

Discussion

The survival rate of carob tree plantlets transferred to *ex vitro* acclimatization treatments was similar to the average values reported for the same cultivars of this species acclimatized at ambient CO_2: 85 and 80 % for Galhosa and Mulata, respectively (Romano *et al*. 2002). In this study enhanced CO_2 during acclimatization did not increase plant survival.

Comparing the specific behaviour of plantlets under each treatment, the most remarkable difference is that AC plants invested mainly on emergence and expansion of

new leaves, whereas EC plants invested more on stem elongation. Despite the fact that a reduction on leaf emergence by EC had been observed, the mortality rate of persistent leaves in both cultivars was slowed down (Fig. 1), and thus their functional leaf life span was increased. The improvement of PL photosynthetic rate measured at saturating PPFD and CO_2 (P_{Nmax}) and the similar P_{Nmax} measured at 125 μmol m^{-2} s^{-1} in PL and NL (Fig. 2), suggest that carob tree behaves as a competent species (Grout 1988). So, during the first weeks of acclimatization, PL play an important role, contributing to a positive carbon balance in the whole plant as described for other species (Van Huylenbroeck *et al*. 1998, Amâncio *et al*. 1999). In EC plants, this role of PL is maintained over longer than in AC plants.

CO_2 concentration did not affect the formation of stomata in NL of this species during acclimatization, which is in agreement with the results of Tay *et al*. (2000). On the other hand, the stability of stomatal index shows that changes in stomata density are not due to differences in the number of stomata developed but chiefly to differences in the spacing of stomata (Tichá 1985).

In this study, when measurements were done at CO_2 saturation, photosynthetic rate of NL at EC or AC followed the same trend, whether at growth or saturating light (Fig. 2). However, when measurements were performed under ambient CO_2 and growth PPFD, a significant decrease in photosynthetic rate on EC plants in comparison of AC plants, was observed in Mulata, whereas in Galhosa no significant change occurred (Table 2). According Ceulemans *et al*. (1997), the measure of net photosynthetic rate (P_N) after plants grown at ambient and elevated CO_2 had been transferred to similar ambient CO_2 atmosphere, can be used in assessing photosynthetic acclimation. Thus, EC down-regulated P_N in both cultivars, albeit photosynthetic acclimation had been more evident in Mulata. Effects on gas exchange similar to those observed in Galhosa are reported for other woody plants growing at elevated CO_2, such as *Quercus alba* (Gunderson *et al*. 1993) and *Fagus silvatica* (Epron *et al*. 1996). Similar results to those of Mulata were found in *Prunus avium* × *pseudocerasus* (Atkinson *et al*. 1997). Although there were some indications of down-regulation of photosynthesis under EC, it does not seem very likely that such acclimation of photosynthesis might be mainly due to stomatal control, since similar values of stomatal density (Table 1), no significant differences in g_s and high value of c_i (*ca*. 300 μmol mol^{-1}) (Table 2) were observed in plants cultured at both CO_2 concentrations. A possible explanation for the down-regulation of photo-synthesis in response to elevated CO_2 can be a feedback mechanism caused by an insufficient demand for sugars to balance the enhanced supply under high CO_2. This imbalance is reflected in the increased accumulation of starch and sucrose in leaves (Van Oosten and Besford 1994). Regardless of the increase in starch content observed at the end of acclimatization treatment (Table 4), which is in agreement with results of Van Huylenbroeck and Debergh (1996) and Amâncio *et al*. (1999), no significant increase in the pool of starch or total soluble sugars in the leaves of EC in comparison to leaves of AC, was detected. However, it is clear a trend to an increase in the pool of starch under EC; thus, a down regulation linked to end product inhibition in carob tree could not be discarded.

After the *ex vitro* transfer, leaves showed an increase in total chlorophyll content relative to *in vitro* leaves, which can be a response to photoautotrophy (Table 3). Similar results were reported after transfer of *Nicotiana* (Pospíšilová *et al*. 1999), *Calathea* (Van Huylenbroeck *et al*. 2000), and *Castanea* (Carvalho *et al*. 2001), to *ex vitro* conditions. Usually, mature leaves of plants exposed to increased CO_2 show a decline in total chlorophyll content and chlorophyll *a*/*b* ratio (see Van Oosten and Besford 1994). Interestingly, EC leaves of carob tree Mulata showed lower total chlorophyll content but significantly higher chlorophyll *a*/*b* ratio than AC leaves. This can indicate that the investment on light harvesting pigments decreased in EC leaves, particularly in Mulata.

In conclusion, the overall aspect of *C. siliqua* micropropagated plants grown for 8 weeks in the environment with enhanced CO_2 or at ambient CO_2 concentration, looked very similar. In spite of this, some differences in growth and functional aspects were found between the two CO_2 concentrations and between the two carob cultivars. As the carob tree is a slow-growing species, and the expansion of new leaves is very slow, the first new leaf was only fully expanded in the eighth week of acclimatization. Such a lengthy period under high CO_2 required to attain this stage can be responsible for a certain down regulation or negative acclimation of photosynthesis. In these circumstances, improvement on photosynthetic competence and growth can be affected. So, acclimatization of carob tree micropropagated plants at 810 μmol(CO_2) mol^{-1} under an irradiance of 125 μmol m^{-2} s^{-1} does not bring any advantage when compared to 350 μmol(CO_2) mol^{-1}. Nevertheless, these results can be useful for a better understanding of the physiological aspects during the transition from *in vitro* to *in vivo*. Possibly, in carob tree micropropagated plants, overcoming the physiological transplantation stress and improvement of acclimatization, require an increase in irradiance simultaneously with an increase in CO_2 concentration, such as has been suggested for other recalcitrant woody species (Carvalho and Amâncio 2002).

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