Biotechnology Research and Innovation, 2020, 4(1): e2019092. https://doi.org/10.4322/biori.2019092

Biotechnology Research & Innovation

www.biori.periodikos.com.br

RESEARCH PAPER

Integrative approach reveals new insights into photosynthetic and redox protection in *ex vitro* tobacco plantlets acclimatization to increasing light intensity

Cinthya F. Vieira[a], Fabricio E. L. Carvalho[a], Yugo Lima-Melo[a], Cristina P. S. Carvalho[a], Milton C. Lima Neto[b], Marcio O. Martins[a], Joaquim A. G. Silveira[a,*]

[a]*Department of Biochemistry and Molecular Biology, Federal University of Ceará – UFC, CEP 60451-970, Fortaleza, CE, Brazil*
[b]*Biosciences Institute, São Paulo State University – UNESP, Coastal Campus, CEP 11380-972, São Vicente, SP, Brazil*

KEYWORDS
Ex vitro acclimatization;
Nicotiana tabacum;
Oxidative stress;
Photosynthesis.

Abstract: Integrative mechanisms involving photosynthetic and antioxidant protection regulated by light intensity during *in vitro* and *ex vitro* plantlets acclimatization are poorly understood. Tobacco plantlets grown under *in vitro* and *ex vitro* environments were exposed to different light regimes to evaluate the role of photosynthesis and antioxidant protection. *In vitro* plantlets displayed a narrow photosynthetic capacity to cope with light as revealed by low net CO_2 assimilation (P_N), decreased actual quantum efficiency of photosystem II (ΦPSII) associated with non-induction of non-photochemical quenching (NPQ). In contrast, acclimated *ex vitro* plants showed strong stimulation in P_N whereas ΦPSII and NPQ remained at high levels. *In vitro* plantlets exposed to moderate light suffered strong oxidative stress associated with increased activities of superoxide dismutases, catalases and ascorbate peroxidases, revealing an ineffective antioxidant system. In contrast, *ex vitro* plants presented lower oxidative damage in parallel to unchanged enzymatic activities, indicating an efficient antioxidant steady state. The levels of reduced ascorbate (ASC) and glutathione (GSH) were also increased only in *ex vitro* plants in response to excess light. An integrative study based on correlation networks and principal component analyses (PCA) corroborate that the two plant groups indeed displayed contrasting acclimation processes. In conclusion, during *in vitro to* ex *vitro* transition, tobacco plantlets exposed to increasing light display physiological adjustments involving photosynthesis and improvement of the enzymatic and non-enzymatic antioxidant systems. These findings highlight the importance of integrative approaches to understand *ex vitro* acclimatization to environmental stimuli.

* Corresponding author:
E-mail: silveira@ufc.br (J. A. G. Silveira).

Highlights

Person's correlation network indicates that *ex vitro* plants display lower density and higher heterogeneity compared to *in vitro* ones

Low photosynthetic efficiency in *in vitro* plants in response to light is associated with low NPQ induction and ineffective antioxidant defence.

Increase of NPQ and PSII efficiency in response to light intensity can contribute to avoid oxidative stress during *ex vitro* plantlets acclimatization.

Introduction

Plant propagation by *in vitro* techniques is important for agriculture and plant science, especially for generation of transgenic plants and production of biotechnological products. However, *in vitro* morphogenesis and organogenesis processes are complex and several mechanisms are still not completely understood (Lando et al., 2016). This complexity is aggravated by the artificial environmental conditions commonly employed during plantlets growth and tissue differentiation, especially exposure to very low light intensities. Indeed, these conditions are extremely contrasting in terms of light regimes, root substrates and other environmental conditions employed for cultivation of seed-plants (Chandra et al., 2010; Sáez et al., 2012). Therefore, acclimatization of *in vitro* plantlets displays several genetic and morphogenetic alterations at tissue, cell and organelle levels, which might affect their morpho-physiological organization and alter plant metabolism as compared to plants grown under normal conditions (Lando et al., 2016).

Chloroplast development and morphogenesis of photosynthetic components are drastically affected under *in vitro* conditions, especially due to the almost complete heterotrophic growth conditions (Sáez et al., 2012). Pre-acclimatization or endurance phase of *in vitro* plantlets throughout *ex vitro* acclimation process is a very important feature for achieving a successful plant micropropagation procedure (Perveen et al., 2013). *Ex vitro* phase encompasses several favorable morphogenetic changes in photosynthetic apparatus, involving photochemical, biochemical and stomatal components (Monja-Mio et al., 2015; Perveen & Anis, 2015). Thus, a progressive increase in the light intensity, combined with adjustments in vapor pressure deficit, are commonly employed during *ex vitro* endurance in order to favor the fitness of morpho-physiological components, including photosynthetic and antioxidant systems.

The rapid and intense water loss is an important problem for acclimatization due to excessive transpiration by *in vitro* plantlets, which is a consequence of undeveloped stomatal systems (Barry-Etienne et al., 2002). This process is controlled by several genes and complex signaling transduction pathways involving light stimulus and metabolic changes, which are still not completely understood (Daloso et al., 2017). In parallel to stomatal organization, the photosynthetic efficiency is gradually improved to face with gradual increase of light intensity (Alvarez et al., 2012; Monja-Mio et al., 2015; Swain et al., 2010). Moreover, the effective quantum yield of PSII (ΦPSII) and non-photochemical quenching (NPQ) are enhanced during *ex vitro* acclimatization in response to a gradual increase of light intensity until accomplishing full photosynthetic capacity (Carvalho et al., 2006; Guan et al., 2008).

