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Effect of Photon Flux Density and Exogenous Sucrose on the Photosynthetic Performance during *In Vitro* Culture of *Castanea sativa*

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Abstract

The low photon flux density (PFD) under *in vitro* conditions and sucrose added to the culture medium negatively limits the photochemical activity and photoprotective mechanisms of microshoots. In this work we hypothesize that decreasing sucrose in the culture medium in combination with increasing irradiance, could improve the photosynthesis and consequently the *in vitro* growth. We evaluated the effect of exogenous sucrose (30 and 5 $g \cdot L^{-1}$, HS and LS, respectively), under different PFD (50 and 150 μmol photons $m^{-2} \cdot s^{-1}$, LL and HL, respectively) on the photosynthetic performance and growth of *Castanea sativa* microshoots. Decreasing sucrose negatively affected the physiological attributes evaluated. Only chloroplast ultrastructure was improved by LS; however this did not lead to an improved in photosynthesis or growth. HL HS produced an increase in photosynthetic activity and chlorophyll contents, reaching under these conditions a higher proliferation rate and biomass production. Additionally, the photochemical activity (electron transport rate and non-photochemical quenching) was improved by HL. Thus, our results suggest that, at least for *C. sativa* HL is beneficial during the *in vitro* culture, improving photosynthetic performance as well as growth, but this is only possible in the presence of moderate concentrations of sucrose added to the culture medium.

Keywords

Sucrose, PFD, Micropropagation, Photosynthesis, Chlorophyll Fluorescence

1. Introduction

Micropropagation technique has become very important in plants production due among others, to its efficiency in the vegetative propagation of species whose macro-propagation is difficult, such as woody species. However, these species also present some problems for its micropropagation [1]. These problems include its relative low *in vitro* growth, significant losses due to contamination, poor rooting and low survival percentages during their *ex vitro* transference [2]. Most of these characteristics are related to the heterotrophic or mixotrophic plant growth in conventional micropropagation systems [3]. In the last years, some of these problems have been reduced through the development of photoautotrophic systems, which had been associated with the promotion of growth as well as improvements in morphological and physiological attributes [4] beside the possibility of scale-up [3]. However, these systems also have disadvantages associated with the complexity of the technique and high costs associated, besides the limitation to apply this multiplication system using multiple buds/shoots. Additionally, the scale-up requires a conventional micropropagation system that provides plants with better morpho-physiological attributes. Thus, the success of micropropagation systems in general, requires further study and optimization of the *in vitro* environment, mainly due to its effects on the processes that determine the *in vitro* plant growth [5].

Usually, *in vitro* culture is carried out in chambers with low photon flux density (PFD), which may limit development of efficient photosynthetic and photoprotective mechanisms [6] [7]. Mechanisms such as heat dissipation and photochemical process that drain the excess electrons accumulated in the inter-system pool allow managing the excess of absorbed light energy [8] [9]. Through these processes, the light energy is used to produce ATP and NADPH in the light reaction and subsequently, in the light independent reaction, carbon is fixed into carbohydrates [10]. Under low PFD, insufficient ATP is produced to allow the carbon fixation and carbohydrate biosynthesis, leading to a reduced plants growth. This makes it necessary to include in the culture medium an external organic carbon source (usually 20 or 30 $g \cdot L^{-1}$ sucrose), which would be crucial for *in vitro* growth [11] [12]. However, it has been reported that sucrose added to the culture medium could have a negative effect on the photosynthetic capacity [13] [14]. This negative effect is based on the concept of balance between processes of sugar consumption and production. Thus, the presence of sugar in the medium decreases the need for sugar production and therefore it should result in lower photosynthetic rates [15].

Despite evidence respect to the negative effect of sucrose on the photosynthetic performance, there is also evidence regarding the profitability of their use. Indeed, Paul and Stitt [16] argued that the lack of down-regulation in photomixothrophically grown tobacco might be due to the low light regime which results in low photosynthetic rates and a source limitation to growth. This would indicate that the presence of sugar in the culture medium may not be causing the low photosynthetic capacity developed *in vitro*. In fact, Ticha *et al*. [17] reported that sucrose increases not only the photosynthetic potential but also the high light resistance of *in vitro* grown plantlets. So, considering that

the PFD used *in vitro* is a limiting factor for photosynthesis, we hypothesize that the negative effect of sucrose on the photosynthetic capacity of *in vitro* plants would be dependent on irradiance at which plants are grown. Therefore, decreasing sucrose in the culture medium in combination with increasing irradiance, could improve the photosynthesis performance and consequently the *in vitro* growth.

Castanea sativa, is a hardwood forest species of valuable agro economic importance [18]. *In vitro* culture of *C. sativa* has allowed obtain a large number of individuals and solve the difficulties observed during the traditional macropropagation, due to the recalcitrant condition of this species. Despite significant advances in its micropropagation, and the studies conducted in order to increase the *ex vitro* survival rates [6] [7] [19], the effect of the sugars added to the culture medium of has not been evaluated. Based on this, the objective of this study was to evaluate the effect of exogenous sucrose, under different light conditions, on the photosynthetic performance and growth of *Castanea sativa* microshoots.

