Scientia Horticulturae 138 (2012) 7–16

Contents lists available at SciVerse ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Increased light intensity during *in vitro* culture improves water loss control and photosynthetic performance of *Castanea sativa* grown in ventilated vessels

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ARTICLE INFO

Article history:
Received 20 August 2011
Received in revised form 6 January 2012
Accepted 3 February 2012

Keywords:
Micropropagation
Photon flux density
Ventilation
Transpiration rate
Photosynthesis

ABSTRACT

The effects of *in vitro* environmental conditions, ventilation of culture vessels and light level, on water loss control and photosynthetic capacity of *Castanea sativa* during *in vitro* culture were assessed. *C. sativa* microshoots were cultured in ventilated (V) and non ventilated (NV) vessels, using two photon flux density (PFD) levels, 50 and 150 μmol photons m^{-2} s^{-1} (L_{50} and L_{150}, respectively). The use of ventilation and the increase in irradiance improved the performance of the microshoots with respect to traditional *in vitro* culture of *C. sativa* (NVL_{50}). Microshoots grown under VL_{150} showed an increase in stomatal density and improved their functional characteristics, showing a more elliptical shape and lower percentage of open stomata. This paralleled a significantly lower transpiration rate and stomatal conductance. Increasing light level and using ventilated vessels increased the microshoots' capacity to harmlessly dissipate excess absorbed energy, water use efficiency and photosynthetic activity, resulting in a greater production of new microshoots. These improvements during *in vitro* culture generate microshoots with anatomical and functional characteristics similar to those observed in seedlings, which could help reduce the stress observed during *ex vitro* transfer.

1. Introduction

Micropropagation has become an important and effective method of propagation for many species. However, the artificial environment culture conditions promote anatomical and physiological anomalies in micropropagated plants, such as thin cuticles with reduced epicuticular wax, poor stomatal control and reduced photosynthetic capacity (Carvalho et al., 2001; Badr and Desjardins, 2007; Sáez et al., 2012). These anomalies may contribute to high mortality during *ex vitro* transfer (Debergh et al., 1992) due to the stress involving drastic change in environmental conditions. Therefore, acclimatization of micropropagated plants to *ex vitro* conditions is crucial to overcome stresses of a variable environment (Osório et al., 2010). Several studies have examined the benefits of environmental control mainly during acclimatization stage. Thus, strategies such a reductions in the amount of carbohydrate applied to the culture medium, the relative humidity inside the vessels (Seon et al., 2000; Fuentes et al., 2005; Deccetti et al., 2008), variation of irradiance levels (Navarro et al., 1994; Carvalho et al., 2001; Lian et al., 2002; Deccetti et al., 2008; Osório et al., 2010), use of natural light (Costa et al., 2009) and increase in gas exchange (Pospísilová et al., 2000; Mosaleeyanon et al., 2004) have been adopted. According to Pospísilová et al. (2000), *in vitro* conditions are those that produce malformations, however, very little have been investigated during the proliferation *in vitro* (Seon et al., 2000; Serret and Trillas, 2000; Brutti et al., 2002). There are some reports on environmental control in shrub and herbaceous species during *in vitro* culture (Serret et al., 1996; Serret and Trillas, 2000; Tichá et al., 1998; Kadlecek et al., 2001; Majada et al., 2001; Mohamed and Alsadon, 2010) but very few studies are available for woody species (Premkumar et al., 2003), which seem to be particularly affected during the acclimatization and *ex vitro* transfer. The advantage of managing the environmental conditions during the proliferation stage is to realize a significant decrease of *ex vitro* acclimatization, which requires time and expenses that can restrict the commercial application of micropropagation technique (Fila et al., 1998; Seon et al., 2000; Kadlecek et al., 2001).

Abbreviations: A_{max}, photosynthetic capacity; IBA, indole 3-butyric acid; BAP, 6-benzylaminopurine; MS, Murashige and Skoog medium; ETR, electron transport rate; LCP, light compensation point; LSP, light saturation point; NPQ, non-photochemical quenching; PFD, photon flux density; PSII, photosystem II; g_s, stomatal conductance; qL, photochemical quenching; 1-qL, excitation pressure; Q_a, primary quinone acceptor of PSII; WUE, water use efficiency.

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doi:10.1016/j.scienta.2012.02.005

The relative humidity and irradiance levels inside culture vessels are important environmental factors that can be addressed during *in vitro* culture. The use of poorly ventilated or closed vessels results in high relative humidity, which can cause an increase in stomata density (Joshi et al., 2006) non-functional (Apóstolo et al., 2005) and scarce epicuticular wax development (Majada et al., 2001). These characteristics restrict water loss control when *in vitro* cultured plants are subjected to sudden changes in environmental conditions, such as during *ex vitro* transfer, which imposes a sudden increase in evapotranspiration demand (Khan et al., 2003). In addition, the low photon flux density (PFD) used in the growth chambers limits photosynthesis, reducing both the ATP and NADPH production rates. This also affects, among other factors, ribulose 1.5 biphosphate (RuBP) regeneration rate (Taiz and Zeiger, 2002) and inhibits the development of photo-protection mechanisms (Sáez et al., 2012). Thus, the substantial increase in irradiance after the plants are transferred to natural environmental conditions can lead to photo-oxidative destruction of the photosynthetic apparatus (Long et al., 1994). So, plants must have both the capability of manage the water loss and a series of mechanisms that prevent excess of light capture and balance the absorbed energy with photosynthetic demand and sink capacities.

With the purpose to produce *in vitro* microshoots with characteristics similar to those found in plants grown under normal conditions (seedlings) before the acclimatization stage, we assessed whether the manipulation of microenvironmental conditions, irradiance and ventilation, from the *in vitro* introduction and proliferation stages of *Castanea sativa*, would improve the anatomical and functional characteristics associated with water loss control, photosynthetic capacity and photo-protection mechanisms. This could improve the micropropagation of this woody species, and especially help to alleviate plant stresses associated with their transfer from *in vitro* culture to *ex vitro* conditions.

