Photosynthetic and leaf anatomical characteristics of *Castanea sativa*: a comparison between *in vitro* and nursery plants

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Abstract

The anatomic and functional leaf characteristics related to photosynthetic performance of *Castanea sativa* growing *in vitro* and in nursery were compared. The irradiance saturated photosynthesis in *in vitro* grown plantlets was significantly lower compared to nursery plants (65 *vs*. 722 μmol m^{-2} s^{-1}). The maximum photosynthetic rate (P_{Nmax}) was 4.0 and 10.0 μmol(CO_2) m^{-2} s^{-1} in *in vitro* microshoots and nursery plant leaves, respectively. Carboxylation efficiency (C_E) and electron transport rate (ETR) were three-folds higher in nursery plants than in microshoots. The non-photochemical quenching (NPQ) was saturated at 80 μmol m^{-2} s^{-1} in microshoots suggesting limited photoprotection by thermal dissipation. The microshoots had wide open, spherical stomata and higher stomatal density than nursery plants and they had almost no epicuticular wax. Consequently, the microshoots had high stomatal conductance and high transpiration rate. These anatomic and functional leaf characteristics are likely major causes of the low survival rates of plantlets after *ex vitro* transfer.

Additional key words: chloroplasts, fluorescence, micropropagation, net photosynthetic rate, stomata, transpiration rate.

Introduction

In vitro culture has been used for the multiplication of species in limited space and time, obtaining a large number of individuals that are free of pathogens (Badr and Desjardins 2007). However, the special environmental conditions inside the culture vessels and heterotrophic or mixotrophic nutrition may generate anomalies of micropropagated plants at both anatomic and functional levels, such as hyperhydricity (Debergh *et al*. 1992), poor water loss control (Badr and Desjardins 2007), low photosynthesis (Pospíšilová *et al*. 1992, Carvalho *et al.* 2001, Badr and Desjardins 2007), difficult rooting and low functionality of the developed roots (Pierik 1990). These anomalies are evident during the transfer of *in vitro* plants to the greenhouse or field, causing their slow establishment and low survival percentage (Pospíšilová *et al*. 2000, Hazarika 2006) in comparison to plants cultured by traditional propagation (Talavera *et al.* 1998).

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Abbreviations: BAP - 6-benzylaminopurine; C_E - carboxylation efficiency; DKW - Driver and Kuniyuki medium; ETR - electron transport rate; g_s - stomatal conductance; IBA - indolebutyric acid; NPQ - non-photochemical quenching; PFD - photon flux density; P_N - net photosynthetic rate; PS 2 - photosystem 2; Q_a - primary quinone acceptor of PS 2; qL - photochemical quenching.
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Transfer from *in vitro* to *ex vitro* conditions requires adaptation to the new conditions (Apóstolo *et al*. 2005). One of the major changes that *in vitro* microshoots must withstand is a substantial increase in irradiance (Osorio *et al*. 2010) which challenges the photo-protective mechanisms of these plants. Excess irradiance can decrease photosynthesis and lead to the photo-oxidative destruction of the photosynthetic apparatus (Long *et al.* 1994). It also inhibited CO_2 assimilation (Krause 1988).

Plants have evolved a series of mechanisms that enable them to manage absorption of excess radiation. These include heat dissipation and photochemical processes that drain excess of electrons accumulated in the inter-system pool. Most of the photo-protective mechanisms are induced and regulated directly by photosynthetic photon flux density (PFD) through internal sensors represented by the excitation pressure of photosystem 2 (PS 2), redox state of the plastoquinone pool and the photo-induced trans-thylakoid ΔpH (Horton and Ruban 2005, Walters 2005). The PFD used in the growth chambers may limit the development of efficient photosynthetic and photoprotective mechanisms. Low PFD reduces both ATP and NADPH production rates, which in turn affects numerous processes including the regeneration rate of ribulose-1,5-bisphosphate (RuBP) (Taiz and Zeiger 2002). In addition, low PFD leads to abnormal chloroplasts development that reduces light capture efficiency (Wetztein and Sommer 1982). In conventional micropropagation, *in vitro* plants are grown heterotrophically or photomixotrophically in a medium containing sucrose as the main carbon source. This negatively affects biochemical processes related to both quantity and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; Koch 1996). The insufficient supply of the enzyme may also be related to an increased plant susceptibility to feedback inhibition, which is possibly associated with an excessive accumulation of hexoses and starch (Le *et al.* 2001). Thus, the promotion *in vitro* plant growth by sugars supplied to the culture medium may be compensated by their negative effect on net photosynthetic rate (Kozai *et al*. 1992). It is possible that low *ex vitro* establishment of the *in vitro* microshoots is related to the low photosynthetic capacity developed *in vitro* (Johansson *et al.* 1992, Fila *et al.* 1998, Apóstolo *et al*. 2005, Joshi *et al.* 2006). Rodríguez *et al*. (2008) classified *in vitro* cultured plants into two types: species that are unable to adapt to the new condition and require replacing all leaves; and those species that are able of gradual changes in their leaves in order to adapt to photoautotrophic conditions. According to Carvalho *et al.* (2001), leaves of chestnut hybrid that formed after *ex vitro* transfer showed higher photosynthetic capacity than leaves formed *in vitro.* Nonetheless, it is well known that these photosynthetic responses are species-specific (Tichá *et al.* 1998).