2. Materials and Methods

2.1. Plant Material

Castanea sativa mature seeds were subjected to a surface asepsis and its embryonic axis was extracted and cultured *in vitro* on a MS medium modified by a 50% reduction in its macronutrients and kept in darkness until its germination [20]. When the embryos germinated reached a height greater than 1.5 cm and the appearance of chlorophyll tissue was observe (approximately 15 days after its introduction), they were carried to MS medium supplemented with 0.22 μM BAP and 0.024 μM IBA, and 7 $g \cdot L^{-1}$ agar, at pH 6.2 and containing: 5 $g \cdot L^{-1}$ sucrose (LS) or 30 $g \cdot L^{-1}$ sucrose (HS), and were cultured under two different PFD, 50 (LL) and 150 μmol photons $m^{-2} \cdot s^{-1}$ (HL). These levels of PFD were choose because correspond to the normal level use in micropropagation of *C. sativa* [18] and is the recommended level by Saez *et al.* [19]; respectively. The culture room environment conditions were 16 h light photoperiod, at 24˚C ± 2˚C and 60% relative humidity. The microshoots were subcultivated every 45 days. After five months under these conditions, well developed microshoots were selected, and their photosynthetic and chlorophyll fluorescence parameters and chloroplast ultrastructure were evaluated. In order to evaluate the possible improvements of physiological attribute under each treatment, the results were compared with one year old grown seedlings. They were cultured in an outdoor nursery 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. Photosynthetic Performance

Light response curves of net CO_2 assimilation at different PFD (from 0 to 1000 μmol photons $m^{-2} \cdot s^{-1}$) were measured using an infrared gas analyzer (Ciras-2, PP System, United Kingdom). The CO_2 concentration was 360 $\mu g \cdot g^{-1}$, with 200 $cm^{-3} \cdot min^{-1}$ of flow, 75% relative humidity and 15˚C to 20˚C of temperature inside the leaf chamber. The

notable points of these curves, such as, light compensation point: light compensation point (LCP), net photosynthesis at light saturation (A_{ls}) and dark respiration rate (R_d) were obtained using Photosynthesis Assistant 1.1 software (Dundee Scientific, United Kingdom). Additionally, CO_2 response curves (from 50 to 1000 ppm) were performed at 15˚C and 20˚C under 500 μmol photons $m^{-2}\cdot s^{-1}$, to obtain the carboxylation efficiency (C_E) and the maximum rate of net photosynthesis under light and CO_2 saturating conditions (A_{max}). The microshoot leaves were photographed inside the cuvette immediately after they were measured and its area estimated with the Sigma Scan Pro 5.0 software (SPSS, Chicago, IL).

2.3. Pigment Content

Pigment determination was made according to Lichtenthaler and Wellburn [21]. Leaf extracts were made using 50 mg fresh weight with 5 cm^3 80% acetone. Following extraction, the samples were centrifuged for 3 min at 12,000 rpm and 4˚C. The *a* and *b* chlorophyll contents, and total carotenoids were determined spectrophotometrically (Spectronic, Genesys 2).

2.4. *In Vitro* Growth

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.

2.5. Fluorescence Parameters

The photochemical activity was analyzed by the kinetics of chlorophyll fluorescence. This was measured with pulse-amplitude fluorimeter (FMS II, Hansatech Instrument, United Kingdom) in expanded leaves, which were previously dark adapted for 30 min. Different pulses of light were applied following standard routine programmed in the machine. According to the terminology of Rosenqvist and Van Kooten [22], minimal fluorescence (F_0) was determined by applying a weak modulated light (6 μmol photons $m^{-2}\cdot s^{-1}$) and maximal fluorescence (F_m), was induced by a short pulse (0.8 s) of saturating light (9000 μmol photons $m^{-2}\cdot s^{-1}$). The fluorescence signals were followed until reaching the steady state (F_s). To determine maximal fluorescence in light ($F_{m'}$) various pulses of saturating light were applied. The minimal fluorescence ($F_{o'}$) was determined after turning off the actinic light and immediately a 2 s far red pulse was applied. The electron transport rate (ETR) was calculated according to Genty *et al.* [23] as: ETR = 0.84 (ΦPSII) (PFD) 0.5; where ΦPSII is effective quantum yield of the PSII, PFD corresponds to incident photosynthetic flux density, 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.* [24]: $qL = ((F_{m'} - F_s)/(F_{m'} - F_{o'}))\ (F_{o'}/F_s)$ and the non-photochemical quenching as: $NPQ = (F_m - F_{m'})/F_{m'}$ [25]. The fluorescence measurements were performed at PFDs of 10, 50, 75,

100, 150, 250, 450, 600 and 900 μmol photons $m^{-2} \cdot s^{-1}$.

2.6. Chloroplast Ultrastructure

Chloroplast ultrastructure was analyzed by transmission electron microscopy (TEM). Leaf sections of 1 mm^2 were fixed in 4% glutaraldehyde, and post fixation with 1% osmium tetroxide. Then, they were analyzed with TEM (MET Jeol, JEM1200 EXII) at a voltage intensity of 60 kV. The photomicrographs were analyzed using Image J software. Chloroplast area (μm^2), grana per chloroplasts (N˚), grana area (μm^2) and thylakoid per chloroplast (N˚) were evaluated.

2.7. Statistical Analysis

The effect of sucrose (5 or 30 $g \cdot L^{-1}$), light intensity (150 or 50 μmol photons $m^{-2} \cdot s^{-1}$) and their interaction on physiological characteristics of *in vitro* cultured microshoots were studied using a factorial experimental design. All experiments were arranged in completely randomized design. Experimental units corresponded to a culture vessel containing a single microshoot and in each analysis at least three random measurements were made. 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 physiological traits among each *in vitro* treatment and seedlings.