2. Materials and methods

2.1. Plant material and culture conditions

Mature seeds of *Castanea sativa* were collected from a seed orchard in the Bío-Bío Region, Chile. Half of the seeds were used for *in vitro* culture and the other half for the seedlings production. For *in vitro* culture, seeds were subjected to surface asepsis and the embryonic axis was extracted and cultured in ventilated (V) and non ventilated vessels (NV) on half-strength MS medium, supplemented with 30 g L^{-1} sucrose and gelled with 7 g L^{-1} agar at pH 6.2. The seeds were kept in darkness until germination. After the embryos reached a height greater than 2 cm (approximately 20 days after the start of *in vitro* culture), they were transferred to MS medium supplemented with 0.22 μM BAP and 0.024 μM IBA and 30 g L^{-1} sucrose. The medium was adjusted to pH 6.2 and gelled with 7 g L^{-1} agar. Microshoots were cultured under two different photon flux densities, 50 and 150 μmol photons m^{-2} s^{-1} (L_{50} and L_{150}, respectively) measured inside of ventilated and non ventilated vessels. Ventilation was accomplished by perforating a hole in magenta caps and covering them with membrane filter disks of 2 cm diameter (Micropore 3M™, Chile). In the non ventilated treatment, the vessels were closed with conventional magenta caps (Sigma Aldrich, St. Luis, MO, USA). The culture room environment conditions were set at 16 h light photoperiod with 24 ± 2 °C and 60% relative humidity. The microshoots were subcultivated every 45 days. After six months under these conditions, well developed microshoots (healthy and without visual contamination) were selected and used for analysis. The *in vitro* growth was evaluated through proliferation rate, determined as the number of new microshoots produced by the initially cultured microshoot, that were well developed and with a height greater than 2 cm and with at least one expanded leaf. Dry mass production was obtained for each treatment after drying leaves at 60 °C for 72 h.

The seedlings were cultured in an outdoor nursery with 85.2% relative humidity on average and 15.2 ± 2 °C and 6.5 ± 2 °C maximum and minimum temperature, respectively. Seeds were grown in black plastic bags filled with organic soil mixed with pine bark compost, maintained beneath a shade cloth (80% solar interception) and irrigated once a day.

2.2. Water loss control and net photosynthesis

Parameters associated with water loss control and photosynthetic performances were measured using an infrared gas analyzer (Ciras-2, PP System, Hitchin, UK). The measurements were made in leaves of seedlings and microshoots. These were removed from the culture vessels and a portion of agar was kept at their base to minimize desiccation. The parameters inside the leaf chamber were: CO_2 concentration 380 ppm with a flow of 200 cm^{-3} min^{-1}, relative humidity 75% and temperature 15–20 °C. The leaves were photographed inside the leaf chamber immediately following the measurements; and the leaf area was estimated using Sigma Scan Pro 5.0 software (SPSS, Chicago, IL). The measured gas exchange values were adjusted for the leaf chamber area/actual leaf area ratio. Light response curves of net CO_2 assimilation were made at different PFD (from 0 to 1500 μmol photons m^{-2} s^{-1}). Values for stomatal conductance (g_s) and transpiration rate (E) inside the leaf chamber were recorded. Using Photosynthesis Assistant 1.1 software (Dundee Scientific, Dundee, UK) light compensation point (LCP), light saturation point (LSP) and dark respiration rates (R_d) were obtained from the light response curves. Additionally, CO_2 response curves were performed at 15–20 °C and 500 μmol photons m^{-2} s^{-1}, to obtain the maximum rate of net photosynthesis under light and CO_2 saturating conditions (A_{max}). Instantaneous water use efficiency (WUE) was calculated as the ratio between net photosynthesis and transpiration rate.

2.3. Stomata frequency and morphology

Stomata frequency and morphology were analyzed with a scanning electron microscope (SEM, Jeol JSM6380 nLV, Tokio, Japan). Fresh leaf samples from expanded leaves were collected and sections of 1 mm^2 were fixed in 4% glutaraldehyde and post fixed in 1% osmium tetroxide. Following the treatments, leaves were analyzed with SEM and photomicrographs were analyzed using Image J software. The length (μm), width (μm), and percentage of open stomata (%) were determined.

2.4. Pigment determination

Leaf discs (3.86 mm diameter) were collected, frozen in liquid nitrogen and stored at −80 °C. Three discs (*ca.* 30 mg fresh weight) were powdered with liquid nitrogen in a cooled mortar. A spatula tip of $CaCO_3$ was added before extracting with 1 mL 100% HPLC-grade acetone at 4 °C under dim light. Pigments were separated and quantified by reversed-phase HPLC as described previously (Garcia-Plazaola and Becerril, 1999). The instrument system consisted of quaternary pump with automatic degassing system and autosampler. Signals from a diode array detector were integrated and analyzed using the software Agilent ChemStation B.04.01 (Agilent Technologies, Waldbronn, Germany). Chromatography was carried out using a Spherisorb ODS-1 reversed phase column (5 μm particle size; 4.6 mm × 250 mm, Atlantil Hilit, Waters, Ireland) and a Nova-pack C-18 (4 μm; 3.9 mm × 20 mm) guard column (Waters, Ireland). The mobile phase consisted of two components: solvent A, acetonitrile:methanol:Tris Buffer (0.1 M, pH 8.0) (42:1:7) and

solvent B, methanol:ethyl acetate (34:16). Pigments were eluted using a linear gradient from 100% A to 100% B within the first 12 min, followed by an isocratic elution with 100% B for the next 6 min. Absorbance was monitored at 445 nm. Retention times and response factors of Chl *a*, Chl *b*, lutein, β carotene, violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) were determined by injection of pure standards purchased from DHI, Hoershholm, Denmark. The epoxidation state (EPS) was determinate as $V + 0.5A/(V + A + Z)$.

2.5. Chloroplast ultrastructure

Chloroplast ultrastructure was analyzed with a transmission electron microscope (TEM, Jeol JEM1200 EXII, Tokio, Japan) at a voltage intensity of 60 kV. Fresh leaf samples from expanded leaves were collected and sections of 1 mm^2 were fixed in 4% glutaraldehyde and post fixed in 1% osmium tetroxide. Following the treatments, leaves were analyzed by TEM and chloroplast area (μm^2) and number of grana per chloroplast were determined from photomicrographs analyzed with Image J software.

2.6. Photochemical activities

Photochemical activity was measured through the kinetics of chlorophyll fluorescence using a pulse-amplitude fluorimeter (FMS II, Hansatech Instrument, King's Lyn, UK) in fully expanded leaves, which were previously darkened for 30 min. Different light pulses were applied following a standard routine program in the equipment. Minimal fluorescence (F_0) was determined by applying a weak modulated light (6 μmol photons m^{-2} s^{-1}) and maximal fluorescence (F_m), was induced by a short pulse (0.8 s) of saturating light (9000 μmol photons m^{-2} s^{-1}) according to Rosenqvist and Van Kooten (2003). The fluorescence signals were applied until they reached the steady state (F_s). To determine the maximal fluorescence in light ($F_{m'}$) various pulses of saturating light were applied. The minimal fluorescence ($F_{0'}$) was determined by turning off the actinic light and immediately applying a 2 s far red pulse. The data for F_v/F_m = variable fluorescence/maximal fluorescence, where $F_v = (F_m - F_0)$ (maximal fluorescence − minimal fluorescence) and $\Phi PSII = (F_{m'} - F_s)/F_{m'}$ were used as indicators of the maximal and effective quantum yield of the PSII, respectively (Genty et al., 1989). The electron transport rate (ETR) was calculated as in Genty et al. (1989) according to the following: ETR = 0.84 (ΦPSII) (PFD) 0.5, where ΦPSII is effective quantum yield of the PSII and PFD corresponds to incident photosynthetic photon flux. The factor 0.5 assumes that the efficiency of both photosystems is equal and that light is equally distributed between them. The factor 0.84 is the mean value of absorbance for green leaves. The fraction of PSII centers in the open state (qL) was calculated as described by Kramer et al. (2004): $qL = ((F_{m'} - F_s)/(F_{m'} - F_{0'}))\ (F_{0'}/F_s)$. Non-photochemical quenching was defined as: $NPQ = (F_m - F_{m'})/F_{m'}$ (Maxwell and Johnson, 2000). The fluorescence measurements were performed at PFDs of 10, 50, 75, 100, 150, 250, 450, 600 and 900 μmol photons m^{-2} s^{-1}.

