Effects of Elevated CO_2 on Acclimatization of *In Vitro*-Regenerated Chestnuts: Growth Analysis

Maria Teresa Coelho, Maria da Graça Diogo and José Carlos Gonçalves
Instituto Politécnico de Castelo Branco, Escola Superior Agrária de Castelo Branco, Laboratório de Biologia Vegetal,
Quinta da Senhora de Mércules, Apartado 119, 6001-909 Castelo Branco, Portugal.

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Abstract

In this study we present the results of growth analysis of *in vitro*-regenerated chestnut hybrid plantlets (*Castanea sativa* x *C. crenata*), during the acclimatization stage, using two CO_2 concentrations (350 and 700 μLL^{-1}) at 250 $\mu mol\ m^{-2}\ s^{-1}$ as irradiance level (PPFD). Elevated CO_2 did not affect the survival rate and it was susceptible to increase progressive autotrophy, expressed by a significant increase in relative growth, shoot/root ratio and leaf area ratio (LAR). The plants under elevated CO_2 showed an higher stomatal frequency but the new leaves developed at the end of acclimatization revealed a gradual normal stomatal morphology and they reduced the stomatal frequency. Their morphology showed an effective water loss control which is one of the most important problem during this critical phase of the autotrophic competence acquiring process. The net photosynthesis rate was similar in both treatments but the plants acclimatized at elevated CO_2 showed an increase in maximum photosynthetic rate (A_{max}), and this can lead to a better physiological development. We think that the gains that we have achieved with the use of elevated CO_2 can be more significant if an higher light intensity can be used instead because they have a better response capacity to an increment of the level of irradiance.

INTRODUCTION

During the acclimatization *in vitro*-regenerated plants have to adapt to the new environmental conditions which allow the acquisition of their autotrophic competence. *In vitro* phases occur in heterotrophic conditions under low irradiances levels, high relative humidity, limited gas exchanges and with presence of sugar in the medium and this environment can affect their capacity for adaptation to greenhouse and field conditions. Increasing photosynthetic capacity of the young plantlets by increasing light intensity and ambient CO_2 concentration can give faster and more successful acclimatization before transfer to natural conditions (Desjardins *et al*., 1987; Matysiak and Nowak, 1998).

In previous work we have showed that the development of an *ex vitro* root system is crucial to provide higher survival rates (Gonçalves *et al*., 1998a) and to allow a significant growth during acclimatization of micropropagated chestnuts (Gonçalves *et al*., 1998b). We think that the good functionality of this root system can assure a healthy water status which can support increasing photosynthetic rates.

We intend to continue this study on the impact of elevated CO_2 in relation to irradiance level on chestnut acclimatization process with the goal to obtain a better vigour in the growth of the microplant and increase their autrotrophic acquiring competence and also study the possibility of the reduction of the period time of acclimatization.

MATERIAL AND METHODS

Plant material and culture conditions

We used an adult *Castanea sativa* x *C. crenata* hybrid clone, M1, resistant to ink disease. The original plant material was obtained from stump sprouts and the apices and nodal segments were established and multiplied *in vitro*. The cultures were kept in a growth chamber at 25/20ºC day/night, with 16 h photoperiod and 45±5 μmol m^{-2} s^{-1} (PPFD) provided by cool-white fluorescent lamps.

Rooting and acclimatization

For rooting, 3-5 cm long shoots were isolated and shoot-tips were removed. Roots were induced by leaving the shoots for 5 days in Murashige and Skoog (1962) medium, with macronutrients reduced to half strength and nitrates to quarter strength, supplemented with 3 mg L^{-1} IBA. After this induction, the shoots developed *ex vitro* root in 60x40x20 cm polystyrene boxes containing peat:perlite (1:2, v:v) substrate. Then the rooted shoots were transplanted to plastic pots filled with 200 cm^3 of peat:perlite (1:2, v:v). Plantlets were acclimatised during the following 4 weeks in controlled chambers (ARALAB ™) at 25±2 ºC, with 16 h photoperiod at 250±5 μmol m^{-2} s^{-1} as irradiance level (PPFD) provided by cool-white and grow-lux fluorescent lamps, and relative humidity produced by an ultrasonic fog system gradually reduced from 95% to ambient relative humidity.

During the acclimatization process, it was used two CO_2 concentrations: 350 μL L^{-1} and 700 μL L^{-1}. The CO_2 concentration was monitored with an IRGA, added from a compressed supply of pure gas and injected automatically by a by-pass valve only during photoperiod.