3. Results

3.1. Photosynthetic Performance

A significant increase in photosynthesis at light saturation (**Figure 1(A)**) as well as in photosynthetic capacity (**Figure 1(B)**) was observed when a high sucrose addition was combined with a higher PFD ($P < 0.05$). Thus, photomixotrophic condition (HL HS) surpassing the other treatments, reached rate of 3.52 and 4.45 μmol CO_2 $m^2 \cdot s^{-1}$ in A_{ls} and A_{max}, respectively. The light saturation (**Figure 1(C)**) and Rubisco carboxylation efficiency (**Figure 1(F)**) showed the same trend. So the treatment with higher photosynthetic capacity (HL HS), reached light saturation above 200 μmol photons m^2 s^{-1}, unlike that seen in other treatments which did not exceed 160 μmol photons m^2 s^{-1}. Regarding the light compensation point (**Figure 1(D)**) and dark respiration rate (**Figure 1(E)**), the factors evaluated did not produce significant differences.

3.2. Pigment Content

The pigment contents responded to sucrose concentration and PFD, as well to an interaction between them (**Table 1**). Apparently, the effect of sucrose on the total chlorophyll content was dependent of PFD. Thus, when plants grown at LL, sucrose had no significant effect on chlorophyll content, while at HL the decrease of sucrose resulted in a decrease of total chlorophyll content. The same trends were observed for both chlorophyll *a* and *b*. Thus, the highest production of chlorophylls (*a* + *b*) was observed in

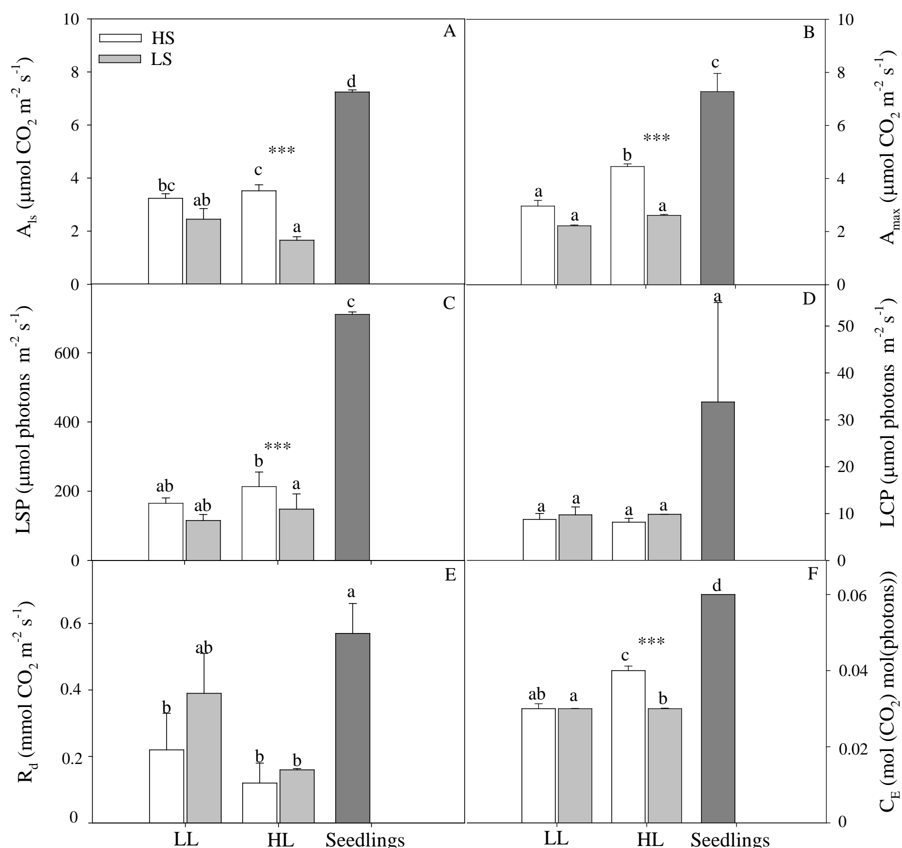

Figure 1. Photosynthetic parameters obtained from light and CO_2 response curves of microshoot of *C. sativa* culture under different sucrose concentrations and PFD. (A) Net photosynthesis at light saturating (A_{ls}); (B) Photosynthetic capacity (A_{max}); (C) Light saturation point (LSP); (D) Light compensation point (LCP); (E) Dark respiration rate (R_d) and (F), carboxilation efficiency (C_E). Means ± S.E., n = 3. ***indicates the significant effect of sucrose factor at $P < 0.05$. Different letters indicate significant differences among each *in vitro* treatment and seedlings at $P < 0.05$ in one way ANOVA. Mean ± S.E., n = 3.

Table 1. Pigment content (mg·g^{-1} FW) of *in vitro* cultured microshoot leaves of *C. sativa* grown under different sucrose concentration and PFD. Different letters indicate significant differences among each *in vitro* treatment and seedlings at $P < 0.05$ in one way ANOVA. Mean ± S.E., n = 4.

	Seedlings	LL HS	LL LS	HL HS	HL LS	S	L	S*L
Chl *a* (mg·g^{-1})	1.74 ± 0.23 a	1.27 ± 0.26 a	1.75 ± 0.06 a	2.67 ± 0.03 b	0.96 ± 0.05 a	*	ns	**
Chl *b* (mg·g^{-1})	0.55 ± 0.11 a	0.39 ± 0.10 a	0.37 ± 0.03 a	1.11 ± 0.21 b	0.26 ± 0.15 a	*	*	*
Chl (*a* + *b*) (mg·g^{-1})	2.29 ± 0.03 a	1.66 ± 0.36 a	2.12 ± 0.09 a	3.78 ± 0.11 b	1.21 ± 0.69 a	*	ns	**
Cart (mg·g^{-1})	0.33 ± 0.04	0.27 ± 0.06	0.37 ± 0.07	0.31 ± 0.07	0.23 ± 0.13	ns	ns	ns

Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total carotenoids (Cart), total chlorophyll (a + b). ***refers to factor's significance at $P \leq 0.001$; **at $P \leq 0.01$; *$P < 0.05$ and ns, no significant in two way ANOVA.

photomixotrophic conditions (HL HS). Under these conditions, the total chlorophyll was about twice that found in other *in vitro* treatments, and even greater than those determined in seedlings. The carotenoids content did not differ among *in vitro* treatments and was also similar to that observed in seedlings.