Other studies indicate that the high mortality rates during transfer to *ex vitro* conditions are mainly associated to the low ability of the plantlets to regulate water loss, due to anatomical abnormalities developed *in vitro*, such as stomatal malfunction (Apóstolo *et al.* 2005), reduced production of epicuticular waxes (Majada *et al.* 2001) and increased stomatal density (Joshi *et al.* 2006). Therefore, both the low photosynthetic capacity and the inability to restrict water loss affect the successful transfer from *in vitro* to *ex vitro* conditions. *Castanea sativa,* a hardwood forest species of considerable agro-economic importance (Carvalho and Amâncio 2002), is highly recalcitrant to macropropagation. *In vitro* culture of *C. sativa* has allowed obtaining a large number of individuals. However, the survival rates of *in vitro* grown plants when transferred to the *ex vitro* conditions are significantly lower than those of seedlings established from seeds in the natural environment (Vieitez *et al*. 1986). Many studies lack a comparison with plants grown under nursery or field. In some papers, plants that look normal are used as reference to hyperhydrated plants (Debergh *et al.* 1992). However, plants grown under *in vitro* conditions are not entirely normal (Majada *et al.* 2001), therefore to assess the photosynthetic performance it is necessary to identify the anatomical and functional characteristics of plants grown in nursery or field and the extent to which they differ to those *in vitro* developed plants. Based on this, the objective of our study was to evaluate the anatomic and functional leaf characteristics related to photosynthetic performance and photo-protection *in vitro* microshoots of *C. sativa* and compare them with plants grown in nursery.

Materials and methods

Mature seeds of *Castanea sativa* Mill. were collected from a commercial orchard in the Bío-Bío Region, Chile. Half of the seeds were used for the production of nursery plants and the other half for the production of microshoots. The nursery plants were grown in plastic bags with composted pine bark substrate in an outside nursery. The second group of seeds, for the *in vitro* culture, was subject to a surface asepsis. The embryonic axis were extracted and cultured *in vitro* on half-strength Driver and Kuniyuki (DKW) medium until germination (Ríos *et al*. 2005). After 45 d of cultivation, the roots were split and the microshoots were carried to DKW medium containing 0.22 μM BAP and 0.024 μM IBA, 30 g dm^{-3} sucrose and 7 g dm^{-3} agar, pH 6.2. Cultures were kept in a growth chamber at temperature of 24 ± 2 ºC, 16-h photoperiod, photon flux density (PFD) of 40 μmol(photon) m^{-2} s^{-1} provided by fluorescent lamps and 60 % relative humidity. The microshoots were subcultured every 45 d for twelve months. Following the *in vitro* culture the well developed microshoots during the

proliferation stage and nursery plants were selected, and their photosynthetic and chlorophyll fluorescence parameters, stomatal apparatus, chloroplast ultrastructure and leaf anatomy were evaluated.

Response of P_N to PFD from 0 to 1 500 μmol m^{-2} s^{-1} were measured using an infrared gas analyzer (*Ciras-2*, *PP System*, Hitchin, UK) in both, nursery plant and *in vitro* microshoot attached leaves. Microshoots were removed from the culture vessels and a portion of agar was kept at their base to avoid desiccation. The parameters inside the leaf chamber were: CO_2 concentration of 360 μmol mol^{-1}, flow rate of 200 cm^{-3} min^{-1}, relative humidity of 75 % and temperature of 15 - 20 °C. The leaves were photographed inside the leaf chamber immediately after the measurements; the leaf area was estimated with the 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. The different light pulses were applied either manually, applying the next pulse after reaching steady state. Each curve takes approximately 45 min. Collected data were adjusted to a rectangular hyperbola using *Photosynthesis Assistant 1.1* software (*Dundee Scientific*, Dundee, UK). The compensation irradiance, net photosynthetic rate at saturation irradiance and dark respiration rate (R_d) were extrapolated from this curve. Additionally, CO_2 response curves were generated at PFD 500 μmol m^{-2} s^{-1}. Data were adjusted to a rectangular hyperbola according to the empirical model of Olson and Leverenz (1994) in order to obtain the carboxylation efficiency (C_E) and the maximum P_N under saturating PFD and CO_2 for both *in vitro* microshoots and nursery plants. The *in vitro* microshoot leaves were photographed inside the cuvette immediately after the measurements were taken; the leaf area was estimated with the *Sigma Scan Pro 5.0* software (*SPSS*, Chicago, USA). Additionally, values for stomatal conductance (g_s) and transpiration rate (E) were recorded.