Photosynthetic acclimation also requires a fine balance between the activities of light-harvesting antenna, photosynthetic linear electron flow and Calvin-Benson cycle reactions (Foyer et al., 2012; Silveira & Carvalho, 2016). Several studies have reported that excessive light, which occurs when the irradiance absorbed by plants exceeds their catabolic capacity, might trigger alterations in gene expression (Li et al., 2009; Gollan et al., 2017; Lima-Melo et al., 2019a). The transcriptomic changes involved with the response to excessive light are dependent on specific signal transduction routes, which are closely related to the redox state of plastoquinone pool in chloroplasts and light perception by specific photoreceptors (Dietz 2014; Li et al., 2009). Therefore, the light intensity and the consequent reducing power generated in photochemical reactions should represent a major factor in acclimation processes of *in vitro* plantlets (Perveen et al., 2013; Lando et al., 2016).

The reducing power that is not totally utilized by CO_2 assimilation and other biochemical processes can induce reactive oxygen species (ROS) accumulation in chloroplasts (Foyer, 2018), which in turn can generate oxidative damage in all plant cell compartments. Moreover, H_2O_2 is believed to be capable of inactivating photosystem II (PSII) reaction center through inhibition of D1 protein *de novo* synthesis (Jimbo et al., 2018). This process is critical for plant growth and survival, as it can lead to chronic photoinhibition and total collapse of all photosynthetic processes (Adams et al., 2013; Ruban, 2015). However, H_2O_2 is capable of reaching the nucleus and activating several important transcription factors, which are crucial for regulation of gene expression related to light acclimation mechanisms (Exposito-Rodriguez et al., 2017).

Deficiency of antioxidant defence in chloroplasts is aggravated during the plantlets acclimation when the redox systems are still incipient. During *ex vitro* acclimation, antioxidant enzymes, especially superoxide dismutase (SOD), ascorbate peroxidases (APX), and catalases (CAT) are vital to regulate ROS levels (Dias et al., 2011; Perveen et al., 2013). The chloroplastic isoforms of SOD and APX are crucial to the water-water cycle, which might contribute to dissipate part of excess energy absorbed in both photosystems I and II (Asada 2006). In parallel, the synthesis and turnover of other non-enzymatic antioxidant components, including ascorbate (ASC) and glutathione (GSH), involving specific synthesis-pathways and the ascorbate-glutathione cycle, are essential for the maintenance of redox homeostasis during *ex vitro* acclimation under increased light conditions (Carvalho et al., 2006; Varshney & Anis, 2012).

Ex vitro acclimation process has been widely studied in terms of changes at morphological, anatomical, and photosynthetic and antioxidant levels (Carvalho et al., 2006; Lando et al., 2016; Sáez et al., 2015), but most of these studies are fragmented and inconclusive. Moreover, the interactive features involving such processes including *in vitro* growth and acclimatization of plantlets have been much less investigated. Indeed, it is still not well known whether the regulation of antioxidative metabolism is important for

effective photosynthetic acclimation during acclimatization of *ex vitro* plantlets, especially when they are challenged by different light regimes. This kind of study is important because the knowledge of these responses is essential to introduce new tools to maximize the endurance of plantlets.

In this study *in vitro* and *ex vitro* tobacco plantlets were exposed to different light regimes in order to understand some relationships between photosynthetic protection by NPQ and electron transport efficiency associated with effective antioxidant defence. We hypothesized that an efficient antioxidant system is crucial to protect and support photosynthesis efficiency during plantlet acclimatization. We demonstrated that an effective NPQ induction could avoid ROS overaccumulation in chloroplasts, thus favoring the photosynthetic efficiency in response to high light in *ex vitro* plants. *In vitro* plantlets were not able to display these responses, which were associated with non-developed photosynthetic and redox systems. An integrated analysis employing a correlation network and principal component analysis (PCA) revealed that the interaction among photosynthetic and redox parameters of *ex vitro* plantlets present a weaker interactive density suggesting higher flexibility probably associated to an effective photosynthetic acclimation process to increasing light, which can reduce the requirement for induction of an antioxidant mechanism. The role and importance of antioxidative metabolism and photosynthetic apparatus regulation during *ex vitro* acclimation process are discussed.

Material and methods

In vitro and *ex vitro* growth conditions

Nicotiana tabacum plants cv. Xanthi obtained from seeds were used as source of explants for *in vitro* cultivation. Plantlets were grown in complete MS medium (Murashige & Skoog, 1962), pH 5.8, supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar. Nodal segments were removed from the plantlets in a laminar flow chamber and inoculated into 200 mL glass bottles, sealed with transparent polyethylene cover (70% PPFD transmittance) and kept in a growth room with 12-hour photoperiod, external light intensity of 50 µmol m^{-2} s^{-1} and 25 °C temperature for 30 days (*in vitro* reference).