3.3. *In Vitro* Growth

Randomly chosen *Castanea sativa* microshoots grown under different sucrose and PFD conditions are shown in Figure 2. Plants grown on HS showed robust growth, mainly in HL HS where in addition there was a higher development of leaf area. In contrast at LS, the plants had a lower growth, mainly in HL LS, where besides a slight yellowing of the leaves was observed. The biomass production measured as dry matter production (Figure 3(A)) was determined by PFD factor ($P < 0.05$), so regardless of the sucrose concentration, the increase in light caused an increase in the dry weight percentage. In contrast, the proliferation rate of new microshoots (Figure 3(B)), evaluated after four months of *in vitro* culture was significantly determined by the sucrose concentration added to the culture medium ($P < 0.001$). Thus, the smallest sucrose addition did not produce increase in this parameter which was even lower that one. However with high sucrose addition there was an active proliferation reaching up three new microshoots by initial microshoot produced.

3.4. Fluorescence Parameters

The photochemical activity was evaluated through of chlorophyll *a* fluorescence. The electron transport rate (ETR) (Figure 4(A)) was significantly higher in microshoots produced with both high sucrose and PFD (HL HS), surpassing values observed in seedlings. In this treatment, ETR increased linearly until 300 μmol photons $m^{-2} \cdot s^{-1}$ peaking close to 110 μmol electrons $m^{-2} \cdot s^{-1}$. Lower values were observed in LS treat-

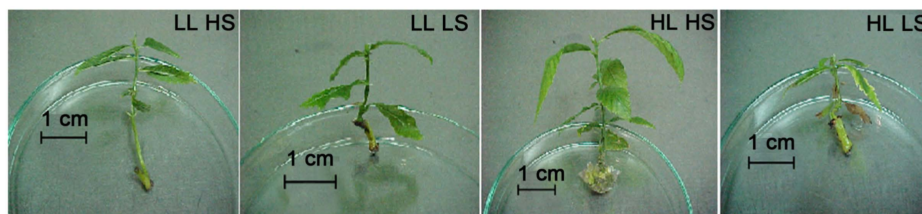

Figure 2. Randomly chosen *Castanea sativa* microshoots grown under low light and high sucrose (LL HS), low light and low sucrose (LL LS), high light and high sucrose (HL HS) and high light and low sucrose (HL LS).

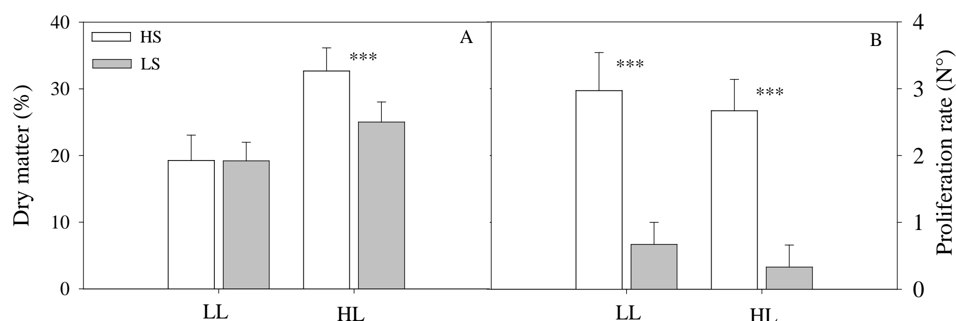

Figure 3. Dry matter (A) and proliferation rate (B) of *Castanea sativa* grown under different sucrose concentrations and PFD. ***Significant differences between LS (5 $g \cdot L^{-1}$) and HS (30 $g \cdot L^{-1}$) at $P < 0.05$.