The kinetic of chlorophyll fluorescence was measured with pulse-amplitude fluorimeter (*FMS II, Hansatech Instruments,* King´s Lyn, UK) on previously darkened (for 30 min) leaves of both plant types. Minimum fluorescence (F_0) was determined by applying a weak modulated irradiance (6 μmol m^{-2} s^{-1}) and the maximum fluorescence (F_m), was induced by applying a short pulse (0.8 s) of saturating radiation (9 000 μmol m^{-2} s^{-1}) according to Rosenqvist and Van Kooten (2003). The fluorescence signals were followed until they reached a steady state (F_s). To determine the maximum fluorescence under irradiance (F_m') various pulses of saturating radiation were applied. The minimum fluorescence (F_o') was determined by turning off the actinic light and immediately applying a 2 s far-red pulse. The variable fluorescence/maximum fluorescence ratio [$F_v/F_m = (F_m-F_o)/F_m$] and $\Phi_{PS2} = (F_m'-F_s)/F_m'$ were used as indicators of the maximal and effective quantum yield of the PS 2, respectively (Genty *et al.* 1989).

The electron transport rate (ETR) was also calculated according to Genty *et al.* (1989) as: $ETR = 0.84 \times \Phi_{PS2} \times PFD \times 0.5$. The factor 0.5 assumes that the efficiency of both photosystems is equal and that radiation is equally distributed between them. The factor 0.84 is the mean value of absorbance for green leaves. The photochemical quenching (qL) was calculated (Kramer *et al.* 2004): $qL = [(F_m' - F_s)/(F_m' - F_o') \times (F_o'/ F_s)]$; and the non-photochemical quenching 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 m^{-2} s^{-1}.

Leaf extracts from the *in vitro* cultured microshoots and nursery plants were prepared using 50 mg fresh tissue in 5 cm^3 of 80 % acetone. The contents of chlorophyll *a* and *b*, and total carotenoids were determined spectrophotometrically (*Genesys2*, *Spectronic*, Rochester, USA) according to Lichtenthaler and Wellburn (1983).

The central portion of leaves was fixed in formaldehyde, acetic acid and ethanol (FAA), for 72 h and preserved in 70 % ethanol (v/v). Transverse sections of 10 μm were stained with safranine fast green and mounted in water-glycerol for microscopic (*Olympus CX31*, Tokyo Japan) observation (Kraus and Arduin 1997). Photomicrographs were analyzed using *Image J* software. The width of the epidermis (upper and lower faces), and the spongy and palisade parenchyma were also measured.

Fresh leaf sections of 1 mm^2 were also fixed in 4 % glutaraldehyde, and post fixed with 1 % osmium tetroxide. Following the treatments, leaves were analyzed with a scanning electron microscope (SEM, *JSM6380 nLV*, *Jeol,* Tokyo, Japan) and a transmission electron microscope (TEM, *JEM1200 EXII*) at 60 kV. The photomicrographs were analyzed using *Image J* software. The length, width, and aperture of stomata as well as chloroplast ultrastructure were determined.

Reported values correspond to the mean of 3 measurements for the photosynthetic parameters, of 5 measurements for fluorescence parameters analyses and pigment contents and of 20 measurements in microscopy analysis. Comparisons were made using Student *t*-test. All data were checked for normality and homogeneity of variances. The differences between values were considered significant at $P \leq 0.05$. The analysis was done with *SAS* software *v.9.0* (SAS Institute, Cary, USA).

Results

In vitro microshoots exhibited a typical shade plant PFD response curve (Fig. 1*A*) with a low compensation PFD (Table 1), high quantum yield and low PFD saturated P_N. Saturation PFD of *in vitro* microshoots was 65 µmol m^{-2} s^{-1}, while in nursery plants 722 µmol m^{-2} s^{-1}. Nursery plants exhibited significantly higher P_N at PFD above 300 µmol m^{-2} s^{-1} than *in vitro* microshoots (Fig. 1*A*). Consequently, the PFD response curves were consistent with the PFD at which plants developed (growth chamber and nursery). The dependence of P_N on internal CO_2 concentration (c_i) indicates that *in vitro* microshoots and nursery plants presented a differential behavior in terms

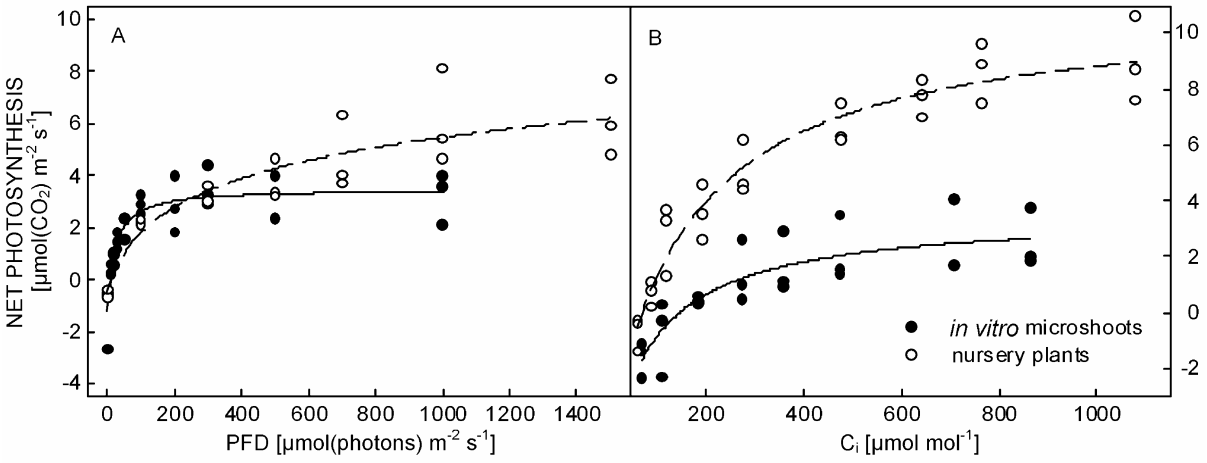

Fig. 1. Response of P_N to irradiance (*A*) and CO_2 (*B*) in leaves from *in vitro* microshoot and nursery plant leaves of *C. sativa*. Data (n = 3 at each PFD and CO_2 intervals) were adjusted to a rectangular hyperbola to obtain photosynthetic parameters.

