Growth Analysis of *In Vitro*-Regenerated Chestnuts During the Acclimatization Stage Using Elevated CO_2

Análisis del crecimiento en castañas Vitro-Regeneradas durante la etapa de la aclimatación usando el CO_2 elevado

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Abstract

Great advances have been made but some cultured plantlets still having a poor performance during the *ex vitro* stages and more specifically during the acclimatization and the nursery establishment stages. The problem is highly complex and requires the fast adaptation of plants growing in artificial conditions when transferred to the natural conditions. Until now, there is no consensus on the better strategy to obtain a higher efficiency of the propagation protocols while optimising acclimatization success. 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 μLL^{-1} 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). For both CO_2 concentrations, the plants are successfully acclimatized and they are in good conditions to be transferred to a greenhouse to continue their development for the rest of the season, and in the next winter/spring they can go to the field. The plants under elevated CO_2 showed a 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 problems during this critical phase of the autotrophic competence acquiring process. The net photosynthesis rate (A) 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. The different analysed leaf types showed a marked increment of the maximum photosynthetic rate as the new leaves developed during the acclimatization stage. Net photosynthesis rate and the maximum photosynthetic rate are light dependent, and are positively affected by the highest irradiance level. We think that the gains that we have achieved with the use of elevated CO_2 can be more significant if a higher light intensity can be used instead because they have a better response capacity to an increment of the level of irradiance.

Keywords: acclimatization, chestnut, growth analysis, CO_2, autotrophy

Resumen

Se han hecho de los grandes avances mas algunas de las plántulas todavía tenían un pobre desempeño durante las etapas *ex vitro* y más específicamente durante las etapas de aclimatación y el establecimiento en vivero. El problema es altamente complejo y requiere la rápida adaptación de las plantas desarrolladas en condiciones artificiales cuando transferidas a las condiciones naturales. Hasta este momento no hay consenso en la mejor estrategia para obtener una más alta eficiencia en los protocolos de propagación a la vez que se optimiza la aclimatación. En este estudio presentamos los resultados del análisis del crecimiento en híbridos vitro-regenerados de la castaña (*Castanea sativa* x *C. crenata*), durante la etapa de la aclimatación, usando dos concentraciones del CO_2 (350 μLL^{-1} y 700 μLL^{-1}) en 250 $\mu mol\ m^{-2}\ s^{-1}$ como nivel del irradiance (PPFD). El CO_2 elevado no afectó la tarifa de la supervivencia y fuera susceptible de aumentar progresivo autotrophia, expresado en un aumento significativo en crecimiento relativo, cociente de aereo/raíz y en el cociente del área de la hoja (LAR). Para ambas concentraciones del CO_2, las plantas se aclimatan con éxito y están en las buenas condiciones para se transferirán a un invernadero para proceder su desarrollo para el resto de la estación, y en el invierno / resorte siguiente pueden ir al campo. Las plantas debajo del CO_2 elevado demostraron una frecuencia stomatal más alta pero las hojas nuevas desarrolladas en el final de la aclimatación revelaron una gradual normal morfología stomatal y redujeron la frecuencia stomatal. Su morfología demostró el control eficaz de pérdida de agua lo cuál es uno dos problemas más importantes durante esta fase crítica de

adquirir la capacidad autotrophica. La tarifa neta de la fotosíntesis (A) fuera similar en ambos tratamientos pero las plantas aclimatadas en el CO_2 elevado demostraron un aumento en la tarifa fotosintética máxima (A_{max}), y ésta puede conducir a un mejor desarrollo fisiológico. Los diversos tipos de hoja analizados demostraron un incremento marcado de la tarifa fotosintética máxima con las nuevas hojas desarrolladas durante la etapa de la aclimatación. La tarifa neta de la fotosíntesis y la tarifa fotosintética máxima son dependientes de la luz y son afectadas positivamente por el nivel más alto del irradiance. Pensamos que los aumentos que hemos alcanzado con el uso del CO_2 elevado pueden ser más significativos si una intensidad de luz más alta puede ser utilizada porque esto plantas tienen una mejor capacidad de respuesta a un incremento del nivel del irradiance.

Palabras claves: aclimatación, castaña, análisis del crecimiento, CO_2, autotrofia

INTRODUCTION
INTRODUCCIÓN

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
MATERIALES Y MÉTODOS
Plant material and culture conditions
Condiciones del material vegetal y de la cultura

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
El arraigar y aclimatación

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
Análisis del crecimiento

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 (A) 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%, atmospheric CO_2 (350±5 µL L^{-1}) 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 (A_{max}) was 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 was 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 repeated 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
RESULTADOS Y DISCUSIÓN

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) (Table 1). 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 (Fig. 1B) 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 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 (A) (Table 4) was similar in both treatments but the plants acclimatized at elevated CO_2 showed an increase in maximum photosynthetic rate (A_{max}) (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
Tablas

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 (µL 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 (µL 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

Fgures
Figuras

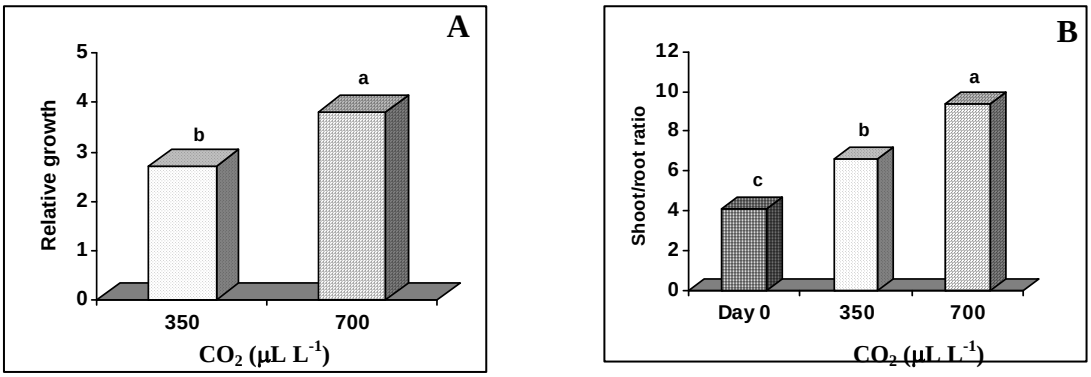

Fig. 1. Effects of CO_2 concentration during acclimatization of in vitro-regenerated chestnut plants on relative growth (A) and shoot/root ratio (B).