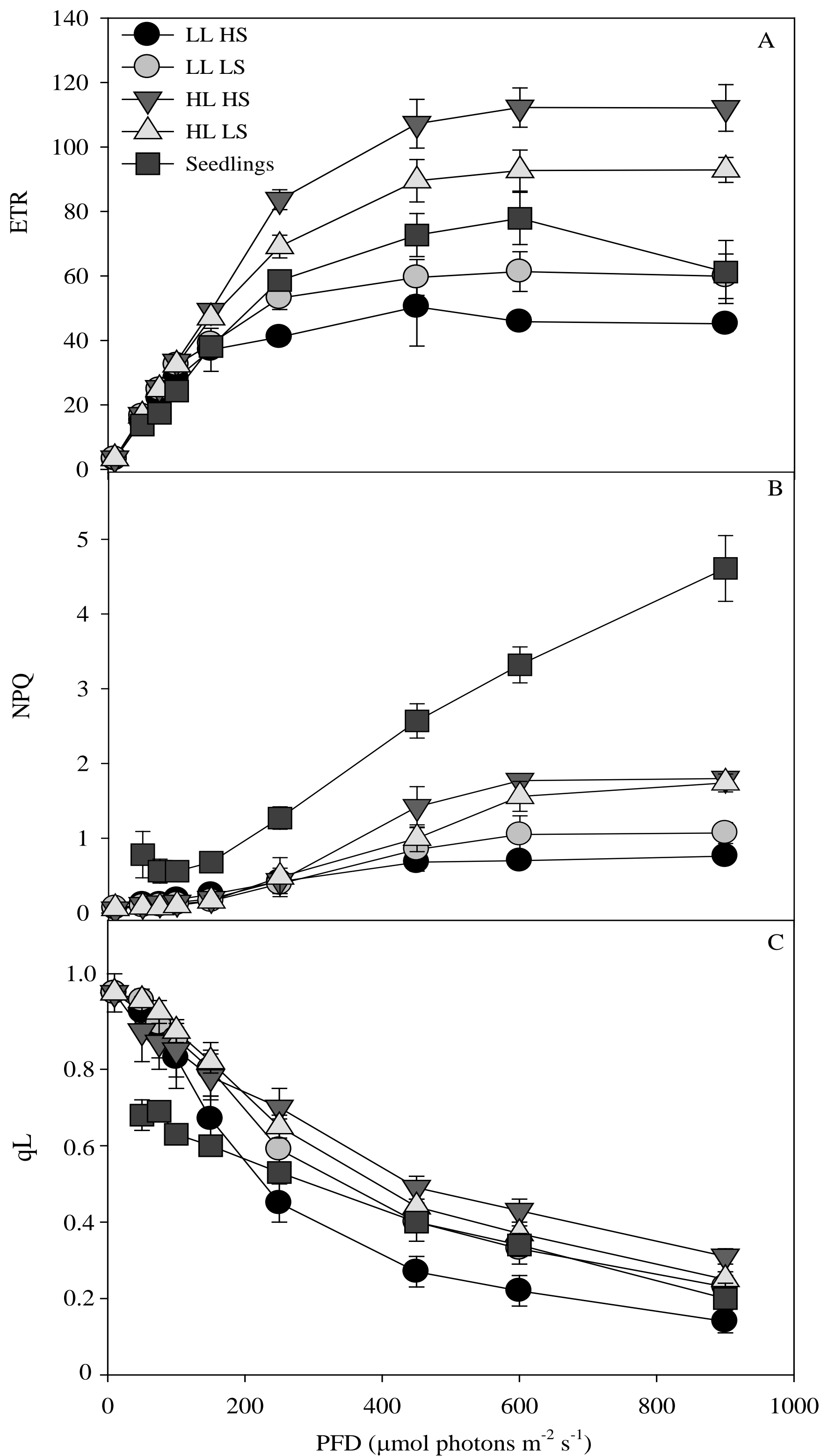

Figure 4. Electron transport rate (ETR) (A), non-photochemical quenching (NPQ) (B) and relative redox state of PSII (qL) (C) from leaves of *C. sativa* grown under different sucrose concentrations and PFD and seedlings. Values are mean ± S.E, n = 5.

ments, where the ETR showed a maximum value near to 60 μmol of electrons $m^{-2} \cdot s^{-1}$. In these treatments the ETR saturation was near 300 μmol photons $m^{-2} \cdot s^{-1}$ and remained constant up to 900 μmol photons $m^{-2} \cdot s^{-1}$. Regarding the thermal dissipation capacity, the NPQ (**Figure 4(B)**) obtained under different *in vitro* culture conditions, were significantly lower than those observed in seedlings. However, it was observed that HL treatments showed an increase in heat dissipation. These latter treatments presented the highest values close to 1.5 which is three orders higher than normally seen in conventional micropropagation systems of *C. sativa* (LL HS). Finally, regarding to photochemical quenching (qL), differences were noted among different treatments (**Figure 4(C)**). In LL HS, the qL showed a rapid decrease from dark to about 200 μmol photons $m^{-2} \cdot s^{-1}$, exhibiting afterwards very low values, which reflects the little photochemical light conversion capacity of these microshoots at light intensities greater than 200 μmol photons $m^{-2} \cdot s^{-1}$, this imply that more than 90% of Qa is maintained in the reduced state creating a high excitation pressure on PSII. The LS treatments showed a similar pattern to seedlings, only being overtaken by microshoots developed under HL HS. It is noteworthy that the excitation pressure at low ACTINIC LIGHT was significantly lower in microshoots THAN in seedlings.

3.5. Chloroplast Ultrastructure

In the chloroplast ultrastructure and organization of thylakoidal membranes, sucrose concentration added to culture medium significantly influenced the parameters evaluate and there was also a significant interaction between sucrose and PFD factors (**Table 2**). The size chloroplast was higher in HS treatments and only in HL HS treatment was similar to those observed in seedlings. Regarding the membranes organization, the effect of sucrose concentration was dependent on PFD in which microshoots were grown. Here the largest number of grana (similar to that observed in seedlings) was obtained in LL LS, and under this treatment was also reached the highest granum size. Additionally, the biggest stacking was obtained in LS treatments, mainly in LL. A clear granal stacking was not possible to distinguish in LL HS treatment; rather a network of stromall lamellas was observed. Starch granules were observed only in LL LS treatment (**Figure 5**). In HL treatments starch deposition was not observed. However, it

Table 2. Chloroplast characteristics of *in vitro* cultured microshoot leaves of *C. sativa* grown under different sucrose concentrations and PFD. Different letters indicates significant differences among each *in vitro* treatment and seedlings at $P < 0.05$ in one way ANOVA. Mean ± S.E., n = 10.