Table 1. Photosynthetic parameters, pigment contents and characteristics of leaf anatomy and chloroplast structure of *in vitro* cultured microshoot and nursery plant leaves of *C. sativa*. Means ± SE. Different letters between columns indicate statistically significant differences at $P \leq 0.05$.

Parameters	Microshoots	Nursery plants
P_{Nsat} [µmol(CO_2) m^{-2} s^{-1}]	3.40 ± 0.33 a	7.23 ± 0.68 b
R_d [µmol(CO_2) m^{-2} s^{-1}]	1.26 ± 0.72 a	0.57 ± 0.09 a
PFD comp. [µmol m^{-2} s^{-1})]	6.47 ± 2.28 a	33.80 ± 21.20 a
P_{Nmax} [µmol(CO_2) m^{-2} s^{-1}]	4.10 ± 1.00 a	10.80 ± 3.00 b
C_E [mol(CO_2) mol(photons)]	0.02 ± 0.00 a	0.06 ± 0.00 b
g_s [mmol(H_2O) m^{-2} s^{-1})]	785.67 ± 43.08 a	88.11 ± 5.03 b
E [mmol(H_2O) m^{-2} s^{-1})]	5.17 ± 0.09 a	1.00 ± 0.06
Chl *a* [mg g^{-1}(f.m.)]	1.15 ± 0.27 b	1.74 ± 0.23 a
Chl *b* [mg g^{-1}(f.m.)]	0.23 ± 0.08 b	0.55 ± 0.11 a
Carotenoids [mg g^{-1}(f.m.)]	0.26 ± 0.05 a	0.33 ± 0.04 a
Chl *a/b*	5.25 ± 0.46 b	3.38 ± 0.28 a
Total parenchyma width [µm]	88.73 ± 1.69 b	109.63 ± 1.79 a
Palisade parenchyma [µm]	14.55 ± 0.45 b	33.55 ± 0.71 a
Spongy parenchyma [µm]	51.90 ± 2.55 a	48.85 ± 2.17 a
Epidermis upper [µm]	9.96 ± 0.40 b	18.63 ± 3.08 a
Epidermis lower [µm]	9.10 ± 0.30 b	13.56 ± 0.59 a
Stomata density [mm^{-2}]	431.32 ± 23.2 b	215.56 ± 12.2 a
Stomata length [µm]	20.03 ± 1.01 b	23.23 ± 0.38 a
Stomata width [µm]	18.45 ± 0.69 b	20.09 ± 0.34 a
Stomata aperture area [μm^2]	48.86 ± 12.1 b	1.19 ± 0.66 a
Chloroplast area [μm^2]	4.87 ± 0.56 a	9.41 ± 1.08 b
Starch granules [$chloroplast^{-1}$]	0 a	1.10 ± 0.10 b
Plastoglobuli [$chloroplast^{-1}$]	2.40 ± 0.30 a	5.90 ± 0.70 b

of CO_2 assimilation (Fig. 1*B*). After 12 months of culture, the maximum photosynthetic rate (P_{Nmax}) of the *in vitro* microshoots was less than half of that observed in plants of the same age cultivated in the nursery (Table 1). Consistently, the carboxylation efficiency (C_E) of *in vitro* microshoot leaves was about a third of that observed in nursery plant leaves (Table 1). Both stomatal conductance (g_s) and transpiration rate (E) differed significantly between *in vitro* cultured microshoots and nursery plants. In nursery plants, g_s was eight-fold lower and E was five-fold lower than the *in vitro* microshoots (Table 1).

The maximum photochemical efficiency of PS 2 (F_v/F_m) was 0.82 and 0.79 in nursery plants and microshoots, respectively; these values were not significantly different. The electron transport rate (ETR) and effective quantum yield of PS 2 (Φ_{PS2}) were significantly lower in microshoots than in nursery plants. For *in vitro* microshoots, ETR reached a maximum of 7 μmol(electron) m^{-2} s^{-1} at a PFD of 150 μmol m^{-2} s^{-1} and remained constant until reaching PFD of 900 μmol m^{-2} s^{-1}. For nursery plants, ETR increased up to PFD 600 μmol m^{-2} s^{-1} and reached values around 60 μmol(electron) m^{-2} s^{-1} (Fig. 2*A*). In nursery plants, the photochemical quenching (qP) (Fig. 2*B*) decreased with PFD remaining about 20 % reaction centers in the open state at about PFD of 900 μmol m^{-2} s^{-1}. For *in vitro* microshoots, there was a similar trend, but with significantly lower values, where less than 10 % of open reaction centers were observed at PFD of 200 μmol m^{-2} s^{-1}. The non-photochemical quenching (NPQ) increased from 1.0 to 5.0 almost linearly with PFD (Fig. 2*C*), showing no saturating response in the studied range of PFD in nursery plants. In the *in vitro* microshoots, the NPQ oscillates from zero to about 1.0 within at the same PFDs range.