For *ex vitro* acclimation, 15 days-old *in vitro* plantlets were carefully transferred to 2 L pots containing a mixture of washed sand and vermiculite 1:1 (v/v), previously autoclaved. The plantlets were kept under greenhouse conditions under 75% shading with an average of 230 µmol photons m^{-2} s^{-1} of maximum photosynthetic photon flux density (PPFD) at midday, 29/25 °C day/night temperature, 62% humidity and 12-h photoperiod. The substrate was watered with MS saline, pH 6.0, every two days and with distilled water in alternated days. In the first two weeks, the nutrient solution was supplied with 10% of its ionic strength and afterwards, with ½ strength solution. The plantlets remained under these conditions for 15 days (*ex vitro* reference).

Determination of gas exchange, chlorophyll *a* fluorescence parameters and photosynthetic pigments contents

CO_2 assimilation rate (P_N), stomatal conductance (g_s) and transpiration (*E*) were measured in fully expanded leaves from *in vitro* and *ex vitro* tobacco plantlets with a portable infrared gas analyzer system equipped with an LED source and a leaf chamber (IRGA LI-6400XT, LI-COR, Lincoln, USA), and expressed as µmol CO_2 m^{-2} s^{-1}, mol H_2O m^{-2} s^{-1}, mmol H_2O m^{-2} s^{-1}, and mmol CO_2 mol air^{-1}, respectively. The internal parameters in the IRGA chamber during gas exchange measurements were 1.0 ± 0.2 kPa vapor-pressure deficit and 38 Pa CO_2, at 28 °C. Actinic light employed for photosynthetic determinations was similar to those in which plants were previously grown for 12 hours, i.e., 100, 400, 500, 600, 800 and µmol m^{-2} s^{-1}. A PPFD of 50 and 200 µmol m^{-2} s^{-1} were utilized for the *in vitro* and *ex vitro* references, respectively. The blue light was set to 10% of the PPFD to maximize the stomatal aperture (Flexas et al. 2008). The P_N-PPFD fitting curves were determined according to models proposed by Lieth & Reynolds (1987).

In vivo chlorophyll *a* fluorescence was measured using an LI-6400-XT fluorometer (LI-COR, Lincoln, NE, USA) coupled with the IRGA equipment. The actinic light utilized to measure chlorophyll *a* fluorescence was equal to the light intensity employed for plant growth, as described for the determination of gas exchange parameters. The fluorescence parameters were measured by the saturation pulse method (Schreiber et al. 1995) in leaves exposed to light and dark-adapted (30 min). The intensity and duration of the saturation light pulse were 8000 µmol m^{-2} s^{-1} and 0.7 seconds, respectively. Maximum quantum yield of photosystem II (PSII) [Fv/Fm = (Fm-Fo)/Fm], effective quantum yield of PSII [ΦPSII = (Fm'-Fs)/Fm'] and non-photochemical quenching [NPQ = (Fm-Fm')/Fm'] were calculated according to Genty et al. (1989) and Schreiber et al. (1995).

Membrane damage and lipid peroxidation

Membrane damage as an indicator of cellular viability was estimated by electrolyte leakage measurement, as previously described by Blum & Ebercon (1981). Twenty leaf discs (1.0 cm diameter) were placed in test tubes containing 20 mL of deionized water. Flasks were incubated in a shaking water bath (25 °C) for 12 h, and the electric conductivity in the medium (L1) was measured. Discs were then boiled (95 °C) for 60 min, cooled to 25 °C, and then the electric conductivity (L2) was measured again. Relative membrane damage (MD) was estimated by MD = L1/L2 × 100 (Blum & Ebercon, 1981). Lipid peroxidation was measured based on the formation of thiobarbituric acid-reactive substances (TBARS) in accordance with Cakmak & Horst (1991). The concentration of TBARS was calculated using its absorption coefficient (155 mM^{-1} cm^{-1}) and the results were expressed as ηmol MDA-TBA g FW^{-1} (Cakmak & Horst, 1991).

In situ and *in vitro* detection of hydrogen

peroxide and superoxide radical

In situ detection of H_2O_2 was performed by 3,3'-diaminobenzidine (DAB) staining as previously described (Thordal-Christensen et al., 1997). Leaf segments were infiltrated under dark conditions with a 0.1% (w/v) DAB solution. Segments were incubated for approximately 16 h in dark conditions and then destained with 0.15% (w/v) trichloroacetic acid in an ethanol/chloroform solution (4:1 v/v) for 48 h before being photographed. Superoxide *in situ* quantification was performed with leaf samples immersed in 6 mM nitroblue tetrazolium (NBT) solution containing 50 mM sodium phosphate (pH 7.5) for 12 h in the dark (Zulfugarov et al., 2014). Subsequently, NBT-treated leaf segments were destained and photographed in the same manner as for DAB methodology described above.

In vitro H_2O_2 accumulation was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, CA, USA), according to Zhou et al. (1997) and following the instructions in the accompanying protocol. For H_2O_2 extraction, 150 mg of fresh leaves were ground in liquid N_2, and 1 mL of phosphate buffer (100 mM, pH 7.5) was added to the frozen tissue. The absorbance was quantified spectrophotometrically using a wavelength equal to 560 ηm, and the total H_2O_2 concentration was calculated from a H_2O_2 standard curve according to the kit manufacturer's instructions and expressed as μmol H_2O_2 g^{-1} FW.