2.7. Statistics

The effect of ventilation (V or NV) and light (150 or 50 μmol photons m^{-2} s^{-1}) and their interaction on morpho-physiological characteristics of *in vitro* cultured microshoots were studied using a factorial experimental design. All experiments were arranged in completely randomized design. Twenty two experimental units, corresponding to a culture vessel containing a single microshoot were installed for each treatment. For photosynthesis measurements three random replications were made, five replications for fluorescence parameters and three replications for pigment determinations. A two-way analysis of variances was used to test for significance at $P \leq 0.05$. Differences among means were established using LSD test. Additionally, one-way ANOVA was used to test significant differences in morpho-physiological traits among each *in vitro* treatment and seedlings.

3. Results

3.1. In vitro growth

The germination rate and expansion of embryonic axis in ventilated vessels (V) was higher than in non ventilated vessels (NV), observing the appearance of the first expanded leaves within 20 days from culture *in vitro*. In NV vessels the appearance of the first leaves was not observed until 45 days in the proliferation medium. After six months *in vitro* culture, the proliferation rate was dependent on the ventilation ($P = 0.005$). While the V treatment exceed proliferation rate was more than 100% of the NV (Fig. 1A). A similar tendency in dry matter production (Fig. 1B) was observed, but in this case, both light level and ventilation had a significant effect ($P = 0.0058$ and $P < 0.0001$, respectively). Although there was not a significant interaction between factors ($P = 0.604$ and $P = 0.950$ in proliferation rate and biomass production, respectively), the use of VL_{150} increased both, the proliferation of new microshoots (up to seven times more than in the traditional culture), and their biomass production with an increased dry mass production over 50% of the traditional culture (NVL_{50}).

3.2. Water loss control

Water loss control capacity was analyzed through transpiration (E) and stomatal conductance (g_s) measured *ex vitro* under CIRAS-2 leaf chamber conditions (see Section 2). E and g_s varied significantly with light levels ($P < 0.0001$) and ventilation ($P < 0.0001$) showing a significant interaction ($P < 0.0001$ and $P = 0.0008$, for E and g_s, respectively). Microshoots growing at L_{50} had higher E (Fig. 2A) and g_s (Fig. 2B) over the microshoots growing at L_{150} independent of ventilation. At L_{50} the use of ventilated vessels reduced by 50% both E and g_s, but the values were higher with respect to seedling. In L_{150} treatments, both E and g_s have values up to seven times lower than in L_{50}. Additionally, at this light level, the ventilation only produced a significant reduction of E. Microshoots cultured under VL_{150} exhibited similar E and g_s values to those observed in seedling, reflecting an improvement of water loss control.

3.3. Stomata frequency and morphology

The density and the anatomical characteristics of stomata responded with a significant interaction ($P < 0.0001$) between light and ventilation factors (Table 1). A high stomatal density was observed in leaves developed in NVL_{50}. Here, the stomatal shape was rounded, mostly open (Table 1), supported on the epidermal cells and had no deposition of epicuticular waxes (Fig. 3A–B). At the same light level in ventilated vessels (VL_{50}), leaf stomatal density did not differ with respect to NV, but exhibited a significantly lower percentage of open stomata that showed an elliptical shape. Additionally, stomata supported on the epidermis and stomata covered by a discontinuous wax deposition were observed (Fig. 3C–D). At the higher light level (L_{150} treatments) the frequency and stomatal morphology varied significantly between V and NV treatments (Table 1). In NV treatments leaves showed less than one third of stomatal density observed in V treatments. Although in both cases the stomata had elliptical shapes, similar to those found in seedling, the appearance of these varied dramatically depending on ventilation. In NV, stomata were covered by epicuticular waxes (Fig. 3E–F) and the ratio between open and closed stomata was similar, as observed in seedlings (Table 1). Whereas in V, slightly dehydrated

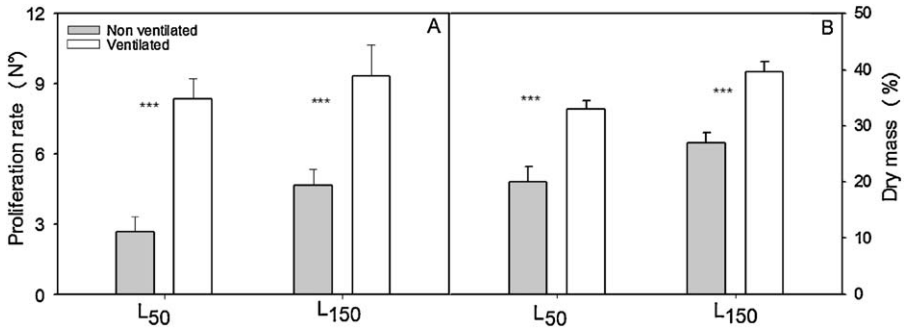

Fig. 1. Proliferation rate (A) and dry mass percentage (B) of *Castanea sativa* grown under 50 μmol photons m^{-2} s^{-1} (L_{50}) and 150 μmol photons m^{-2} s^{-1} (L_{150}) in non ventilated and ventilated vessels. ***Significant differences between ventilated and non ventilated vessels.

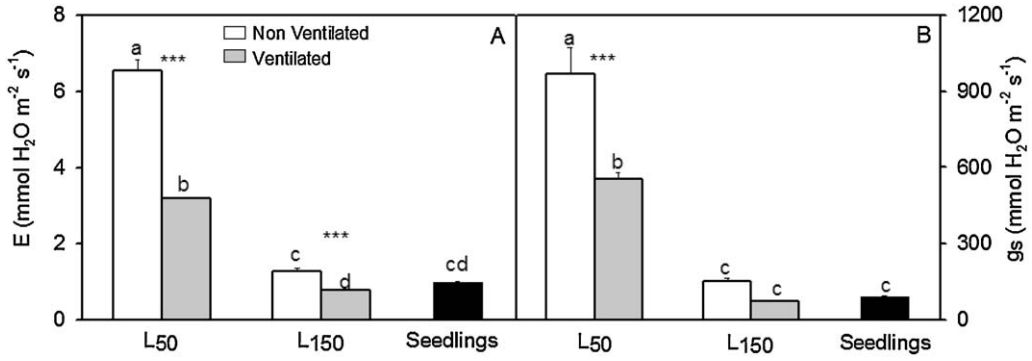

Fig. 2. Transpiration rate (A) and stomatal conductance (B) of microshoot leaves of *Castanea sativa* grown in non ventilated and ventilated vessels at 50 μmol photons m^{-2} s^{-1} (L_{50}) and 150 μmol photons m^{-2} s^{-1} (L_{150}). Different letters indicate significant differences among each treatment and seedlings (control) at $P < 0.05$ in one way ANOVA. Each columns represent the average ($n = 3$) and SE bars. ***Significant differences between ventilated and non ventilated system.

tissue was observed, but there was also evidence of waxes deposition. In this treatment (VL_{150}), the stomata were small and mostly closed (Fig. 3G–H).