	Seedlings	LL HS	LL LS	HL HS	HL LS	S	L	S*L
Chlp (μm^{-2})	9.41 ± 1.08 c	13.35 ± 1.52 d	4.51 ± 0.27 a	7.92 ± 0.65 bc	5.54 ± 0.51 ab	***	*	**
G_r $chlp^{-1}$ (N°)	7.00 ± 1.16 cd	0 ± 0 a	8.13 ± 0.85 d	3.14 ± 1.50 b	4.43 ± 1.17 bc	***	ns	**
G_r (μm^{-2})	0.88 ± 0.17 b	0 ± 0 a	0.07 ± 0.00 a	0.04 ± 0.02 a	0.05 ± 0.01 a	**	ns	*
T_{hyl} G_r^{-1} (N°)	20.40 ± 2.82 d	0 ± 0 a	10.55 ± 0.40 c	4.48 ± 1.13 ab	7.43 ± 1.95 bc	***	ns	**

Chloroplast area (Chl), grana per chloroplasts (G_r $chlp^{-1}$), grana area (G_r area), thylakoid per chloroplast (T_{hyl} G_r^{-1}). ***refers to factor's significance at $P \leq 0.001$; **at $P \leq 0.01$; *$P < 0.05$ and ns, no significant in two way ANOVA.

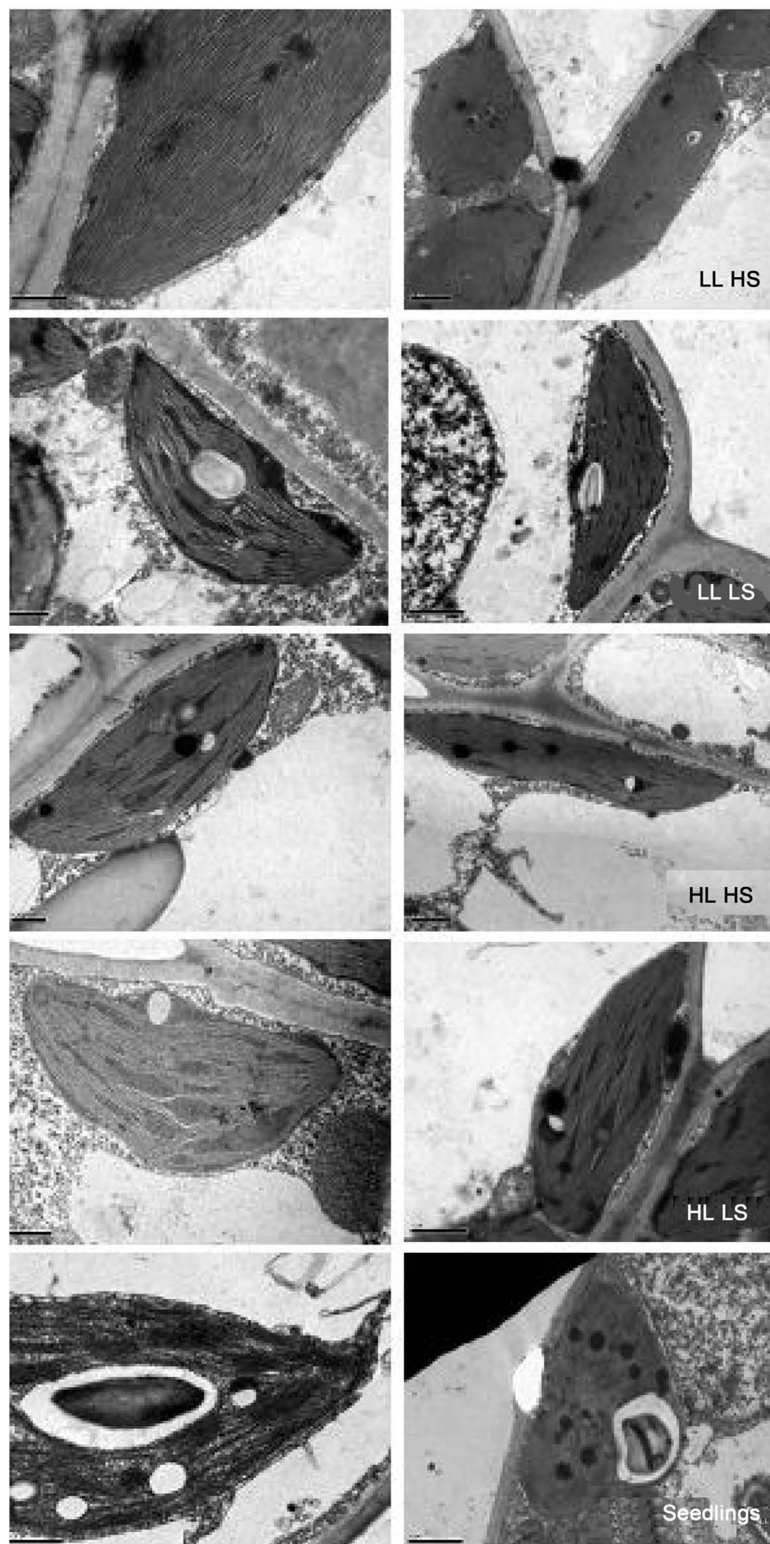

Figure 5. Electron micrographs of mesophyll cell chloroplasts of microshoot leaves of *Castanea sativa* grown under: low light and high sucrose (LL HS), low light and low sucrose (LL LS), high light and high sucrose (HL HS), high light and low sucrose (HL LS) and seedlings. Bars in left and right represent 1 and 0.5 μm, respectively.

was possible to observe the presence of plastoglobuli, similar to those observed in chloroplasts of seedlings (**Figure 5**).