Growth analysis

Plant survival was determined at the end of acclimatization (survival was considered when plants formed new leaves). Plants were separated into persistent leaves (PL, leaves expanded during the multiplication stage and maintained during rooting and acclimatization process), new leaves, stem and roots. Leaf area and rooting evaluations were obtained by computer image analysis (WinRhizo V:3.9 b, ©Régent Instruments Inc, Québec, Canada). Dry weight was evaluated after dryness at 80 ºC until constant values. The leaf stomata surfaces were examined by optical microscopy and the epidermal impressions were made by applying a thin pellicle of transparent fingernail polish on the leaf surface and allowing it to dry for 10 min. The imprints were removed from the leaf and glued on a microscope slide. Three samples for each leaf and treatment were examined.

Net photosynthetic rates were measured using a portable CO_2 Gas Analyser (LI-COR, LI-6400 Portable Photosynthesis) with a circular assimilation chamber with 6 cm^2 working in open system. The environment conditions were: RH 40±5%, 350±5 μL L^{-1} , atmospheric CO_2 and 250 μmol m^{-2} s^{-1} as irradiance level. For each assay were used 15 plants and the experience was repeated twice.

The maximum photosynthetic rate were calculated by the O_2 evolution rate at saturated CO_2 using a Hansatech leaf disc oxygen electrode. The leaf chamber was maintained at 25ºC. Illumination was from an overhead tungsten light source (Björkman light) through different filters. Oxygen evolution was measured as a function of light intensity as described by Walker (1990). A non-linear regression model using the best

fitting reply curve to light calculated the photosynthetic rate at saturated light. For each assay were used 3 plants and the experience was repeated twice.

Classical growth analysis were calculated according to Hunt (1990). Growth analysis of the plantlets at day zero (beginning of acclimatization) was also determined The experiment was designed as being completely randomised and was repetead twice. The statistical significance of the treatments was tested by one-way analysis of variance with individual plants as replicates. In figures and tables, means with different letters are significantly different according Duncan's multiple range test at $P \leq 0,05$.

RESULTS AND DISCUSSION

In this experiment, the elevated CO_2 does not affect the survival rate (Table 1) and gives rise to a significant increase in relative growth (Fig. 1A), shoot/root ratio (Fig. 1B) and leaf area ratio (LAR) (Table 1). These indicators are important to know the well-balanced plant development and they are associated with a vigorous growth.

For both treatments, the plants are successfully acclimatized, 97% of survival for the plants at ambient CO_2 (350 μL L^{-1} CO_2) and 94% for the plants at elevated CO_2 (700 μL L^{-1} CO_2). The two CO_2 concentrations seems to be able to increase progressive autotrophy.

The shoot/root ratio showed a less developed root system by the plants under the elevated CO_2, in spite of the greatest diameter of their roots, which is confirmed by the root system analysis (Table 2). Actually, those plants showed a significantly different shoot/root ratio related to plants under lower CO_2 concentration and to day zero.

Self comparing the leaf dry weight (Fig. 2A) and root dry weight (Fig. 2B) no significant differences were registered between the two concentrations of CO_2, but the significative increments relatively to the acclimatization zero day values must be stated which shows the radicular and aereo systems proper functioning. This allows a greater survival warranty to the plants during this critical phase of the autotrophic competence acquiring process.

The leaf area (Fig. 3) was not significantly affected by the CO_2 concentration. The increase in leaf area is usually associated with increased branching or tillering but the plants at 350 and 700 μL L^{-1} CO_2 showed an higher expansion of the leaves themselves more than the number of new developed leaves. Most of persistent leaves also remain active during all the acclimatization period. The leaf weight ratio (LWR) (Fig. 4A) showed that all the plants had invested more in leaves than in stem and/or roots.

Specific leaf area (SLA) (Fig. 4B) of the acclimatized plants at 700 μL L^{-1} CO_2 was significantly different from those at 350 μL L^{-1} CO_2. The plants acclimatized at ambient CO_2 had a continued foliar expansion but not followed in weight which may lead the thought that maybe the cells have increased intercellular spaces.

The relative growth rate (RGR) (Table 1) also shows the plant new material production efficiency, both in 350 and 700 μL L^{-1} CO_2. No differences in relative growth rate between the two plant groups may be explained as a consequence of a favourable carbon balance between the photosynthetic gains and the respiratory losses by shoot and roots, measured as net assimilation rate (NAR) (Table 1).

Plants acclimatized at 700 μL L^{-1} CO_2 showed a significantly different leaf area ratio (LAR) (Table 1). This parameter allows to characterizate the assimilating apparatus relative dimension and, in wide sense, represents the relation between the photosynthetic material and the respiratory tissues.

We also analysed the stomatal morphology and we saw that during acclimatization, stomata adapted to a reducing relative humidity and that the wide stomatal aperture present in persistent leaves was diminishing as new leaves were formed. At the end of acclimatization the stomata were closed and had an ellipsoidal shape revealing full water loss control.

The stomatal frequency (Table 3) showed significant differences between the two treatments (Table 3) with the highest ones occurring at 700 μL L^{-1} CO_2 on the first and second expanded leaves but no differences were revealed between the two CO_2 concentrations on the third new leaf (Fig. 5).