3.4. Net photosynthesis and water use efficiency

Maximal photosynthetic capacity, light and CO_2 saturated (A_{max}), was dependent on the interaction of light and ventilation (Table 2). At L_{50}, A_{max} was less than half of the observed at L_{150}. When combining VL_{150}, a significant increase in photosynthetic capacity reaching similar values to those obtained in seedlings was observed. However, light saturated net photosynthesis at 380 ppm CO_2 (A_{sp}) remains low in all treatments (Table 2). VL_{150} also produced a significant increase in the light saturation point (LSP), which reached two-fold higher values than in the other treatments (Table 2). Only a slight increase was observed in dark respiration rate (R_d) under VL_{150}, being also closer to that of seedlings (Table 2). This is associated with the slight increase found in the light compensation point (LCP). The improved photosynthetic parameters found in microshoots culture under VL_{150} clearly imply an increased capacity for photosynthesis regarding to the traditional culture (NVL_{50}).

With respect to the water use efficiency (WUE), the tendency was similar to that observed in the photosynthetic performance. WUE (Fig. 4) was influenced by a significant interaction between light and ventilation factors ($P = 0.0046$). Thus, the increase of light

Table 1
Stomatal and chloroplast characteristics of *in vitro* cultured microshoot leaves of *Castanea sativa* grown under different conditions of ventilation and light.

	Stomatal characteristics				Chloroplast characteristics	
	Density (N° mm^{-2})	Length (μm)	Width (μm)	Open stomata (%)	Area (μm^2)	Grana (N°)
Seedlings	215.5 ± 12.1 cd	23.2 ± 0.3 a	20.0 ± 0.3 a	48.8 ± 2.1 b	9.4 ± 1.0 ab	7 ± 1.0 a
NV L_{50}	380.5 ± 27.0 b	20.4 ± 0.9 b	20.8 ± 0.8 a	90.7 ± 5.2 a	13.3 ± 1.5 c	0.1 ± 0.0 c
V L_{50}	315.1 ± 22.5 bc	20.6 ± 0.6 b	17.5 ± 0.5 b	64.8 ± 5.8 b	4.8 ± 1.0 ab	0.6 ± 0.0 c
NV L_{150}	129.3 ± 10.3 d	21.0 ± 0.3 b	19.3 ± 0.7 a	41.0 ± 4.3 b	8.0 ± 1.7 b	4.7 ± 1.3 b
V L_{150}	540.7 ± 22.9 a	14.2 ± 1.4 c	10.0 ± 0.8 c	18.5 ± 2.3 c	2.1 ± 0.2 a	6.5 ± 1.1 a
L	ns	**	***	***	*	**
V	**	***	***	***	**	*
L*V	***	***	***	**	ns	ns

Abbreviations: NVL_{50} non ventilated vessels at 50 μmol photons m^{-2} s^{-1}; VL_{50} ventilated vessels at 50 μmol photons m^{-2} s^{-1}; NVL_{150} non ventilated vessels at 150 μmol photons m^{-2} s^{-1} and VL_{150} ventilated vessels at 150 μmol photons m^{-2} s^{-1}.
Different letters indicate significant differences among each treatment and seedlings (control) at $P < 0.05$ in one way ANOVA. Means ± SE, $n = 20$.
ns, no significant in two-way ANOVA.
* Refers to factor's significance at $P < 0.05$
** Refers to factor's significance at $P \leq 0.01$.
*** Refers to factor's significance at $P \leq 0.001$.

