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Effect of light intensity and CO_2 concentration on growth and the acquisition of *in vivo* characteristics during acclimatization of grapevine regenerated *in vitro*

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Summary

This study reports the effects of light intensity and CO_2 concentration during the acclimatization of *in vitro* plantlets of grapevine (*Vitis vinifera* L. cv. Touriga Nacional) on growth, chlorophyll and soluble carbohydrate contents, stomatal index and regulation of water loss. After *in vitro* phases at 45 μmol m^{-2} s^{-1} PFD, plantlets were transferred directly from *in vitro* to *ex vitro* acclimatization at different irradiation intensities (150 and 300 μmol m^{-2} s^{-1}) and CO_2 concentrations (350 and 700 μl l^{-1}). Growth, especially total biomass, was closer associated with light than with CO_2. *Ex vitro* leaves, expanded at high light and high CO_2, developed fully autotrophic characteristics, mainly with regard to specific leaf area, chlorophyll a/b ratio and down-regulation of sucrose accumulation. In all treatments stomatal indices of leaves were lower than those of *in vitro* leaves although regulation of water loss was reduced at high CO_2.

Key words: acclimatization, chlorophyll, growth analysis, *in vitro* propagation, relative water content, soluble sugars, *Vitis vinifera* L.

Abbreviations: BAP, benzylaminopurine; LAR, leaf area ratio, NAA, α-naphthaleneacetic acid; NAR, net assimilation rate, PFD, photon flux density; RGR, relative growth rate; RWC, relative water content; SI, stomatal index; SLA, specific leaf area.

Introduction

Plant *in vitro* culture occurs under heterotrophic conditions, in the presence of high amounts of sucrose, under low photon flux density (PFD) and CO_2 concentration, in tightly closed vessels where relative humidity (RH) can be close to saturation (Kozai 1991). Generally, heterotrophic or photomixotrophic plantlets show abnormal leaf anatomy, low photosynthetic rates (Galzy and Compan 1992; Pospís ilová *et al.* 1992; Chaves 1994) and inadequate water control (Brainerd and Fuchigami 1982). Consequently, an acclimatization step is necessary to avoid the low survival and growth rates of most species upon transfer of *in vitro* plantlets to *ex vitro*.

During acclimatization, environmental conditions most promising to be manipulated are light intensity and CO_2 concentration. For most species, increasing irradiance leads to higher survival rates and a faster acquisition of autotrophic structures (Donnelly and Vidaver 1984; Amâncio *et al.* 1999; Carvalho *et al.* 2001). On the other hand, by increasing CO_2 concentration higher growth rates and autotrophic characteristics were obtained in radish (Usuda and Shimogawara 1998), tobacco (Pospís ilová *et al.* 1999) and grapevine (Lakso *et al.* 1986; Carvalho and Amâncio 2001). Moreover, a positive interaction between the effects of irradiance and CO_2 concentration on *ex vitro* growth of tissue-cultured plants has been reported (Wolf *et al.* 1998).

In previous trials with *Vitis vinifera* cv. Touriga Nacional, photosynthetic efficiency was improved after acclimatization by doubling the *in vitro* irradiance (Amâncio *et al.* 1999). When irradiance was raised to 3- and 6-fold the *in vitro,* photosynthesis parameters were even higher although slight photoinhibition symptoms were detected at the highest irradiance (Carvalho *et al.* 2001). However, this irradiance combined with increased CO_2 concentration counteracted the photoinhibition symptoms (Carvalho and Amâncio 2002).

The main objective of the present research was to study the effects of acclimatization treatments combining high irradiance with high CO_2 concentration on growth, chlorophyll and soluble carbohydrate contents, stomatal index and relative water content of micropropagated *V. vinifera* cv. Touriga Nacional plantlets.

Material and Methods

Plant material and *in vitro* culture conditions: *In vitro* plantlets of *Vitis vinifera* L. cv. Touriga Nacional regenerated from *in vitro* stock cultures were used. *In vitro* establishment, stock shoot multiplication and root induction were adapted from Neves *et al.* (1998). MS (Murashige and Skoog 1962) medium was supplemented with 0.5 μM α-naphthaleneacetic acid (NAA), 5.0 μM 6-benzylaminopurine (BAP) and 88 mM sucrose for 4-week multiplication cycles; 1.67 μM benzylaminopurine (BAP) and 59 mM sucrose for 2-week shoot elongation periods; 2 μM α-naphthaleneacetic acid (NAA) and 44 mM

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sucrose for 4 d root induction. The cultures were kept in a growth chamber under light from cool-white fluorescent lamps at a photon flux density (PFD) of 45±5 µmol m^{-2} s^{-1} and a photoperiod of 16 h. Temperature was 25±1 ºC during the light period and 22±1 ºC during the dark period.