Superoxide *in vitro* quantification was performed according to Elstner & Heupel (1976). The methodology is based on nitrite formation from hydroxylamine in the presence of $O_2^{-\bullet}$. One g of frozen leaf segments was homogenized with 3 mL of 65 mM potassium phosphate buffer (pH 7.8) for superoxide extraction. The reaction media contained 0.9 mL of 65 mM potassium phosphate buffer (pH 7.8), 0.1 mL of 10 mM hydroxylamine hydrochloride and 1 mL of extracts. The media was incubated at 25 °C for 20 min followed by addition of 17 mM sulfanilamide and 7 mM α-naphthylamine to the mix. The solution was kept at 25 °C for more 20 min and then an ethyl-ether solution at the same volume of the reaction mixture was added to stop the reaction. After centrifugation of 1,500 *g* for 5 min, the absorbance was measured spectrophotometrically at 540 ηm of wavelength (Elstner & Heupel, 1976). The $O_2^{-\bullet}$ content was calculated from standard curves using NO_2^- and the results were expressed as ηmol of $O_2^{-\bullet}$ g^{-1} FW.

Protein extraction and enzymatic activity assays

For the preparation of crude extracts, fresh leaf samples (300 mg) were ground to a fine powder in the presence of liquid N_2 with mortar and pestle and extracted in ice-cold 100 mM K-phosphate buffer, pH 6.8, containing 0.1 mM ethylenediamine tetraacetic acid (EDTA) and 2 mM ascorbic acid. The protein content in each fraction was measured by the Bradford (1976) method using bovine serum albumin (BSA) as standard.

APX activity (E.C. 1.11.1.11) was measured following the ascorbate oxidation by decrease of absorbance at 290 ηm (Nakano & Asada, 1981), with minor modifications. The activity was assayed in a reaction mixture containing 0.5 mM ascorbate and 0.1 mM EDTA dissolved in 100 mM K-phosphate buffer pH 7.0 and enzyme extract. The reaction was started by adding 3 mM H_2O_2. The temperature was adjusted to 25 °C and enzyme activity was measured by the decrease in absorbance at 290 ηm over 300 s. In order to avoid interference of type III peroxidases activity, two parallel determinations were performed: (A) in the absence and (B) in the presence of p-chloromercuribenzoate, a specific APX inhibitor (Amako et al. 1994). Net APX activity was calculated by the difference A - B and expressed as mmol H_2O_2 mg protein^{-1} min^{-1}.

CAT activity (E.C. 1.11.1.6) was measured following the oxidation of H_2O_2 at 240 ηm. CAT activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) and 20 mM H_2O_2. The reaction took place at 30 °C and the absorbance was monitored at 240 ηm over 300 s (Havir & McHale, 1987). CAT activity was calculated according to H_2O_2 molar extinction coefficient (36 mM^{-1} cm^{-1}) and expressed as mmol H_2O_2 mg protein^{-1} min^{-1}.

SOD activity (E.C. 1.15.1.1) was determined by inhibition of blue formazan production via NBT photoreduction. SOD activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 2 μM riboflavin and 75 μM NBT in the dark. The reaction was carried out under illumination at 25 °C for 6 min. The absorbance was measured at 540 ηm (Giannopolitis & Ries, 1977). One SOD activity unit (U) was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction and the activity was expressed as U mg protein^{-1} min^{-1} (Beauchamp & Fridovich, 1971).

Statistical analysis and experimental design

The experiments were arranged in a completely randomized design with or without factorial arrangement, with three replicate, each one represented by an individual plant. The variance analysis and mean comparison were performed by one way ANOVA and Tukey's test.

Correlation's network analysis

In order to estimate the relationships between some physiological parameters, selected variables measured from *in vitro* and *ex vitro* plantlets were correlated by Pearson´s coefficient (r). Subsequently, these correlations were fitted in a network arrangement according to edge-weighted spring embedded layout using the Cytoscape software (version 3.7.1), considering an arbitrary threshold of $r < -0.9$ (negative correlation) or $r > 0.9$ (positive correlation). This layout is based on the "force-directed" paradigm as described in Kamada & Kawai (1989) and this algorithm sets the positions of the nodes in a way that minimizes the sum of forces in the network (connections). The clustering coefficient, network diameter and radius, network centralization, number of nodes, network density and heterogeneity were calculated by the Cytoscape software. The global network connectance (Cg) was calculated in order to better understand the integrated responses of plants during *ex vitro* acclimation. First, the correlation coefficients (r) between each paired variable were normalized to z-values, as $z = 0.5 \ln [(1 + |r|) / (1 - |r|)]$ and afterwards Cg was calculated as the average of z-values formerly obtained (Souza et al., 2009).