Chlorophyll (Chl) *a* and Chl *b* contents were significantly higher in nursery plants than in *in vitro* microshoots. However, the Chl *a/b* ratio was lower in nursery plants than in the *in vitro* microshoots (Table 1).

The leaves of nursery plant were thicker (Table 1), with greater development of both upper and lower epidermis. The palisade parenchyma found in nursery leaves showed typical elongated cells with large presence of peripherically located chloroplasts (Fig. 3). In contrast, the *in vitro* microshoots had very dense parenchyma with few chloroplasts poorly developed palisade, and no air spaces in the spongy parenchyma (Fig. 3).

Stomatal density was more than double in *in vitro* microshoot leaves compared to leaves developed in nursery. Differences were also observed in both the size and number of stomata (Table 1). The *in vitro* microshoots had a greater proportion of open stomata than those from nursery. In addition, the stomata of *in vitro* microshoots were spherical, superficial and supported by the epidermis cells (Fig. 4). In contrast typical elliptical stomata covered by epicuticular wax depositions were found in nursery leaves (Fig. 4).

The size of the chloroplasts in nursery plants was double that observed in *in vitro* cultured microshoots (Table 1). Grana were easily delimitable in nursery plants. The average area of grana was 0.8 μm^2 with seven grana per chloroplast and 20.4 thylakoids per granum. On the other hand, it was not possible to distinguish clear granal stacking in *in vitro* leaves; but a network of stromal lamellas was rather observed (Fig. 5). In addition large starch granules were observed in the leaves of nursery plants, covering on average 1.5 μm^2 per chloroplast, while *in vitro* microshoots chloroplasts were devoid of starch. Furthermore, a significantly higher number of plastoglobuli was observed per chloroplast in nursery plants as compared with *in vitro* leaves (Table 1). The average area occupied by plastoglobuli was 0.27 and 0.01 μm^2 per chloroplast for nursery and *in vitro* cultured leaves, respectively.

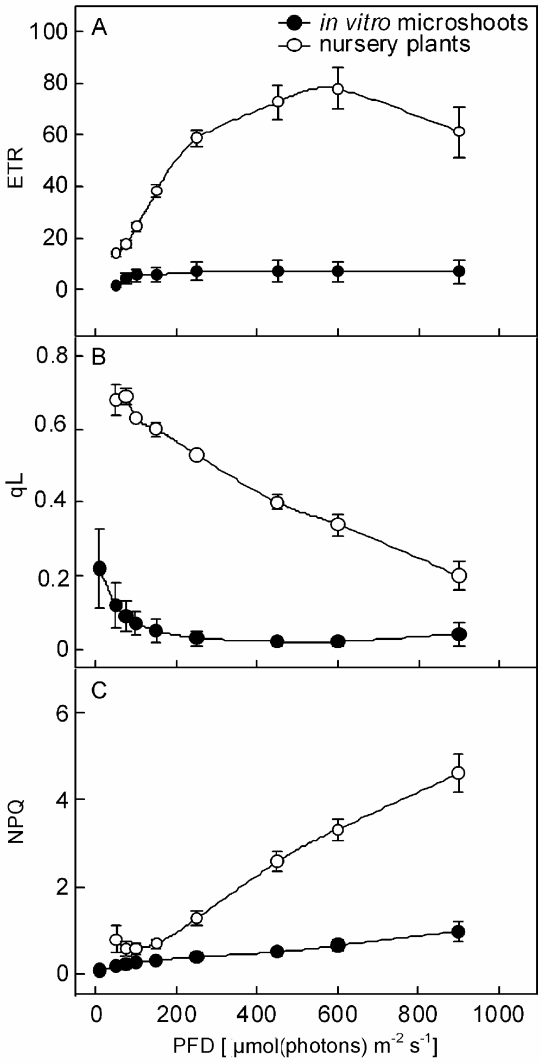

Fig. 2. Electron transport rate (*A*), photochemical quenching (*B*) and non-photochemical quenching (C) of *in vitro* microshoot and nursery plant leaves of *C. sativa*. Each data point represents the average ± SE (n = 5).