The net photosynthetic rate (Table 4) was similar in both treatments but the plants acclimatized at elevated CO_2 showed an increase in maximum photosynthetic rate (Table 5) and is clear that this increment happens as the new leaves developed during the acclimatization stage. We think that the gains that we have achieved with the use of elevated CO_2 can be more significant if we use an higher light intensity, once the saturation light level of this plants is near the 400 μmol m^{-2} s^{-1}, and the stimulatory effects of increased CO_2 concentrations on photosynthesis are much more pronounced under higher than low light conditions. This photosynthetic increase may have a substantial impact on subsequent growth and yield potential.

We intend to continue this study on the impact of elevated CO_2, in relation to irradiance level on the chestnut acclimatization process in order to obtain a better vigour in the growth of the microplant and also allowing the reduction of the period time of acclimatization in a successful micropropagation system.

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Tables

Table 1. Effects of CO_2 concentration during acclimatization of *in vitro*-regenerated chestnut plants on survival, relative growth rate (RGR), net assimilation rate (NAR) and leaf area ratio (LAR).

CO_2 ($\mu L\ L^{-1}$)	**Survival** (%)	**RGR** ($g\ g^{-1}\ day^{-1}$)	**NAR** ($g\ m^{-2}\ day^{-1}$)	**LAR** ($m^{-2}\ g^{-1}$)
350	97 a	0,703 a	60,1 a	0,0188 b
700	94 a	0,732 a	61,5 a	0,0223 a

Table 2. Effects of CO_2 concentration during acclimatization of *in vitro*-regenerated chestnut plants on root system.

CO_2 ($\mu L\ L^{-1}$)	**Total lenght** (cm)	**Area** (cm^2)	**Volume** (cm^3)	**Diameter** (mm)
350	284,5 a	76,0 a	1,61 a	0,82 b
700	223,7 b	62,0 b	1,35 a	0,86 a

Table 3. Effects of CO_2 concentration during acclimatization of *in vitro*-regenerated chestnut plants on stomatal frequency.

CO_2 ($\mu L\ L^{-1}$)	**Stomatal frequency**
350	291,1 b
700	345,6 a

Table 4. Effects of CO_2 concentration on net photosynthetic rate (A) at the end of acclimatization.

CO_2 ($\mu L\ L^{-1}$)	**A** ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)
350	4,93 a
700	4,72 a

Table 5. Effects of CO_2 concentration on maximum photosynthetic rate (A_{max}).

	A_{max} ($\mu mol\ O_2\ m^{-2}\ s^{-1}$)		
CO_2 ($\mu L\ L^{-1}$)	**PL**	**L2**	**L3**
350	3,66±0,10	7,99±0,11	6,95±0,15
700	6,31±0,17	11,89±0,78	11,31±0,90

Figures

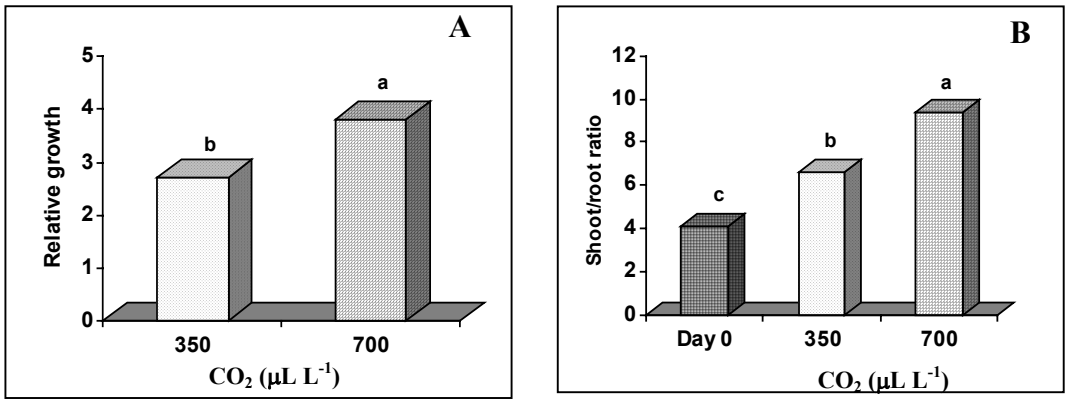

Fig. 1. Effects of CO_2 concentration during acclimatization of *in vitro*-regenerated chestnut plants on relative growth (A) and shoot/root ratio (B).