4. Discussion

As outlined, the effect of sucrose was dependent on light intensity to which the culture was developed. Thus, our results suggest that at least in *Castanea sativa,* sucrose has a positive effect. Although it has been hypothesized that sucrose causes a down-regulation of photosynthesis [14] [26] [27] our result shown that the photosynthetic performance was improved by sucrose (**Figure 1(A)** and **Figure 1(B)**). However, this positive effect was only significant ($P < 0.05$) when the culture was carried out under higher PFD (HL HS). According to Paul and Stitt [16] the down-regulation as a consequence of the sucrose addition to the culture medium might be due to the low light regime which result in low photosynthetic rates and a source limitation to growth. However, in this case, down-regulation was always given by a lower sucrose addition, even when light regime was increased, and became more evident under this condition. This according to Ticha *et al.* [17] indicates that the source may not be a limiting factor. Kovtun and Daie [28] observed that the addition of sucrose accelerates the sink to source transition, suggesting that this effect prevents the down-regulation of photosynthesis. Thus, it is likely that the problem is not the source but rather sink limitation, and the enhanced development of photo mixotrophically grown plants increases the capacity to use carbohydrates.

It is well established that the initial slope of CO_2 response curve, is an indirect measure of Rubisco carboxylation efficiency [29]. In this regard, carboxylation efficiency (**Figure 1(F)**) showed the same trend that A_{ls} and A_{max} (**Figure 1**). The high C_E determined in HL HS coincided with the increased carbon assimilation observed in this treatment. This disagrees with Fuentes *et al.* [14], who informs that lower value of C_E was found in plantlets with high exogenous sucrose, suggesting their lower ability to assimilate CO_2. In fact, several researches suggest that the negative effect of sucrose on plantlet´s photosynthesis is a result of a decrease in Rubisco efficiency [14] [30] [31]. However, our result indicates that besides the presence of sugar in the culture medium, the activity exhibit by Rubisco in tissue culture systems could be modulated by other factors, like light. Some results indicated PFD as a primary factor in the low activity of Rubisco [31]. Low light level may have an effect on trans-thylakoid ΔpH, which affects Rubisco activase, leading to a reduce capacity to displace inhibitors bound to the carbamate [32] [33].

It has been shown that the plantlets *in vitro* can achieve photoautotrophic growth by increasing Light intensity without sucrose [14] [34] [35]. Our result shown that the increase PFD (from 50 to 150 μmol photons $m^{-2}\cdot s^{-1}$) is not enough for increase photosynthesis when sucrose added to the culture medium is reduced (from 30 to 5 $g\cdot L^{-1}$). Low sucrose concentration at low PFD (LL LS) had no effect on chlorophyll content, similar to the result found in the photosynthetic performance (**Table 1**), but when PFD increased, sucrose was necessary to increase chlorophyll content. Thus, higher chloro-

phyll content was determined by HL HS treatment, with similar values than those observed in seedlings. This was similar to that reported by Ticha *et al.* [17] who found higher chlorophyll content in tobacco plantlets photomixotrophically growth. The chlorophyll content may influence the plants ability to maximize the light harvesting capacity [32] [36]. It is one of the most important factors in determining the photosynthetic rates [37]. In fact, under photomixotrophic conditions (HL HS) higher chlorophyll content was concomitant with higher A_{ls}, A_{max}, LSP and C_E (**Figure 1**).

The increase in photosynthetic activity promoted by the HL HS produced also an increased in microshoots growth. The explants development, mainly leaf area (**Figure 2**) and the biomass production (**Figure 3(A)**) were greater when larger amount of exogenous sucrose and high PFD were used. Previous studies already revealed the importance of sucrose for culture growth, accounts for 75% to 85% of the biomass increase [38]. According to Eckstein *et al.* [39] which accounts increasing sugar concentration has a visibly stronger influence on leaf area than on leaf number and according these authors; sugar present in the medium would be use mainly as a carbon source for biomass production and does not play a major signaling role in development. The same authors reported that plants grown on medium without sugar were small and their development was delayed, independent of the sugar kind (sucrose or glucose) or its concentration (10 or 30 $g \cdot L^{-1}$). However plants developed slightly different phenotypes depending on the irradiance. Thus, plants growth at irradiances lower than 30 µmol photons $m^{-2} \cdot s^{-1}$ had smaller leaves, but plants grown at higher irradiance (100 to 300 µmol photons $m^{-2} \cdot s^{-1}$) were well developed and reached the biggest dimensions, like in our results. Proliferation rate (**Figure 3(B)**) was also positively affected by HS, this supports the idea of greater utilization of exogenous sugars in growth, as new microshoots formation (**Figure 3**) as well microshoots photosynthetically more competent (**Figure 1**). Additionally, sugars provide the energy source and the building blocks for plant's metabolism and act as regulatory molecules controlling physiology, metabolism, development, and the expression of genes in all living cells [40] [41]. Several researches have suggested that sugars act as active messengers promoting or inhibiting plant genes implicated in many fundamental processes including photosynthesis, respiration, carbon and nitrogen metabolism, pathogen defense, wounding response, control of cell cycle, and senescence [42] [43]. However, in *in vitro* culture these phenomena are still poorly investigated [39] [40].

On the hand, it is probably that at low sucrose, the increase in PFD result harmful to growth plant, because the low chlorophylls contents produce under these conditions would reduce the photosynthesis *in vitro* by decreasing light energy absorption [12]. Thus leaf yellowing was observed in HL LS (**Figure 2**) suggesting that this light intensity may occasion damage or inactivation of the photosynthetic system, or according to the low LSP found (**Figure 1(C)**), this condition likely provided excess light causing an energy imbalance and promoting photoinhibition or photodamage. Given that the increase irradiance produced no apparent damage when was combined with high sucrose, this means that sugar works as photoprotective, allowing the plants to overcome the

stress and photodamage caused by higher irradiances [41]. Additionally, the higher growth observed in HL HS would help to replace the damage photosystems and to consume a surplus of assimilate [44].