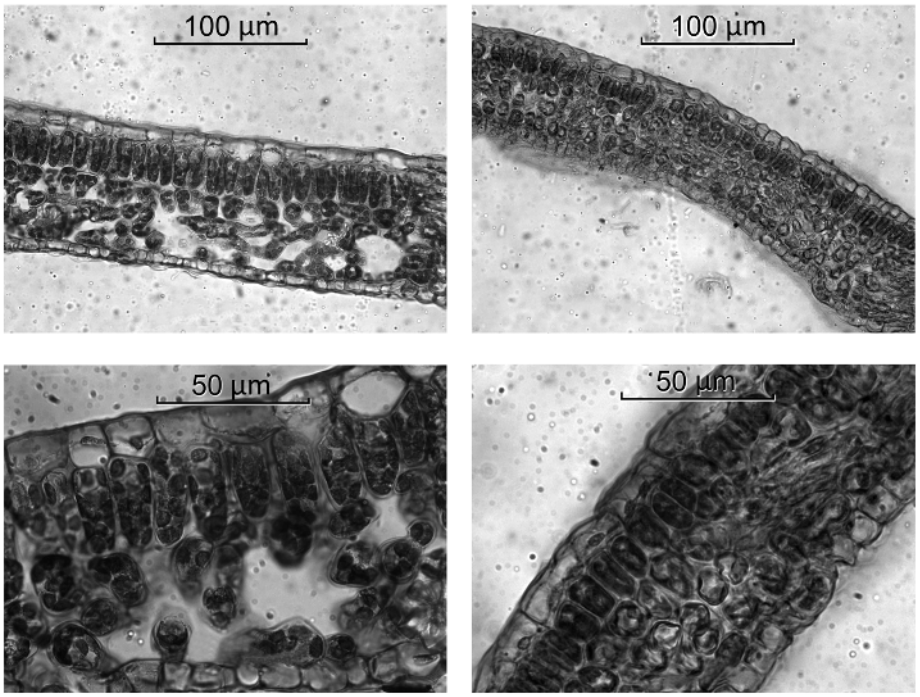

Fig. 3. Transverse section of *C. sativa* leaves (40× and 100×, top and bottom, respectively) from *in vitro* cultured microshoots (*on the right*) and nursery plants (*on the left*).

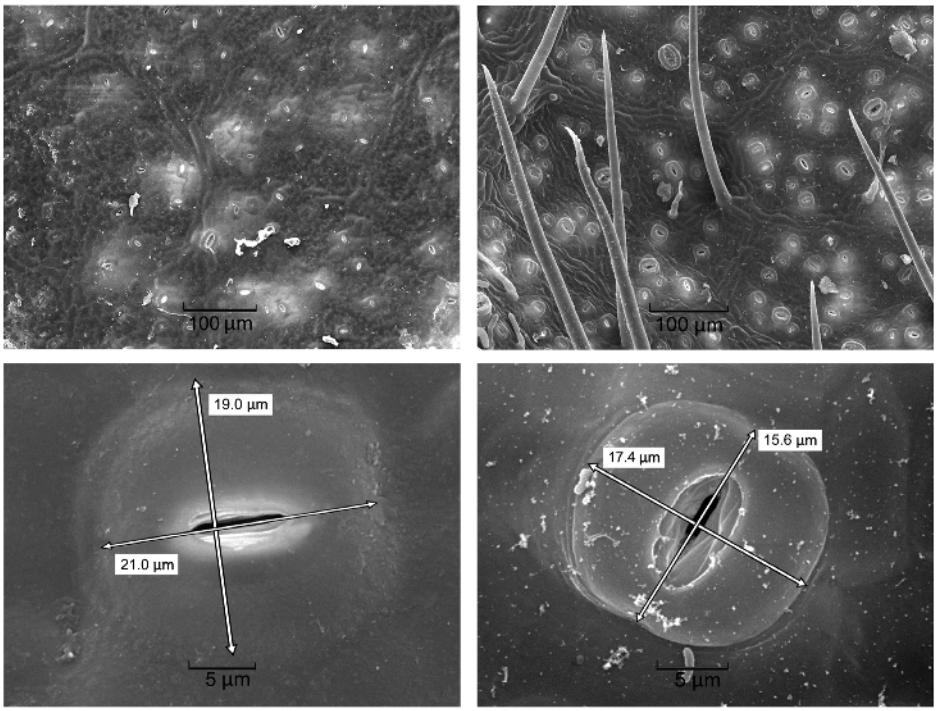

Fig. 4. Stomata of *in vitro* cultured microshoot (*on the right*) and nursery plant (*on the left*) leaves of *C. sativa*.

Discussion

All leaf characteristics that affect the photosynthetic rate and anatomy were significantly different in *in vitro* microshoots compared with the plants grown in the nursery. In the *in vitro* cultured microshoots, P_{Nmax} was