Results

In vitro plantlets exhibit impaired stomatal closure regulation and decreased photosynthetic activity

To investigate the effects of micropropagation techniques on tobacco photosynthetic performance, regenerated plantlets were grown for 30 days under *in vitro* conditions or for 15 days under *in vitro* conditions followed by 15 days under shade conditions in a greenhouse (*ex vitro* ; Figure S1). The photosynthesis capacity during 12 h of continuous light, as indicated by CO_2 assimilation rates, was much higher in *ex vitro* plants as compared to *in vitro* plantlets (Figure 1A). This response was especially prominent at reference and moderate light conditions (ML; 500 µmol m^{-2} s^{-1}), where the *ex vitro* P_N was approximately twice that exhibited by *in vitro* plantlets. Under low light conditions (LL; 200 µmol m^{-2} s^{-1}), P_N was also higher in *ex vitro* compared to *in vitro* plantlets (around 20%), but on a smaller scale compared to that observed for reference and ML conditions (Figure 1A).

The *in vitro* plantlets presented very high and atypical stomatal conductance (g_s) (maximum value near to 1.5 mol m^{-2} s^{-1}) values, which were positively modulated by light (Figure 1B). In contrast, *ex vitro* plantlets showed very low g_s, reaching maximum values near to 0.16 mol m^{-2} s^{-1}. Differently from *in vitro* , the *ex vitro* plantlets exhibited effective stomatal closure in response to increased light (Figure 1B). These contrasts in g_s were well-correlated with stomatal opening and density (Figure S2). The values of transpiration rate were also well-correlated with g_s under reference, LL and ML conditions (data not shown). *In vitro* plantlets displayed values much higher (around 9.5 mmol H_2O m^{-2} s^{-1}) compared to the *ex vitro* ones (1.5 mmol H_2O m^{-2} s^{-1}). PSII activity, represented by the effective quantum yield of PSII (ΦPSII), showed a similar trend to CO_2 assimilation, in which *ex vitro* tobacco plants displayed higher values than *in vitro* plantlets (Figure 1C). The non-photochemical quenching (NPQ) also exhibited tendencies virtually equal to CO_2 assimilation over the studied photoperiod (Figure 1D).

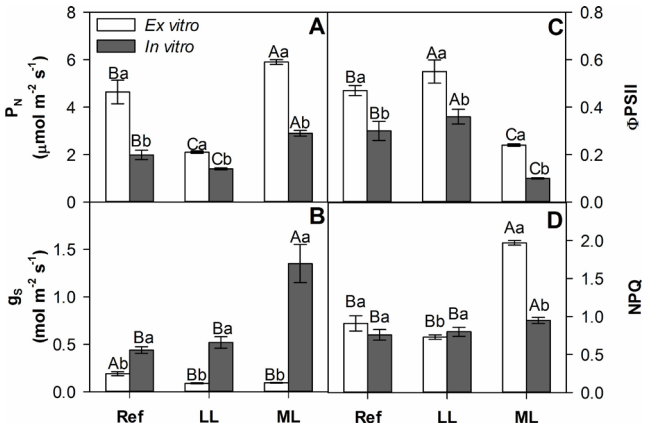

Figure 1. Photosynthetic parameters. (A) Net photosynthesis, (B) stomatal conductance, (C) quantum efficiency of PSII and (D) non-photochemical quenching in leaves from plants grown under *in vitro* and *ex vitro* conditions and acclimated to different light conditions. Reference seedlings consist of leaves collected immediately from the previous growth conditions, i.e., *in vitro* and *ex vitro*, respectively. Subsequently, plants were acclimated to low light conditions (100 µmol photons m^{-2} s^{-1}) and moderate light regimes (500 µmol photons m^{-2} s^{-1}) for 12 hours. Different capital letters mean significant difference (Tukey test, $p < 0.05$) among different light regimes and different lowercase letters represent significant difference between *in vitro* and *ex vitro* growth conditions.

In vitro plantlets exhibited high dark respiration, low photosynthetic efficiency to light and are incapable to induce NPQ

Aiming to understand the photosynthetic dynamics exhibited by *ex vitro* and *in vitro* plantlets in response to light, gas exchange- and photochemical-light curves were performed. Interestingly, the saturation point was very similar for both plantlets studied, which corresponded to approximately 200 µmol photons m^{-2} s^{-1} (Figure S3). On the other hand, light compensation point was much lower in *ex vitro* (15.1 µmol m^{-2} s^{-1}) compared to *in vitro* plantlets (68.0 µmol m^{-2} s^{-1}), as well as maximum light CO_2 assimilation, which were 2.81 and 6.85 µmol m^{-2} s^{-1} in *in vitro* and *ex vitro* plantlets, respectively (Figure S3B-C). In addition, the photosynthetic efficiency (assimilated CO_2 molecules per quantum unit) for *ex vitro* plantlets was significantly higher (20%) in comparison to those *in vitro* (Figure S3D). *In vitro* plantlets also presented remarkably higher dark respiration in comparison to *ex vitro* plants.