HL HS also promotes the accumulation of chlorophylls, which determines the dry mater production [45]. According to Sestak [46], this is product particularly of chlorophyll *a* content, which is more involved in determining photosynthetic activity. This is important because the photosynthesis, how responsible for the plant`s energy and carbon incorporation into the plant, is crucial for its survival [47], for example during the *ex vitro* transference [14].

Regarding photochemical activity, our result indicates that increase in PFD produced an increase in electron transport rate (**Figure 4(A)**), and this increase was more pronounced when the sucrose addition was higher. Despite the high ETR observed in HL treatments, which exceeded those found in seedlings, carbon assimilation never reached rate obtained in seedlings (**Figure 1(B)**). According to Mohamed and Alsadon [12], the chloroplasts could have light stimulated electron transport but lower level of photosynthetic activity resulting in low carbon assimilation in *in vitro* plants. Respect to mechanism for managing the excess light energy, Demmig-Adams *et al.* [48] reported that once light has been absorbed, this may be achieved by thermal dissipation of excess absorbed energy (non photochemical quenching, NPQ) or through the photochemical use of the energy (photochemical quenching, qL). In NPQ (**Figure 4(B)**) the same behavior that ETR was found, showing higher values in HL treatments, without difference between high or low sucrose. Additionally, in these treatments it was possible to observe plastoglobuli accumulation, similar to that found in seedlings. However, NPQ was significantly lower that seedlings, reflecting the already reported poor ability of *in vitro* plants to handle the excess light energy [6] [49] [50]. Probably because to develop these mechanisms is not only necessary to grow under stable levels of high irradiance, but also the occurrence of increased light intensity events (PFD variability), that triggers the development of photoprotective mechanisms as adaptive strategy. It may be also possible that light signals from different light quality other than that provided by light bulbs in growth chamber and that would be necessary to trigger photoprotective mechanisms. In fact, in nature, the high variability in photon flux density and their spectral composition, trigger dynamics acclimation responses, among them NPQ, state transitions and long-term response, that allows respond to a very broad range of conditions [51].

The photochemical quenching (qL) is associated with the potential at a given PFD to execute photochemical conversion of light energy to drive electrons through the intersystem pool. In LLHS, the qL showed a rapid decrease from dark to about 200 μmol photons $m^{-2}\cdot s^{-1}$, exhibiting afterwards very low values, which reflects the little photochemical light conversion capacity of these plants at light intensities greater than 200 μmol photons $m^{-2}\cdot s^{-1}$ (**Figure 4(C)**). This implies that more than 90% of Qa are maintained in the reduced state creating a high excitation pressure for PSII [52]. These results are consistent with the lower ETR observed at saturating PFD. In contrast, HL HS treatment, qL remained high, even higher than that observed in seedlings, maintaining a lower excitation pressure on PSII than the other treatments.

Additional to functional attributes, sucrose and PFD affected anatomical attributes related to chloroplast ultrastructure. Thus, as size as well membrane organizations into the chloroplast were affected (**Table 2**). As reported by Lee *et al.* [53] chloroplasts of *in vitro* plants cultured under low light (50 μmol photons $m^{-2} \cdot s^{-1}$) were flattened and devoid of organized grana, and when PFD increased (300 μmol photons $m^{-2} \cdot s^{-1}$) well organized granas were observed (**Table 2** and **Figure 5**). However, our result indicated that thylakoid's organization varied according to sucrose and PFD used. Thus at LS, grana number per chloroplast increased. These grana had a greater area and thylakoids number, these features were enhanced at LL. Similar results were report by Serret and Trillas [54] working with different sucrose and light level in *Gardenia jasminoides*. Additionally, in TEM analysis (**Figure 5**) was only possible observe starch accumulation under LL LS treatment. According to Capellades *et al.* [55], the starch might be responsible for the lower photosynthesis rate. However these authors report that the size and number of starch granules increased with the level of sucrose. So, when the sugar export from the leaf is reduced, it will be stored in these organs. An explanation for the inhibition of photosynthesis might be the low rate of regeneration of the carboxylation substrate RuBP due to the accumulation of soluble sugar in the leaves [56]. Our results indicate that in LL LS an increase in the starch granules was concomitant with lower net photosynthesis. By contrast at HS starch accumulation was not observed, it is likely that the storage here is unnecessary because of the continued presence of a carbon source, which are used in growth.

5. Conclusion

In conclusion, although low exogenous sucrose produced an improvement on the thylakoidal organization membranes, which could imply an improvement of the photosynthetic apparatus, structure did not produce an improvement on photosynthetic performance and negatively affected the *in vitro* growth of *Castanea sativa* plantlets. Thus, none of functional and growth parameters evaluated were improved by decreasing sucrose, even when this decrease was accompanied by increases in PFD. Additionally, no assessed treatments produce more competent microshoots from the point of view of their similarity to plants grown in nursery conditions (seedlings). The results confirmed that using sucrose as external source of carbohydrates during proliferation stage of *Castanea sativa* microshoots had a positive effect on photosynthesis, chlorophyll content and growth, similar to that reported by Cournac *et al.* [56]; Paul and Stitt [16]; and Ticha *et al.* [17]. With this knowledge it is possible to establish strategies of mixotrophic culture that lead to production of competent microshoot during *in vitro* culture and, consequently, minimize the transfer stress and maximize the *ex vitro* survival rates.

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