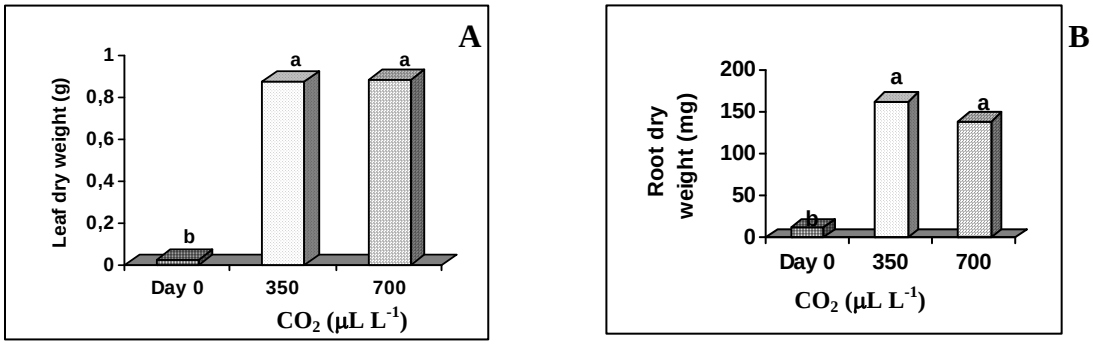

Fig. 2. Effects of CO_2 concentration during acclimatization of in vitro-regenerated chestnut plants on leaf dry weight (A) and root dry weight (B).

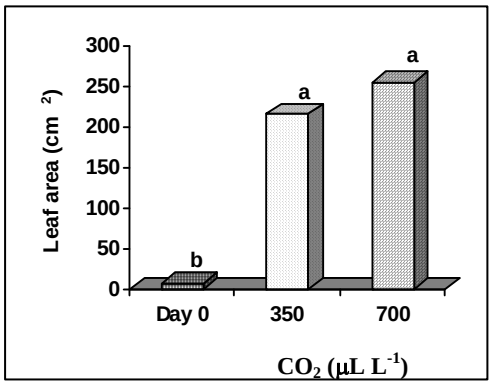

Fig. 3. Effects of CO_2 concentration during acclimatization of in vitro-regenerated chestnut plants on leaf area.

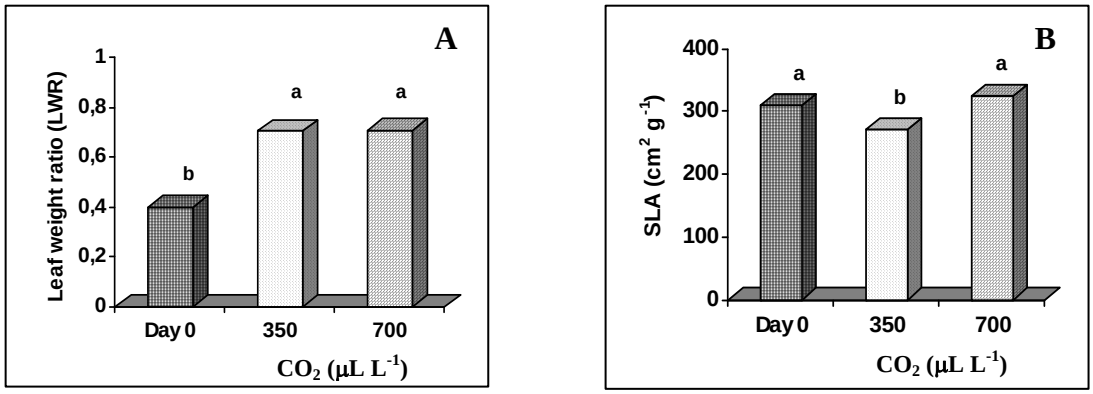

Fig. 4. Effects of CO_2 concentration during acclimatization of in vitro-regenerated chestnut plants on leaf weight ratio (LWR) (A) and specific leaf area (SLA) (B).

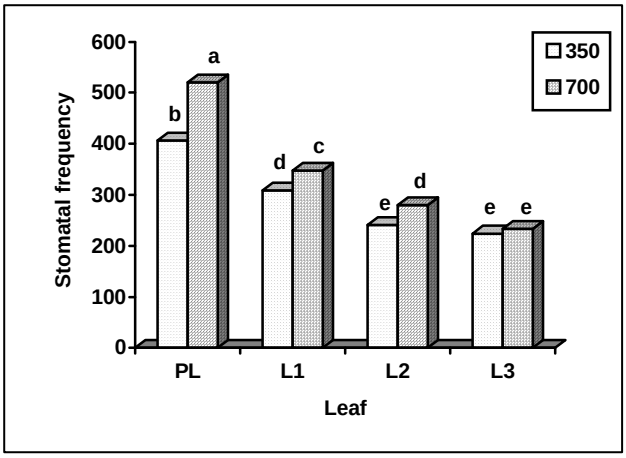

Fig. 5. Effects of CO_2 concentration during acclimatization of in vitro-regenerated chestnut plants on stomatal frequency, in different leaves in each treatment.