In addition to the responses concerning gas exchange parameters, *ex vitro* plantlets also exhibited decreased quantum yield of PSII (ΦPSII) dynamics in response to light compared to *in vitro* plantlets (Figure 2A). These responses were accompanied by a very limited capability of NPQ induction in response to light presented by *in vitro* plantlets (Figure 2B). Interestingly, no significant differences in maximum fluorescence in dark-adapted leaves (Fm) were noted for *ex vitro* and *in vitro* plantlets (Figure 2D), which should reject the hypothesis of underestimated NPQ due to PSII down-regulation. On the other hand, minimum fluorescence in dark-adapted leaves (Fo) was significantly lower in *in vitro* , as compared to *ex vitro* plantlets (Figure 2C).

In order to confirm the limited responsiveness of *in vitro* plants to progressively-increased light, we conducted an experiment involving the whole plant acclimation to increasing light exposure, which is physiologically more relevant than common light curves with a limited area of actinic light exposure, as is the case in IRGA's chamber. The gas exchange data show that P_N in *ex vitro* plants gradualy increased from approximately 2.2 to 5.9 µmol CO_2 m^{-2} s^{-1} within the three firts days of experiment (which corresponded to 200, 400, and 600 µmol photons m^{-2} s^{-1}, respectively), maintaining the same rate on the fourth day (800 µmol photons m^{-2} s^{-1}) (Figure 3A). A similar trend was observed for *in vitro* plantlets, but with lower P_N values, which increased from approximately 1 to 3.2 µmol CO_2 m^{-2} s^{-1}. The P_N values decreased in both groups on the fifth day of experiment (1000 µmol photons m^{-2} s^{-1}), probably as a consequence of some level of photoinhibition (Figure 3A). During the whole experiment, the g_s values were much higher in *ex vitro* plants than in *in vitro* plantlets (Figure 3C). This resulted in the observed responses for E

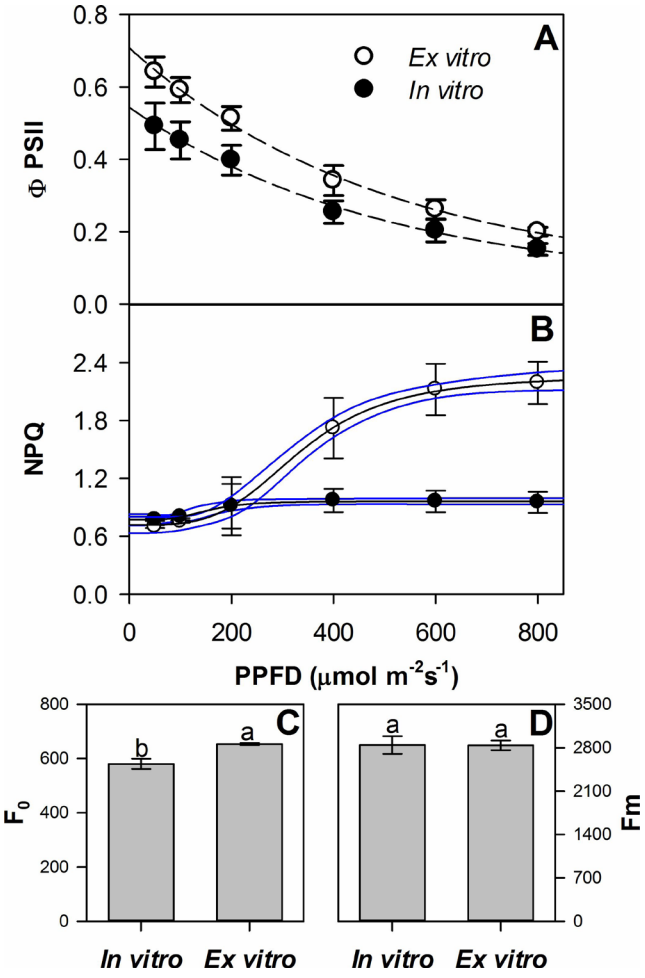

Figure 2. Photochemical responses under increasing light intensity. (A) quantum efficiency of PSII, (B) non-photochemical quenching, (C) minimum dark fluorescence of PSII, and (D) maximum fluorescence of PSII in the dark in leaves from plants grown under *in vitro* and *ex vitro* conditions Different letters mean significant difference ($p < 0.05$) between *in vitro* and *ex vitro* growth conditions.