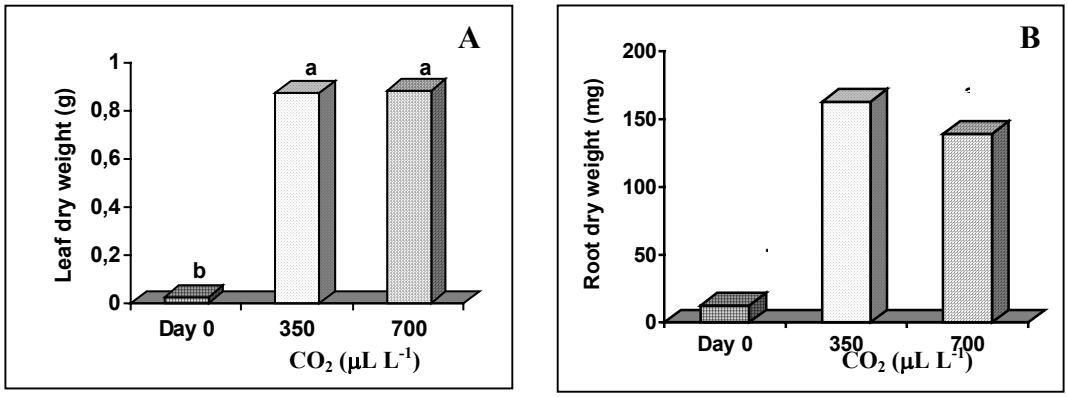

Fig. 2. Effects of CO_2 concentration during acclimatization of *in vitro*-regenerated chestnut plants on leaf dry weight (A) and root dry weight (B).

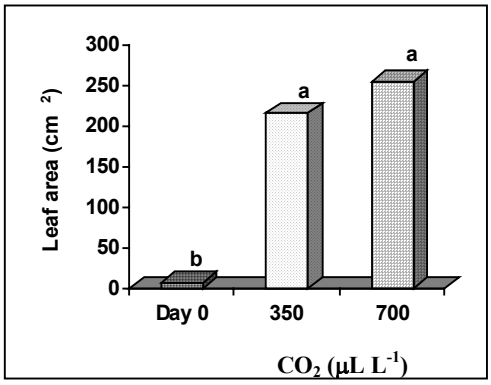

Fig. 3. Effects of CO_2 concentration during acclimatization of *in vitro*-regenerated chestnut plants on leaf area.

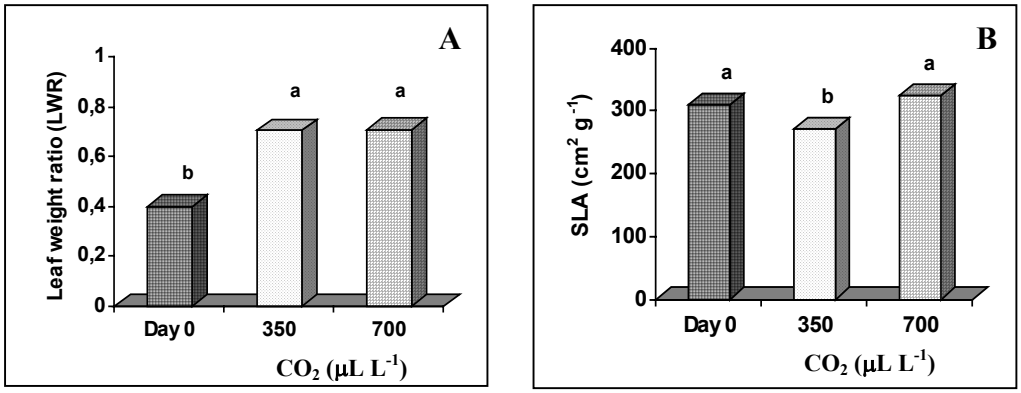

Fig. 4. Effects of CO_2 concentration during acclimatization of *in vitro*-regenerated chestnut plants on leaf weight ratio (LWR) (A) and specific leaf area (SLA) (B).

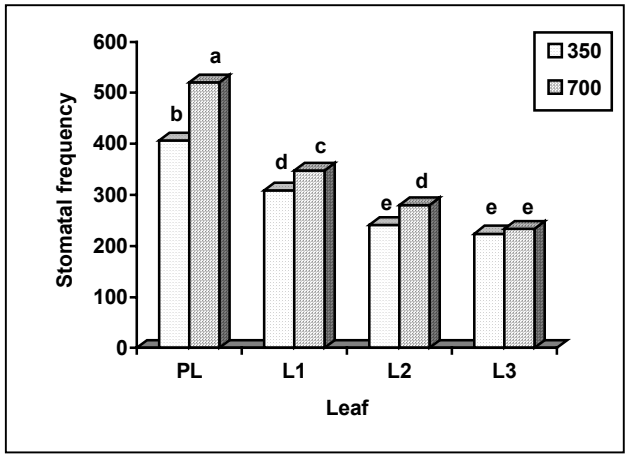

Fig. 5. Effects of CO_2 concentration during acclimatization of *in vitro*-regenerated chestnut plants on stomatal frequency, in different leaves in each treatment.