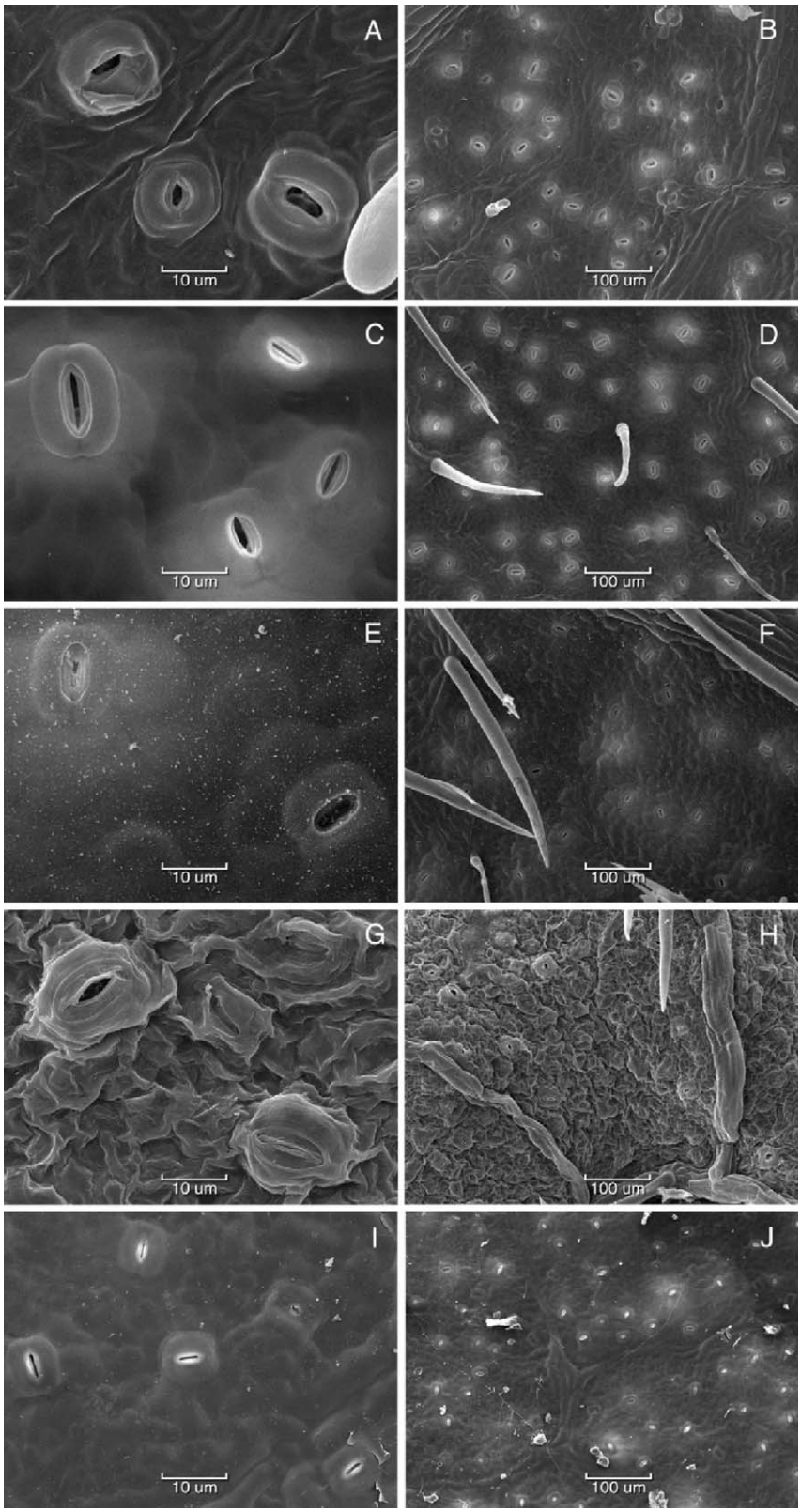

Fig. 3. Stomata of *in vitro* cultured of *Castanea sativa* grown in non ventilated vessels at 50 μmol photons m^{-2} s^{-1} (A–B), ventilated vessels at 50 μmol photons m^{-2} s^{-1} (C–D), non ventilated vessels at 150 μmol photons m^{-2} s^{-1} (E–F), ventilated vessels at 150 μmol photons m^{-2} s^{-1} (G–H) and seedlings (I–J).

produced an increase in WUE of over 100% with respect to the L_{50} treatments. Additionally, at L_{150} ventilation produced a significant increased of WUE, reaching values as high as those observed in the seedlings.

3.5. Pigment content

Chlorophyll (Chl) content was significantly influenced by light level (Table 3). Thus, an increase of up to 100% in both Chl *a* and

Table 2
Photosynthetic parameters obtained from light and CO_2 response curves of *in vitro* cultured microshoot of *Castanea sativa* grown under different conditions of ventilation and light.

	A_{max} (μmol CO_2 m^{-2} s^{-1})	A_{sp} (μmol CO_2 m^{-2} s^{-1})	LCP (μmol m^{-2} s^{-1})	LSP (μmol m^{-2} s^{-1})	R_d (μmol CO_2 m^{-2} s^{-1})
Seedlings	10.80 ± 3.00 a	7.23 ± 0.68 a	33.80 ± 21.20 a	722.02 ± 26.43a	0.57 ± 0.09 a
NVL_{50}	2.97 ± 0.38c c	3.24 ± 0.17 b	9.97 ± 0.70 a	164.92 ± 15.73 c	0.22 ± 0.02 b
VL_{50}	1.39 ± 0.12 d	1.75 ± 0.07 c	9.50 ± 0.00 a	193.67 ± 2.94 c	0.20 ± 0.02 b
NVL_{150}	4.45 ± 0.15 b	3.52 ± 0.23 b	8.17 ± 0.81 a	213.16 ± 12.19 c	0.12 ± 0.06 b
VL_{150}	8.72 ± 1.35 a	3.7 ± 0.19 b	15.46 ± 0.00 a	414.47 ± 15.26 b	0.37 ± 0.03 ab
V	**	**	***	*	ns
L	***	***	**	**	ns
L × V	***	***	***	***	ns

Abbreviations: Photosynthetic capacity (A_{max}), net photosynthesis at saturating light (A_{sp}), light compensation point (LCP), light saturation point (LSP), dark respiration rates (R_d). NVL_{50} non ventilated vessels at 50 μmol photons m^{-} s^{-1}; VL_{50} ventilated vessels at 50 μmol photons m^{-2} s^{-1}; NVL_{150} non ventilated vessels at 150 μmol photons m^{-2} s^{-1} and VL_{150} ventilated vessels at 150 μmol photons m^{-2} s^{-1}.
Different letters indicate significant differences among each treatment and seedlings (control) at $P < 0.05$ in one way ANOVA. Means ± SE, $n = 3$.
ns, no significant in two way ANOVA.
* Refers to factor's significance at $P \leq 0.05$
** Refers to factor's significance at $P \leq 0.01$.
*** Refers to factor's significance at $P \leq 0.001$.

Table 3
Effect of culture conditions, ventilation and light level, on pigments composition of *in vitro* cultured microshoot leaves of *Castanea sativa.*

	Seedlings	NVL_{50}	VL_{50}	NVL_{150}	VL_{150}	L	V	LV
Chl *a*	674.2 ± 169.69 a	1311.3 ± 161.0 a	1353.3 ± 50.6 a	3435.1 ± 305.6 b	3323.0 ± 662.4 b	***	ns	ns
Chl *b*	224.8 ± 47.9 a	400.4 ± 38.5 a	399.6 ± 18.9 a	1105.9 ± 84.5 b	1245.7 ± 92.8 b	***	ns	ns
Chl *a/b*	2.9 ± 0.2 ab	3.2 ± 0.1 ab	3.4 ± 0.2 b	3.1 ± 0.0 ab	2.6 ± 0.3 a	ns	ns	ns
Neoxanthin	58.4 ± 6.5 b	nd	36.1 ± 0.1 a	32.0 ± 3.2 a	42.3 ± 8.0 a			
Lutein	219.1 ± 13.3 b	189.2 ± 8.1 ab	225.4 ± 26.6 b	145.9 ± 8.3 a	147.6 ± 23.4 a	*	ns	ns
β Carotene	187.4 ± 13.2 ab	166.9 ± 9.4 a	172.1 ± 6.2 ab	178.3 ± 1.6 ab	205.5 ± 19.0 b	ns	ns	ns
Violaxantin	216.4 ± 26.4 b	nd	63.4 ± 1.6 a	56.5 ± 5.4 a	61.9 ± 10.9 a			
Anteraxanthin	44.5 ± 5.9	nd	nd	nd	nd			

Abbreviations: Non ventilated vessels at 50 μmol photons m^{-2} s^{-1} (NVL_{50}), ventilated vessels at 50 μmol photons m^{-2} s^{-1} (VL_{50}), non ventilated vessels at 150 μmol photons m^{-2} s^{-1} in (NVL_{150}), ventilated vessels at 150 μmol photons m^{-2} s^{-1} (NVL_{150}). Pigments are in $\mu g\,g^{-1}$ FW. Carotenoids data are expressed in relation to Chl *a* + *b*.
Different letters indicate significant differences among each treatment and seedlings (control) at $P < 0.05$ in one way ANOVA. Means ± SE, $n = 3$.
nd, no detected pigments.
ns, no significant in two way ANOVA.
* Refers to factorĭs significance at $P < 0.05$
** Refers to factorĭs significance at $P \leq 0.01$.
*** Refers to factorĭs significance at $P \leq 0.001$.