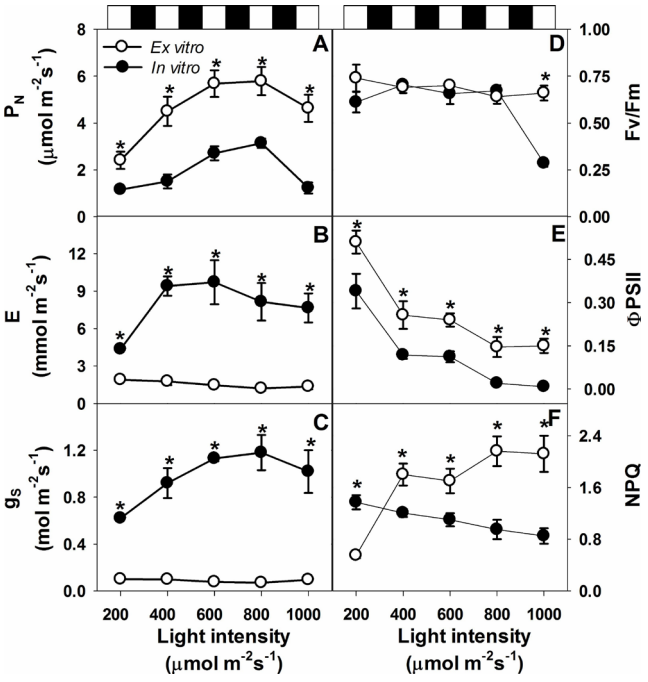

Figure 3. Photosynthetic parameters in tobacco leaves grown under increased light conditions. (A) CO_2 assimilation, (B) stomatal conductance, (C) leaf transpiration, (D) maximum photosynthetic quantum potential, (E) effective photosynthetic quantum yield and (F) non-photochemical quenching in plants grown under *in vitro* conditions for 30 days (closed circles) and plants grown *in vitro* for 15 days followed by 15 days under *ex vitro* conditions (open circles). Plants were acclimated to progressively increased light regimes (200, 400, 600, 800 and 1000 µmol photons m^{-2} s^{-1}). The light intensities were supplied for 12 hours followed by 12 hours of dark before the next light regime. The values are average of three independent measures (n = 3) and error bars represent standard deviation. Asterisks represent significant differences (Tukey's test, $p \leq 0.05$) between *in vitro* and *ex vitro* plants.

(Figure 3B), which were also higher in *ex vitro* plants than in *in vitro* plantlets.

The photochemical data show that Fv/Fm values were similar for both growth conditions during the whole experiment, except for the last day, on which the 1000 µmol photon m^{-2} s^{-1} treatment induced a strong decrease in this parameter only for the *in vitro* plantlets (Figure 3D). Although the ΦPSII parameter was higher in *ex vitro* plants (approximately 0.52) compared to *in vitro* plants (approximately 0.36) for all light conditions, it changed in a similar pattern along the experiment in both conditions (Figure 3E). The ΦPSII decreased approximately 0.25 units in both growth treatments from the first to the second day of treatment and then slightly decreased from the second to the fifth day of experiment, reaching 0.15 in *ex vitro* plants and almost 0 in *in vitro* plantlets (Figure 3E). The increasing light intensity of 200 µmol photons m^{-2} s^{-1} from the first to the second day of experiment dramatically induced NPQ in *ex vitro* plants, which increased from 0.6 to 1.8 units, and then slightly increased to 2.1 units from the second to the fifth day of experiment (Figure 3F). Contrary, NPQ values in *in vitro* plantlets slightly and gradually decreased from 1.4 to 0.9 units from the first to the fifth day of experiment, meaning that NPQ was higher in in vitro plantlets than in ex vitro plants only in the first day of the experiment, in which the light intensity was equivalent to 200 µmol photons m^{-2} s^{-1} (Figure 3F). The results obtained from this experiment corroborated the observations in light curves performed employing IRGA's light source, in which *in vitro* plantlets exhibit loss of stomatal closuring control, impaired CO_2 assimilation and PSII activity, combined with inability for NPQ induction by light (Figure 3).

Ex vitro plantlets effectively triggered antioxidant protection to avoid oxidative stress during light acclimation

Reactive oxygen species (ROS) scavenging mechanisms were assessed during *in vitro* and *ex vitro* acclimation to low (LL; 100 µmol photons m^{-2} s^{-1}) and moderate (ML; 500 µmol photons m^{-2} s^{-1}) light for 12 h of continuous illumination.

In presence of LL, *in vitro* plantlets displayed intense accumulation of H_2O_2 (Figure 4) and radical ion superoxide (Figure 5) in leaf tissues, as indicated by DAB and NBT staining, and these ROS were over accumulated in ML. Interestingly, the respective references of each treatment– approximately 30 µmol m^{-2} s^{-1} for *in vitro* and for *ex vitro* pots consisting of an oscillating light with a maximum of 230 µmol m^{-2} s^{-1} at noon of a typical day, accumulate low ROS levels (Figure 4 and 5). These qualitative results were corroborated by chemical analysis in both light intensities (Figure 6A-B) and closely correlated with lipid peroxidation (TBARS levels; Figure 6C) and membrane damage (Figure 6D). The higher oxidative stress in *in vitro* plantlets was related to lower SOD activity but, unexpectedly, these phenotypes displayed higher CAT and APX activities compared to *ex vitro* in all treatments (Figure 7). Despite this apparent improved performance at LL, *in vitro* leaves exhibited a strong decrease in these activities as light intensity was increased, whereas in *ex vitro* plants they decreased slightly (Figure 7).

In parallel to the main antioxidant enzymes, the current study also addressed the non-enzymatic antioxidants ascorbate and glutathione redox states. *In vitro* plantlets exhibited 30% lower total ascorbate accumulation in response to ML compared to the *ex vitro* plants (Figure 8A). In addition, *in vitro* plantlets exhibited also a decreased percentage of ASC in the reduced form under ML conditions (76%), as compared to *ex vitro* plantlets (90%) (Figure 8A). Similarly, total glutathione accumulation was also stimulated in response to light intensity, reaching slightly higher values in *ex vitro* plantlets (200 ηmol g^{-1} FW) compared to *in vitro* plantlets (Figure 8B). However, differently from the ascorbate response, there were no differences between the glutathione redox states of *ex vitro* and *in vitro* plantlets at any studied light regime (Figure 8B), indicating that ASC synthesis and its redox state were more important for oxidative protection than GSH during the *ex vitro* acclimation.