Acclimatization *ex vitro* and variation of acclimatization parameters: After *in vitro* root induction the plantlets were transferred to *ex vitro* conditions for root expression during the acclimatization phase. Microcuttings were placed into 6 cm diameter pots containing a sterilised mixture of hydrated peat and perlite (1:1, v/v). The pots were placed in a 450 l glass chamber (500E, Aralab, Portugal). Light was provided by fluorescent lamps (Gro-Lux F18W/GRO) placed over the transparent top of the chamber: low light (LL, 150±10 µmol m^{-2} s^{-1}) and high light (HL, 300±10 µmol m^{-2} s^{-1}). Light intensity at the plant level was altered by changing the distance of the plants to the light source and the number of lamps switched on. An Infra Red Gas Analyser (IRGA) connected to the CO_2 supply controlled the atmospheric CO_2 level in the chamber. CO_2 was set to 350 µl l^{-1} (LCO_2) or 700 µl l^{-1} (HCO_2) during the light period except for the first 7 d and during the night in all treatments where CO_2 was maintained at 350 µl l^{-1}. Thus the 4 acclimatization treatments were $LLLCO_2$, $HLLCO_2$, $LLHCO_2$ and $HLHCO_2$. Relative humidity (RH) was controlled by an ultrasonic fog system. The initial value was set to 98 % and decreased daily from the second to the fourth week, until RH in the glass chamber attained ambient values. Temperature was kept at 25±2 ºC during the light period and 22±1 ºC during the dark period.

Growth analysis: Plantlets were harvested at day 0, 7, 14, 21 and 28 in the middle of the light period, washed to remove peat particles and separated into leaves, shoot and roots. Leaves were divided into *in vitro* leaves (corresponding to day 0 of acclimatization, IV), persistent leaves (differentiated *in vitro* and maintained during acclimatization, PL), first (L1) and second (L2) new leaves expanded during acclimatization and analysed from day 14 or 21 onwards, respectively. Fresh and dry weights and leaf area were obtained with random samples. For dry weight determination samples were dried at 80 ºC until constant weight was reached. Leaf area was measured with a LI-COR portable area meter (LI-3000).

From the above determinations, the following parameters were calculated: total biomass; shoot/root ratio; specific leaf area (SLA) *i.e.* leaf area per g dry weight; relative growth rate (RGR), the increase in biomass per unit of biomass and day; net assimilation rate (NAR), the increase in biomass per unit leaf area and day; leaf area ratio (LAR), leaf area per unit dry weight.

Chlorophyll: Chlorophyll a and b and total chlorophyll were quantified in samples formed by two leaf discs (0.77 cm^2) cut from the leaves as referred above. After extraction with 80 % cold acetone and determining the absorbance at 645 and 663 nm, calculations were made using the MACKINNEY (1941) formulae. Results were expressed in mg chlorophyll g^{-1}dry weight.

Soluble carbohydrates: Glucose, fructose and sucrose were analysed in samples, *i.e.* leaf disks, prepared as for chlorophyll determinations and were quantified according to STITT *et al.* (1989).

Stomatal index (SI): The abaxial surfaces of new leaves (L1 and L2) at day 28 were used to prepare imprints with transparent fingernail polish. After drying for 10 min, the imprints were removed, glued on a slide and observed under a light microscope at 100x magnification. Stomatal frequency (SF) and epidermal cell frequency (EF), *i.e.* the number of stomata and epidermal cells per unit leaf area, were used for calculating SI (CONNER and CONNER 1984):

$$SI\ (\%) = [SF/(SF + EF)] \times 100$$

Relative water content (RWC): Six leaf discs (1 cm diameter each) were cut from the same leaf on day 28. Discs were placed in water for 30 min in order to calculate saturated weight (SW). The discs were then exposed to the ambient air at room temperature (25±2 ºC) and ambient relative humidity (about 50 %). Leaf RWC was determined at 10 min intervals for 2 h and then at 20 min intervals for 1 h. RWC was calculated as:

$$RWC = (FW - DW)/(SW - DW) \times 100,$$

where FW, DW and SW are fresh, dry and saturated weights, respectively (CONNER and CONNER 1984).