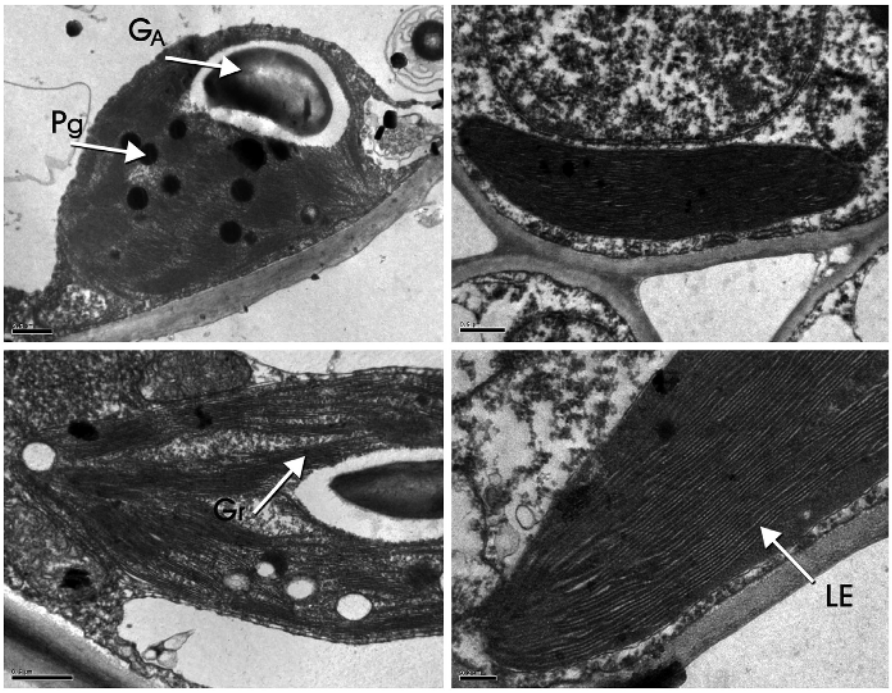

Fig. 5. Electron micrographs of mesophyll cell chloroplasts of *in vitro* cultured microshoots (*on the right*) and nursery plant (*on the left*) leaves of *C. sativa*. Note differences in development of grana as well as differences in size and presence of starch and plastoglubuli between the two groups. Pg - plastoglobuli, G_A - starch, Gr - grana, SL - stroma lamellas.

half and C_E a third of that observed in plants of the same age cultivated in nursery (Table 1). Previous studies have argued that low P_N observed in *in vitro* could result from the low CO_2 concentration inside the culture vessels caused by the restricted air exchanges between the culture headspace and the outer environment (Kozai *et al.* 1992, Zobayed *et al*. 2001, Hazarika 2006). Consequently, low contents of CO_2 limit carboxylase activity of Rubisco (Ishibashi *et al.* 1997). Thus, the CO_2 availability becomes a limiting factor for photosynthesis soon during daily hours (Navarro *et al.* 1994). However, in our study, the CO_2 concentrations remain relatively high inside the culture vessels (data not shown), probably due to the carbon source in the medium and slightly higher rates of dark respiration than observed previously. Even if the majority of stomata remained open (Fig. 4), the P_{Nmax} was lower than that observed in nursery plants (Fig. 1). Considering high g_s and E (Table 1) together with similar values of P_{Nmax} and P_N at saturating PFD (Table 1) observed in *in vitro* microshoots, it is unlikely that limited CO_2 diffusion may explain low P_N found in *in vitro* microshoots. Therefore, the results suggest that the P_N of *in vitro* microshoots of *C. sativa* is limited by Rubisco carboxylation capacity and/or RuBP regeneration at the Calvin cycle. This is consistent with the observed low C_E (Fig. 1, Table 1). Furthermore, low ETR was also found for *in vitro* microshoots (Figs. 1 and 2*A*). This is most likely to be the result of acclimation to low PFD. According to Ishibashi *et al.* (1997), a decrease in ETR and the *in situ* activity of Rubisco represent the main factors that reduce P_N.

Regarding the photo-protective mechanisms, Demmig-Adams *et al*. (1996) reported that this may be achieved by dissipation through the photochemical use of the energy (qL) or by thermal dissipation (NPQ) of excess absorbed light in the pigment that could potentially lead to damage (Grouneva *et al.* 2008). In microshoots, the qP showed a rapid decrease during transition from dark to 200 μmol m^{-2} s^{-1}, and subsequently exhibited very low values. This reflects the low capacity for photochemical radiation energy conversion in these plants at PFD greater than 200 μmol m^{-2} s^{-1} (Fig. 2*B*) where more than 90 % of plastoquinone pool (Q_a) was maintained in the reduced state, which reflect a high excitation pressure (1 - qP) (Gray *et al.* 1998). This result is consistent with the lower ETR observed at saturating PFD in microshoots (Fig. 2*A*).

The low values of NPQ (about 1.0) and its independence of PFD (Fig. 2*C*) indicate that the degree of photoprotection by thermal dissipation could be insufficient at moderate and high PFD, such as those found in greenhouses or the field, respectively. This lack of photo-protection mechanisms may lead to the formation of reactive oxygen species in *in vitro* microshoots once these plants are transferred to *ex vitro* conditions (Müller *et al.* 2001). The brown spots and some necrotic area observed on the *in vitro* microshoots after measurements, indicate their poor capacity to withstand photooxidative damage. In contrast, the greater NPQ values obtained in nursery plants (Fig 2C) indicate

that these plants are capable of adjusting the photochemical processes to higher PFD. The poor development of dissipation mechanisms for excess radiation is likely related to the permanent growth under low PFD in *in vitro* culture. The high F_v/F_m values (about 0.8) obtained under *in vitro* culture corroborate the lack of excess absorbed energy. This would explain the poor development of excess energy dissipation mechanisms. Additionally, TEM studies (Fig. 5) revealed fewer plastoglobuli in *in vitro* microshoot chloroplasts. Plastoglubuli are an important source of α-tocopherol (Steinmüller and Tevini 1985), a potential antioxidant (Munné-Bosch 2005), as well as plastoquinones and carotenoids (Bréhélin *et al*. 2007). Therefore, low platoglobuli occurrence under *in vitro* conditions may reflect a low capacity of *in vitro* microshoots for preventing oxidative damage at the chloroplast membrane systems of these plants.