b was observed in L_{150} treatments, independent of the ventilation. Both the chlorophyll *a* and *b* contents were more than 100% higher than those found in seedlings, independent to the *in vitro* treatments. The Chl *a/b* ratio was similar in all *in vitro* treatments and seedlings, remaining close to 3.0. Although the chlorophyll content was lower in seedlings, the proportion of photoprotective pigments with respect to the total chlorophyll content was higher than *in vitro*. Thus, carotenoids (per unit of chlorophyll) in seedlings exceeded by almost 100% that was determined *in vitro*, independent of treatment used (Table 3). A significantly larger proportion of xanthophyll pools were observed in seedlings, more than three-fold higher than *in vitro*. This is due mainly to a higher proportion of violaxanthin and antheraxanthin. This latter pigment was not detected *in vitro*. Zeaxanthin was not detected in seedlings nor in *in vitro* microshoots. These microshoots displayed a 100% epoxidation state, showing the lack conversion of violaxanthin to antheraxanthin and zeaxanthin in any of the *in vitro* treatments.

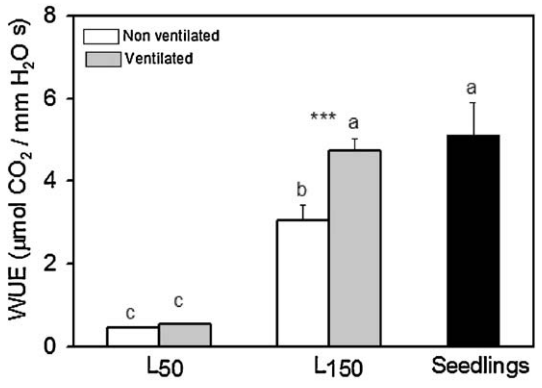

Fig. 4. Water use efficiency response curve (WUE) from *in vitro* microshoot of *Castanea sativa* grown in non ventilated and ventilated vessels at 50 μmol photons m^{-2} s^{-1} (L_{50}) at 150 μmol photons m^{-2} s^{-1} (L_{150}). ***Refers to differences between ventilated and non ventilated treatments. Different letters indicate significant differences among each treatment and seedlings (control) at $P < 0.05$ in one way ANOVA. Each columns represent the average ($n = 3$) and SE bars.

3.6. Chloroplast ultrastructure

Chloroplast characteristics were affected by the light level and ventilation. Chloroplast area in NV treatments was two or three-fold larger than in V treatments, and dependent on light level (Table 1). In NVL_{50} the chloroplasts were flat and devoid of grana, showing a network of stromal lamellae (Fig. 5A–B). Chloroplasts area was significantly decreased under high light and was similar to that observed in seedlings (Table 1). Increased light induced grana formation in *in vitro* microshoots (Fig. 5E–G) reaching similar number of grana per chloroplast as found in seedlings. However, in all cases, the grana size was smaller than those of seedlings (Fig. 5). Here, chloroplasts store large starch granules (Fig. 5I–J), differing to observed in all *in vitro* treatments.

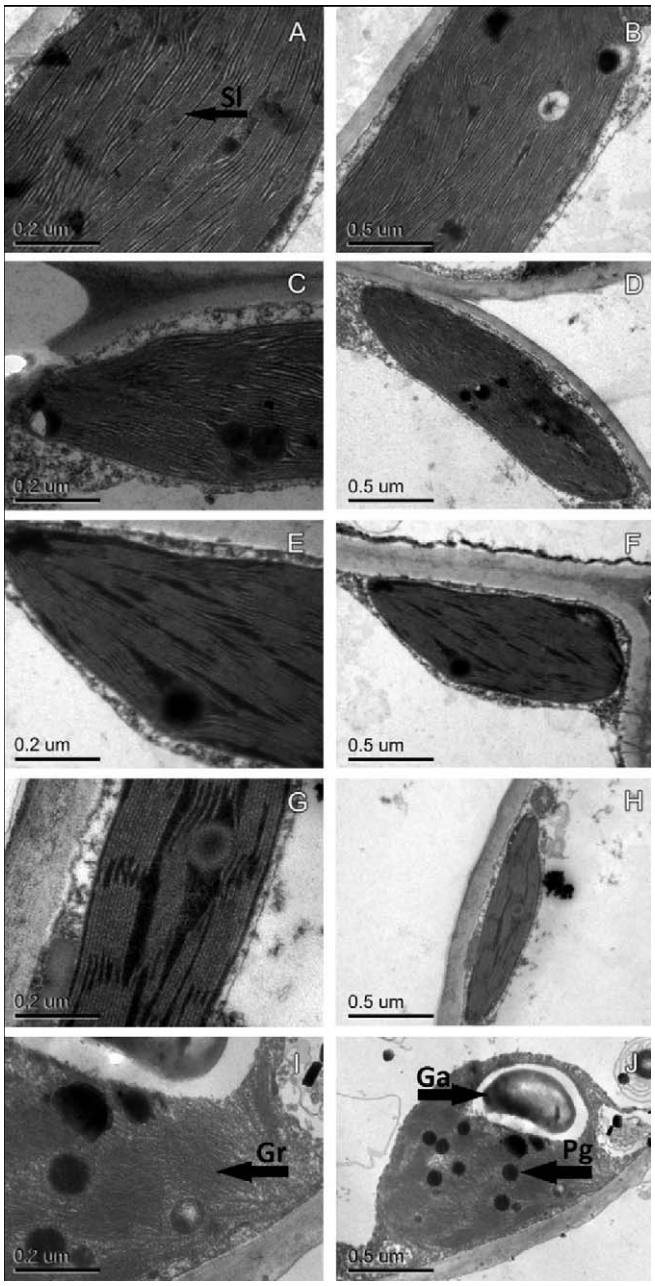

Fig. 5. Electron micrographs of mesophyll cell chloroplasts of *in vitro* cultured microshoot leaves of *Castanea sativa* grown in non ventilated vessels at 50 μmol photons m^{-2} s^{-1} (A–B), ventilated vessels at 50 μmol photons m^{-2} s^{-1} (C–D), non ventilated vessels at 150 μmol photons m^{-2} s^{-1} (E–F), ventilated vessels at 150 μmol photons m^{-2} s^{-1} (G–H) and seedlings (I–J). Left and right bars indicated 0.2 and 0.5 μm, respectively. Sl: stromata lamellas Gr: grana; G_{Al}: starch; Pg: plastoglobuli.