Statistics: Each acclimatization treatment was performed twice. All determinations were obtained with randomly chosen plants. The number of replications was: 30 (biomass and leaf area) or 10 (chlorophyll, soluble sugars, stomata and RWC). The results were statistically evaluated by variance analysis comparing the treatments and the leaf type. Means were separated by the Duncan test for $p < 0.001$ for significant values. Data are presented ± standard error (SE).

Results

Growth: Total biomass data are shown in Fig. 1. Initially high CO_2 under low light led to an increase of biomass; on day 28 the biomass under $HLLCO_2$ had more than doubled in comparison to $LLLCO_2$ and was significantly higher than that at both HCO_2 treatments. Before the application of high CO_2 (day 7) HL had no influence on the pattern of root growth (Tab 1). However, on day 28, plants from $HLLCO_2$ showed a lower shoot/root ratio, indicating higher contributions of roots.

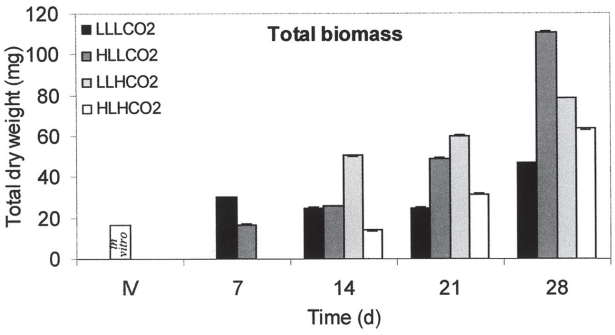

Fig. 1: Total biomass of *in vitro* (IV) plantlets and of plants subjected to the 4 acclimatization treatments. Arrows indicate the start of HCO_2 application. Bars represent SE values. $LLLCO_2$, $HLLCO_2$, $LLHCO_2$, $HLHCO_2$: see Material and Methods.

T a b l e 1

Shoot/root ratio after 7 and 28 d of acclimatization

Treatment	Time (d)	Shoot/root ratio
LL	7	3.4 c
HL	7	2.9 c
LL LCO_2	28	1.9 b
LL HCO_2	28	1.7 b
HL LCO_2	28	1.3 a
HL HCO_2	28	2.1 b

Values followed by different letters (a, b, c) are significantly different, $p<0.001$, n = 30.

Specific leaf area (SLA) of new leaves was higher than that of persistent ones with even slightly higher values under LL, irrespective of CO_2 level (Fig. 2).

Tab. 2 shows the values of relative growth rate (RGR), net assimilation rate (NAR) and leaf area ratio (LAR), on day 28. Under low light, RGR increased due to HCO_2 while under LCO_2 it increased more than 50 % when LL was compared to HL. The same trend was observed for NAR.

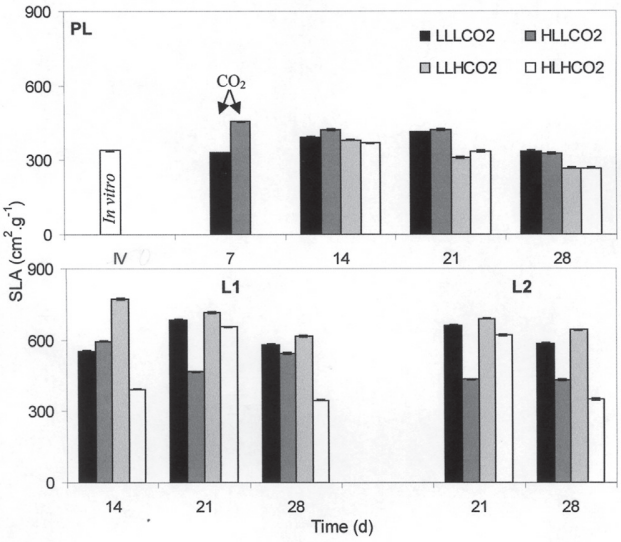

Fig. 2: Specific leaf area (SLA) of *in vitro* (IV) and persistent (PL) leaves, the first (L1) and the second (L2) new leaf of plants subjected to the 4 acclimatization treatments. For details: Fig. 1

C h l o r o p h y l l : The total chlorophyll content of persistent leaves was not significantly different and decreased towards the end of acclimatization (Fig. 3). Both types of new leaves under $HLHCO_2$ showed lowest values, especially on day 28. Lower chlorophyll contents in persistent and new leaves on day 28 may be due to first symptoms of senescence. The ratio chlorophyll a/b was not significantly affected by the treatments (Fig. 4).