Although the chlorophyll content is not directly related to the photosynthetic capacity (Fujiwara *et al.* 1992), it is a good indicator of the status of the photosynthetic apparatus (Seon *et al.* 2000). The high ratio Chl *a/b* found in *in vitro* microshoots leaves is not typical of shade plants (Fila *et al.* 2006) despite their low growth irradiance. On the other hand, the higher Chl *a/b* ratio and lower chlorophyll *b* of microshoots compared to nursery seedlings (Table 1), are in agreement with a less developed photosynthetic apparatus and low photosynthetic activity in the *in vitro* microshoots (Serret *et al.* 1996). The results shown in our study would indicate the existence of small antennas, probably poorly organized, and a large quantity of reaction centers in the chloroplasts of the *in vitro* developed leaves. As a result the capture of light photons and energy absorption at the reaction centers is affected (De las Rivas 2003). This contradict the ultrastructure commonly found in plants growing at low irradiance which typically have characteristics that promote light capture and transfer to the reaction centers, showing larger light harvesting complex and a lower complement in electron transport. On the other hand the sun plants favor the electron transport machinery and CO_2 fixation, showing significantly higher contents of Rubisco and photoprotective systems, such as xanthophylls cycle pigments and antioxidants (Herbinger *et al*. 2005). Considering the low irradiance at which the *in vitro* microshoot leaves developed, the low proportion of chlorophyll *b* that was found in these plants is interesting. The relative content of chlorophyll *a* and *b* is related to the degree of thylakoid stacking. The grana that have the greatest proportion of PS 2 have more light harvesting complexes (LHC) 2, where chlorophyll *b* is preferentially found (Pospíšilová *et al*. 2000). Indeed, granal delimitation in chloroplasts from *in vitro* cultured microshoot leaves was difficult; instead a network of stromal lamellas was observed (Fig. 5). The chloroplast ultrastructure found in *in vitro* microshoots explains the results obtained in the respective photosynthetic studies, probably as a consequence of cultivation under very low irradiance. In this sense, Serret *et al.* (1996), report that by increasing the PFD inside the growth chambers it is possible to observe greater granal stacking and clearer delimitation of grana. However, changes in the efficiency of the photoprotective mechanisms in *in vitro* cultured plants have not been reported.

Sucrose mediated mechanism have been proposed to control down-regulation of photosynthesis by sink (Franck *et al.* 2006). The low rate of regeneration of the carboxylation substrate RuBP due to the accumulation of soluble sugar in the leaves possibly results in inhibition of photosynthesis (Azcón-Bieto 1983). However, our results indicate that a greater amount of starch granules were found in chloroplasts of nursery leaves probably as part of the storage product. On the contrary, *in vitro* growing plantlets did not show starch granules (Table 1 and Fig. 5), probably because photosynthetic rate is too low or exogenous sucrose cause a negative feedback on plastidic enzymes for starch biosynthesis (Krapp and Stitt 1994). The anatomy of *in vitro* microshoot leaves, such as reduced mesophyll differentiation and thin cuticle with scarce wax deposition, exhibited typical features described in other *in vitro* plants (Johansson *et al.* 1992, Apóstolo and Llorente 2000). The nursery plant leaves had both, a thick and well developed palisade parenchyma that according to Costa *et al.* (2009) indicates an adaptation to greater PFD. The leaves of *in vitro* microshoots developed a dense and thin palisade parenchyma without air spaces in the spongy mesophyll (Fig. 3) which could be the outcome of high sugar content in the culture medium. According to Rizzini (1976) higher concentration of sugar reduce cell elongation and increase thinning of the cell wall, resulting in the formation of fewer cells.

Additionally, the poor ventilation of the culture vessels results in a high relative humidity inside them, which forces the microshoots to keep the stomata open (Fabbri *et al.* 1986). Leaves from plants developed in nursery had similar proportions of open and close stomata, while in the *in vitro* cultured microshoots more than 90 % of the stomata were open (Fig. 4). These stomata were observed supported on epidermis cells protruding from the cuticle and had an unusual round shape. This shape is characteristic of stomata endowed with abnormal function, whereas the elliptical shape is frequently associated with stomata that do function normally (Brutti *et al*. 2002).

Malformations in stomata morphology affect aperture and closing, which results in a high stomata conductance (Table 1). Therefore, the excessive water loss would contribute to the high mortality observed during the transfer to *ex vitro* conditions.

The anatomical and functional leaf characteristics found in *in vitro* microshoots of *C. sativa*, suggest that the survival rates *ex vitro* are likely low and this may limit the application of micropropagation techniques for

this species. Given the photosynthetic characteristics present during *in vitro* culture and the degree to which they differ from those found in nursery plants, it is possible to develop methods that allow steering *in vitro* culture towards obtaining photosynthetic characteristics similar to those present in nursery plants.

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