3.7. Photochemical activities

Photochemical activity was affected by both ventilation and light level, but there was not a significant interaction between them ($P > 0.05$). The maximum electron transport rate (ETR_{max}) (Fig. 6A) was significantly lower in L_{50} compared to L_{150}. L_{50} reached its maximum ETR below 200 μmol photons m^{-2} s^{-1} and did not exceed 40 μmol of electrons m^{-2} s^{-1}. Increasing the light level, there was a significant increase in ETR_{max} in both V and NV treatments. Thus, ETR_{max} was close to 100 and 85 μmol electrons m^{-2} s^{-1} in NV and V treatments, respectively; similar to those observed in seedlings. The maximal thermal dissipation capacity of excess light energy

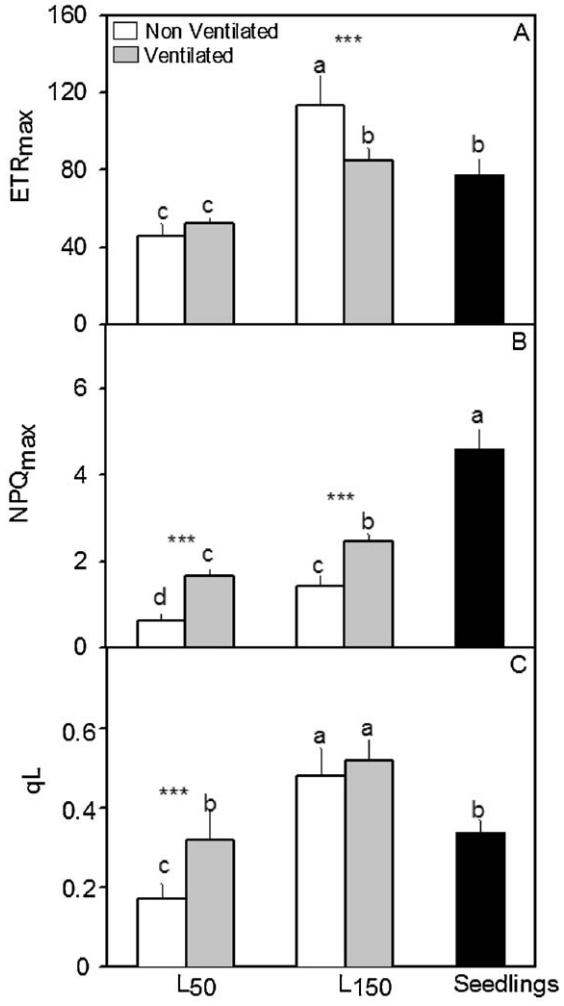

Fig. 6. Maximal electron transport rate (A), maximal non photochemical quenching at saturating light (B) and relative redox state of PSII at saturating light (C) of *in vitro* microshoot leaves of *Castanea sativa* grown in non ventilated and ventilated vessels at 50 μmol photons m^{-2} s^{-1} (L_{50}) and 150 μmol photons m^{-2} s^{-1} (L_{150}). ***Refers to differences between ventilated and non ventilated treatments at $P < 0.05$ in two way ANOVA. Different letters indicate significant differences among each treatment and seedlings (control) at $P < 0.05$ in one way ANOVA. Each columns represent the average ($n = 5$) and SE bars.

(NPQ_{max}) (Fig. 6B) found *in vitro* was low compared with those found in seedlings, where the NQP_{max} oscillates from zero to about 5.0 within the range of studied PFDs. The highest NPQ_{max} was reached in the VL_{150} treatments exhibiting the highest dissipating capacity respect to the other *in vitro* treatments and exceeding by more than 100% the NPQ developed under traditional *in vitro* condition (NVL_{50}). Regarding photochemical use of the energy, the proportion of reaction centers in the open state (qL), was close to 50% in L_{150} treatments (Fig. 6C), exhibiting a lower excitation pressure (1-qL) than the L_{50} treatment and even lower than that observed in seedlings.

4. Discussion

As expected, the evaluated factors, photon flux density (PFD) and ventilation in vessels had significant effects on morpho-physiological variables of *C. sativa*. But it was their interaction that directly affected the characteristics associated with the ability to control water loss, as well the photosynthetic performance. Thus, the combined use of a higher light level (L_{150}), with respect to the normally used (L_{50}) and ventilated vessels (V), produced

improvements in these characteristics and parameters associated with *in vitro* growth. The culture under VL_{150} promotes a greater number of new microshoots with higher dry matter proportion (Fig. 1A and B), which should be influenced by a greater ability to control the transpiration rate (Fig. 2A) showing similar values to those obtained in seedlings. These suggest that microshoots grown under these conditions are adapted to higher irradiances and use stomatal closure to decrease water loss; this is also reflected in an increase in water use efficiency (Fig. 4). These results are consistent with that observed at the stomatal apparatus level, where the combination of factors, VL_{150} influenced both the shape and the stomatal density (Table 1). Regarding the shape, only the increase in light enhances the development of more elliptical stomata, which is generally associated with higher functionality (Brutti et al., 2002; Khan et al., 2003). Additionally, the significant increase in stomatal density, showed that *in vitro* cultured microshoots have the same trend as the plants developed in other environments which have an increase in the stomata frequency under conditions of high light and CO_2 availability (Deccetti et al., 2008).

Regarding photosynthetic performance, the positive effect of higher light in the photosynthetic capacity was enhanced by ventilation (Table 2). Thus an increase over 100% in photosynthetic capacity was observed in microshoots growing in VL_{150}, which was the only treatment that promoted photosynthetic performance similar to seedlings. The improvements in photosynthetic performance agree with the previous reports of Lambers et al. (1998) and Larcher (2003) who noted that the acclimatization to high irradiances usually results in an increased of A_{max}, LSP, LCP and R_d. This improvement is not only associated with the idea of more carbon available for growth but also light promotes photomorphogenetic responses (Larcher, 2000) and structural modifications that are needed for a better plant adaptation to the external environment (Whatley and Whatley, 1982).

The improvements promoted by VL_{150} on the ability to control water loss and photosynthetic performance, were reflected in the water use efficiency (WUE), where microshoots grown under these conditions showed a WUE as high as those found in seedlings (Fig. 4). This behavior was mainly due to the improvement of stomatal characteristics and a more strict transpiration control, rather than a direct effect on net photosynthesis, because the increase in photosynthesis in response to light was only slightly higher in L_{150} treatments (Table 2). The higher competence at the stomatal level could be related to a decrease in relative humidity inside the ventilated vessels. According to Lai et al. (2005), this could account for the decrease of proliferation and hyperhydricity rate of plant tissue. However, in this study, the proliferation rate was significantly greater when using ventilation (Fig. 1 A) together with a high dry mass production (Fig. 1 B). Therefore, it is possible to conclude that when the culture is done under a combination of moderate light intensities and ventilation, such as that used in this study, the increased proliferation not necessarily imply hyperhydricity. Thus, the greatest number of new microshoots and biomass production in VL_{150} treatment reflected the greater metabolic activity, which is verified with its dark respiration rate similar to those found in seedling and higher that those under *in vitro* treatments (Table 2). High biomass production can play important roles until plantlets are able to produce new organs *ex vitro* (Van Huylenbroeck and Debergh, 1996). Additionally, the increase in dark respiration may be also associated simply with a higher substrate availability produced by higher photosynthetic rates. It is likely related with ability of plants to survive under unfavorable conditions (Bravo et al., 2007) such as the *ex vitro* ambient.