S o l u b l e c a r b o h y d r a t e s : Leaf glucose + fructose and sucrose contents on day 28 are shown in Tab. 3. In PL glucose + fructose increased significantly in response to HL but not to HCO_2. The first new leaf had the

T a b l e 2

Relative growth rate (RGR), net assimilation rate (NAR) and leaf area ratio (LAR) at the end of acclimatization (day 28)

	RGR $g\,g^{-1}\,d^{-1}$	NAR $g\,m^{-2}\,d^{-1}$	LAR $m^2\,g^{-1}$d.w.
$LLLCO_2$	0.034 a	1.421 a	0.024 c
$HLLCO_2$	0.053 c	5.088 c	0.010 a
$LLHCO_2$	0.046 b	2.758 b	0.017 b
$HLHCO_2$	0.042 b	2.456 b	0.017 b

In each column, values followed by different letters (a, b, c) are significantly different, $p<0.001$, n = 30.

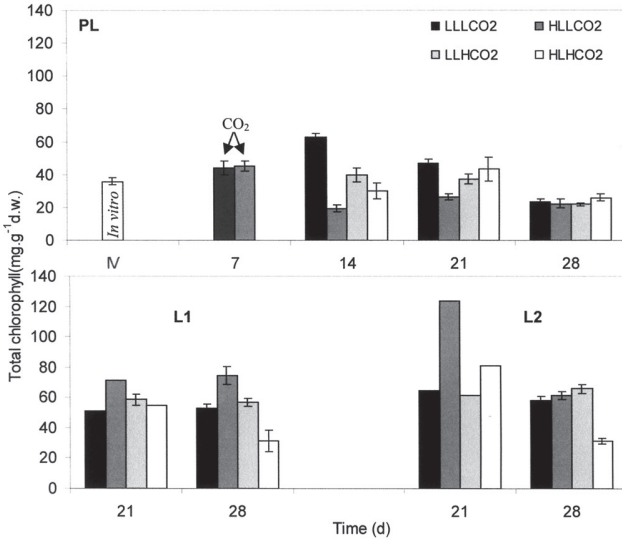

Fig. 3: Total chlorophyll of *in vitro* (IV) and persistent (PL) leaves, the first (L1) and the second (L2) new leaf of plants subjected to the 4 acclimatization treatments. For details: Figs 1 and 2.

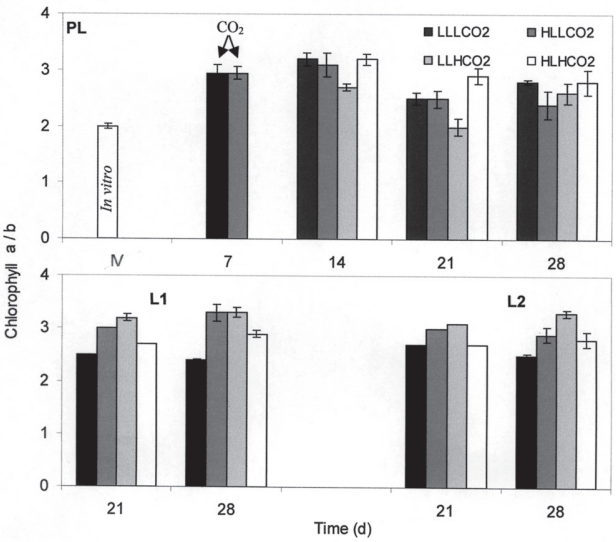

Fig. 4: Chlorophyll a/b ratio of *in vitro* (IV) and persistent (PL) leaves, the first (L1) and the second (L2) new leaf of plants subjected to the 4 acclimatization treatments. For details: Fig. 1.

Table 3

Glucose + fructose and sucrose content of *in vitro* leaves (IV) and at the end of acclimatization (day 28), in persistent leaves (PL), the first new leaf (L1) and the second new leaf (L2)

IV	Glucose + Fructose µmol g^{-1}d.w. 524*)			Sucrose µmol g^{-1}d.w. 251*)		
	PL	L1	L2	PL	L1	L2
$LLLCO_2$	1803 b, d	3542 c, e	3321 b, e	77 a, d	132 a, e	90.4 a, d
$HLLCO_2$	2795 c, d	3513 c, e	2452 a, d	94 a, d	160 a, e	86.4 a, d
$LLHCO_2$	1143 a, d	2172 b, e	2412 a, e	109 a, d	320 c, f	205.0 b, e
$HLHCO_2$	1743 b, d	1405 a, d	3677 b, e	346 b, f	205 b, e	61.0 a, d

In each column a, b, c indicate means significantly different by treatment and in each row d, e, f indicate means significantly different by leaf type, $p<0.001$, n = 10.
*) Values of *in vitro* (IV) material at day 0 of acclimatization.

highest glucose + fructose content under both LCO_2 treatments. $HLHCO_2$ gave rise to maximum contents of sucrose in PL and, typical for autotrophic leaves, minimum contents in the second new leaf.

Stomatal index: SI-values of new leaves expanded during the 4 treatments were between 10 and about 12, *i.e.*, were lower than those of *in vitro* leaves (SI=13) (Tab. 4).

Relative water content: Fig. 5 shows the RWC measured in the first and second new leaf on day 28. At the onset of the experiment leaf discs were fully turgid (RWC=100 %) and stomata fully opened. Under both LCO_2 treatments the water content of L1 and L2, especially L1, was maintained at significantly higher values than under HCO_2. However, under $LLHCO_2$, RWC of the youngest leaf, L2, was close to the values measured under LCO_2.

Table 4

Stomatal index measured at the end of acclimatization (day 28) in the first (L1) and the second (L2) new leaves

	L1	L2
$LLLCO_2$	11.9 b, c	11.7 b, c
$HLLCO_2$	11.8 b, d	10.0 a, c
$LLHCO_2$	10.3 a, c	10.6 a, c
$HLHCO_2$	11.5 b, c	11.6 b, c

In each column a, b indicate means significantly different by treatment and in each row c, d indicate means significantly different by type of leaf, $p<0.001$, n = 10.

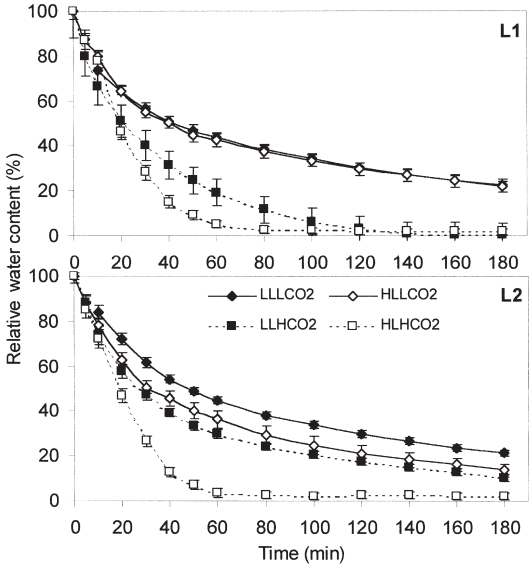

Fig 5: The relative water content (RWC) of disks excised from the first (L1) and the second (L2) new leaf after 28 d of acclimatization. For details: Fig. 1.

Discussion

This is one of the first reports on growth, chlorophyll and soluble carbohydrate contents, stomatal index and relative water content of *in vitro* grapevine following acclimatization treatments combining high irradiance and high CO_2 concentration. Unlike chestnut under the same acclimatization treatments (CARVALHO *et al.* 2001), for grapevine biomass the root system accounted for almost as much dry weight as the shoot (Tab. 1). High CO_2 applied after root initiation (day 7), apparently did not inhibit root formation, although the lower shoot/root ratio of plants under $HLLCO_2$ can correspond to a higher investment in root growth in the absence of high CO_2 (FOURNIOUX and BESSIS 1993). Similarly to grapevine, in *Raphanus sativus* CO_2 enrichment induced an initial increase of biomass followed by a significant enhancement due to high light (USUDA and SHIMOGAWARA 1998). An increase of light during acclimatization of other species, *e.g. Rubus idaeus*, also accounted for significant increases of growth parameters (DONNELLY and VIDAVER 1984).