The higher photosynthetic capacity (A_{max}) developed in L_{150} treatments, may be also associated with a higher chlorophyll content (Table 3), mainly Chl *a*, which is directly involved in the photosynthetic activity (Sestak, 1996). The increase found in chlorophyll content due to increased PFD agrees with that reported by Dai et al. (2009) who mention that the lower Chl contents observed in shaded plants may partially explain the lower photosynthetic rates found in these plants leaves. The above changes can lead to a greater chance of survival, better growth and development during acclimatization (Mohamed and Alsadon, 2010). Despite high chlorophyll content, the Chl *a/b* ratio was similar in all treatments, and was unaffected by light level, which is different than the results reported by Lee et al. (2000), who observed a reduction of Chl *a/b* ratio following exposure to a high PFD in two tree species native to Asia. At lower irradiance (L_{50} treatments), lower pigment content was determined, perhaps because the processes of synthesis and degradation (photo-oxidation) of chlorophyll are directly correlated with irradiance. So, lower irradiance can lead to pronounced reduction on chlorophyll content (per mass unit and, or area). The changes observed in the chlorophyll content at different light levels, also are related to changes in chloroplast ultrastructure. In this sense, a thylakoid organization similar to seedlings (Fig. 5I–J) was observed in microshoots developed in L_{150} treatments, with demarcated grana (Fig. 5E–F and G–H), contrasting with L_{50} treatments (Fig. 5A–B and C–D), where non-stacked thylakoids were observed. This agreed with Lee et al. (1985), who found that chloroplasts of *in vitro* cultured plantlets are flattened and devoid of starch with disorganized grana when grown at low PFD (50 $\mu mol\ m^{-2}\ s^{-1}$) but contain well organized grana when grown at higher PFD (300 $\mu mol\ m^{-2}\ s^{-1}$).

Additionally, in a previous study ventilation has been shown to improve *in vitro* plantlets photosynthetic capacity (Kubota and Kozai, 1991) and the increase in PFD *in vitro* positively affected the chloroplast structure and light capture (Serret et al., 1996). This may affect the energy transfer and electron flow around PSII (Schnettger et al., 1994) and also the form in which the excitation energy is channeled into the reaction centers (De las Rivas, 2003). In this sense, the electron transport rate (ETR) differed significantly between L_{50} and L_{150} treatments; in L_{150} the ETR (Fig. 6A) was significantly higher, even higher than for seedlings, which could be explained by an improvement in the organization of membranes inside the chloroplast (Fig. 5F and H). This is consistent with Mohamed and Alsadon (2010), who claimed that *in vitro* chloroplasts have light stimulated electron transport but low carbon assimilation. This corroborates the whole act of ventilation and the increase in PFD since microshoot growing in NVL_{150} despite having a high ETR, have half of the carbon assimilation determined in VL_{150}.

With respect to the photo-protective mechanisms, non photochemical quenching (NPQ) and photochemical quenching (qL), are decisive in the maintenance of positive photosynthetic rates in environments that promote photo-inhibition, such as during the acclimatization and transfer stages. Low values of NPQ were found in all treatments compare to seedlings (Fig. 6B), however, microshoots grown in VL_{150} were able to develop a greater capacity for thermal dissipation, surpassing by more than 100% the NPQ found in traditional *in vitro* culture conditions (NVL_{50}). Therefore, the microshoots grown under VL_{150} might be less susceptible to photo-inhibition and photo-damage. Regarding the latter Demmig-Adams et al. (1996) found that carotenoids (carotenes and xanthophylls) are efficient in photo-protection, the carotenes act as effective quenchers of chlorophyll triplets and of singlet oxygen and xanthophyll cycle pigments (violaxanthin, antheraxanthine and zeaxanthin) are involved in thermal dissipation. Despite this the proportion of carotenoids per chlorophyll unit was similar among *in vitro* treatments and two-fold lower than in seedlings. This indicates that nursery plants have a greater capacity to synthesize carotenoids as an adaptive strategy to protect photosynthetic machinery. Probably, this capability does not need to be developed

in microshoots, because of permanent low light conditions use under *in vitro* culture.

Regarding photochemical quenching, measured as the percentage of open reaction centers (qL), in both NVL_{150} and VL_{150}, decreased by 40%, similar to that observed in seedlings (Fig. 6C), which indicates the ability of these microshoots to maintain the electron transport, even under moderate light intensities (Fig. 6A). The high qL detected in microshoots grown at L_{150}, indicates that a high proportion of the absorbed energy was directed toward photochemistry. Thus, these microshoots were able to maintain a low proportion of primary quinone acceptor of PSII (Q_a) in the reduced state, reflecting a low excitation pressure on PSII (1-qL) (Gray et al., 1998). This low excitation pressure is consistent with the high ETR observed at saturating PFD in these treatments (Fig. 6 A). On the contrary, in L_{50} there were significantly lower qL values; less than 20% of open reaction centers were observed, which reflects the low photochemical light conversion capacity of these plants at greater light intensities.

Additionally, although we use open pollinated seeds as source material, which can involve a great genetic variability and therefore imply that these results are applicable to a wide range of genotypes, it is important to keep in mind that responses in *in vitro* culture may be affected by species-specific or genotypic factors (Valero Aracama et al., 2007). It is likely that using isolated genotypes or particular selected lines that respond better to light and ventilation treatments may improve even better their morphophysiological traits and performance *ex vitro.*

In conclusion, *in vitro* microshoots of *C. sativa* cultured under higher PDF and in ventilated vessels exhibited significant morphophysiological differences with respect to the traditional *in vitro* cultured (NVL_{50}). These changes lead to competent microshoots similar to plants grown in traditional field conditions (seedlings). Specifically, the culture at high light in ventilated vessels (VL_{150}) enhanced the response capacity of the stomata and increased the plants' capacity to control water loss. This may favor acclimation to *ex vitro* conditions, preventing excessive desiccations of the micropropagated plants after transplanting and make more favorable its adaptation to *ex vitro* conditions. Additionally, the greater photochemical and photo-assimilation activity enhance the metabolic activity and growth of the microshoots, which is vital during the transfer stage, when plantlets must be able to support growth and the formation of new organs *ex vitro*. Experiments are currently being undertaken in our lab exploring this hypothesis.

Acknowledgements

The authors would like to thank Projects INNOVA BIOBIO N°4 B3- 234 and DIUC 210.142.029-1.0 for the financial support and Charles L. Guy for review of the manuscript. Patricia L. Sáez thanks CONICYT for her doctoral fellowship.

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