In our plant system an increase of light, especially $HLLCO_2$, seems to be responsible for a better equilibrium

between leaf area and weight, as demonstrated by the higher SLA values of new leaves formed under both LL treatments. $HLLCO_2$ also induced the highest relative growth rates. However, Lakso *et al.* (1986) reported for a *Vitis* hybrid acclimatized under two different CO_2 levels at the same irradiance, higher RGR under high CO_2. RGR is the product of NAR and LAR; since the trend of RGR and NAR was similar, the net gain in dry weight per leaf area (NAR) has been the main contributor to RGR; this was previously reported for *Malus pumila* cv. Greensleeves (Diaz-Perez *et al*. 1995) and *Fragaria* x *Ananassa* (Desjardins *et al*. 1987). Therefore, the increase of growth due to HL can be attributed to a more positive carbon balance between the photosynthetic gain and respiratory loss at the whole plant level rather than by an extra investment in leaves (Amâncio *et al.* 1999).

The initial increase and final decrease in total chlorophyll content noticed in persistent leaves irrespective of acclimatization conditions seems to be a common acclimatization response reported by several authors (Rival *et al.* 1997; van Huylenbroek *et al.* 1998; Pospís ilová *et al.* 2000). The chlorophyll a/b ratio of new leaves was almost constant except under $LLLCO_2$, where leaves seem to behave like shade leaves. In tobacco plantlets acclimatized in conditions similar to our $LLHCO_2$ treatment, Pospís ilová *et al.* (2000) also report a chlorophyll a/b ratio characteristic of sun leaves. On the other hand, sun leaves induced by HL invested more in the light harvesting apparatus under LCO_2 than under HCO_2 (Levitt 1980).

The high sucrose concentration of *in vitro* leaves reflects the accumulation of the carbon source from the culture media. After transfer to *ex vitro* and until the expansion of new leaves, photomixotrophic persistent leaves are the main source of carbohydrates for plant growth (Galzy and Compan 1992). When new leaves are photosynthetically active they become the source of sucrose for the growing parts of the plant (van Huylenbroeck and Debergh 1996; Amâncio *et al.* 1999). In this study, the grapevine sucrose content of leaves expanded *ex vitro*, especially the youngest leaf under $HLHCO_2$, corresponds to autotrophic leaves.

An inverse relationship between leaf stomatal index and the partial pressure of ambient CO_2 has been reported for most C_3 species (Woodward 1987; Royer *et al.* 2001). In our system application of high CO_2 for short periods (28 d) did not induce equivalent relation on new leaves. As expected, after all treatments SI was lower than in *in vitro*; however, no significant differences were associated with HCO_2 treatments. For open air-grown grapevine plants, Düring and Stoll (1996) report stomatal closing when CO_2 concentration was raised above a certain threshold. However, stomata of *in vitro* apple (Brainerd and Fuchigami 1982) or *in vitro* grapevine (Düring and Harst 1996) remained almost completely open. From the curves reproduced in Fig. 5 it is evident that, mainly in the first leaf expanded *ex vitro*, high CO_2 concentration caused a negative effect on stomatal closure capacity. Apparently, in the present system and time scale, HCO_2 impairs to some extent the signal for the development of the stomatal closure mechanism. However, if water loss is controlled by environmental conditions, open stomata of new leaves are compatible with apparent photosynthesis rates being higher than in photomixotrophic leaves (Carvalho *et al.* 2001).

To summarize, the growth and acquisition of an autotrophic behaviour of grapevine plantlets take advantage of acclimatization at 300 µmol m^{-2} s^{-1}, irrespective of CO_2 concentration. However, although stomatal water loss was higher under HCO_2, $HLHCO_2$ produced the best effects on photosynthesis and on repairing symptoms of photoinhibition (Carvalho and Amâncio 2002). Two main points can be concluded: further investigation is necessary to analyse the effects of CO_2 on the stomatal closure mechanism; both acclimatization protocols ($HLLCO_2$ and $HLHCO_2$) are worth to be tested with different woody species.

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