Ex vitro and *in vitro* plantlets exhibited contrasting correlation network and principal component analysis (PCA) in response to increasing light

In order to reach a deeper and more integrative understanding of the contrasting photosynthetic and redox

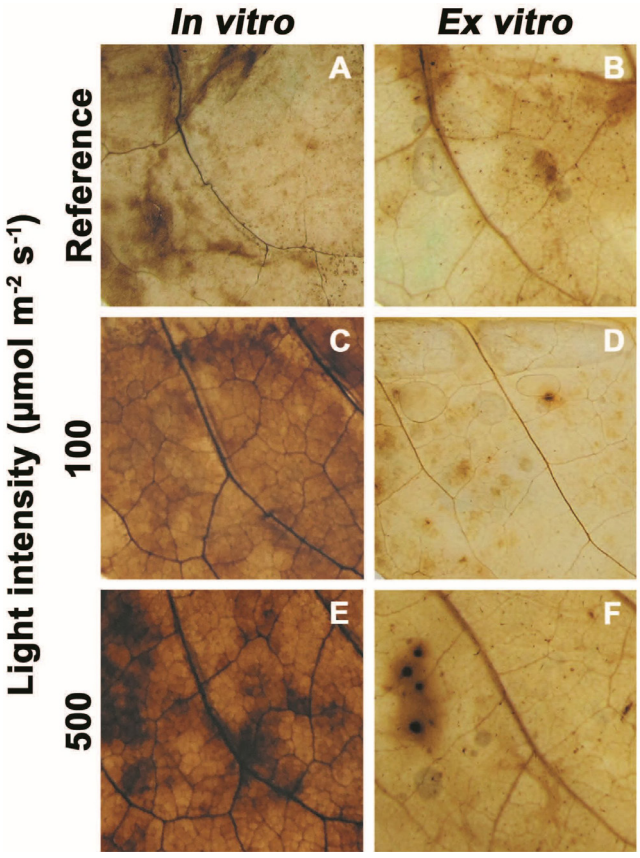

Figure 4. *In situ* hydrogen peroxide DAB staining in leaves. Assays were performed in leaves from plants grown under *in vitro* and *ex vitro* conditions. Reference seedlings (A and B) consist of leaves collected immediately before the light treatments (i.e. from the previous growth conditions *in vitro* and *ex vitro* , respectively). Subsequently, plants were acclimated to low light (C and D; LL; 100 µmol photons m^{-2} s^{-1}) and moderate light (E and F; ML; 500 µmol photons m^{-2} s^{-1}) conditions for 12 hours. Pictures are the most representatives of five independent replicates.

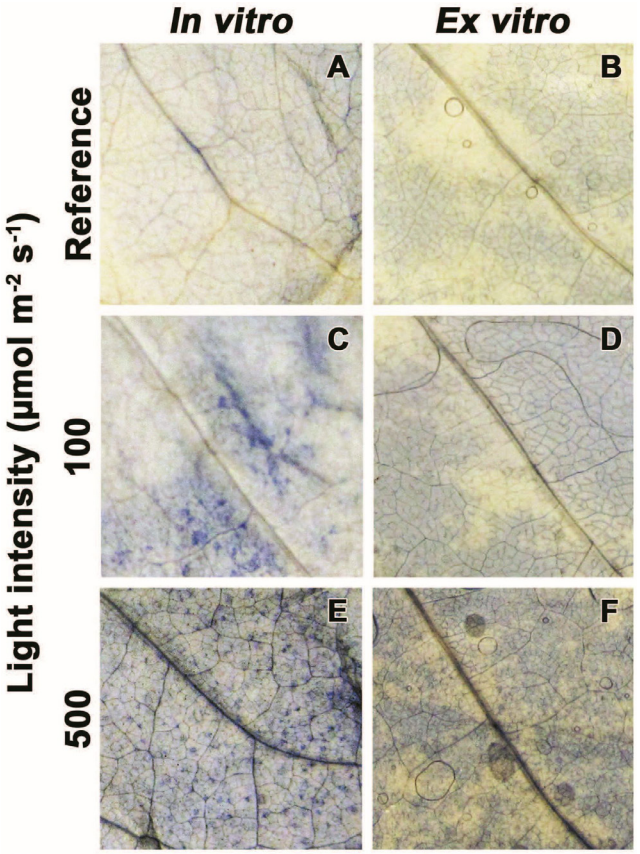

Figure 5. *In situ* radical superoxide NBT staining in leaves. Assays were performed in leaves from plants grown under *in vitro* conditions for 30 days and plants grown in *ex vitro* conditions. Reference seedlings (A and B) consist of leaves collected immediately before the light treatments (i.e. from the previous growth conditions *in vitro* and *ex vitro* , respectively). Subsequently, plants were acclimated to low light (C and D; LL; 100 µmol photons m^{-2} s^{-1}) and moderate light (E and F; ML; 500 µmol photons m^{-2} s^{-1}) for 12 hours. Pictures are the most representatives of five independent replicates.

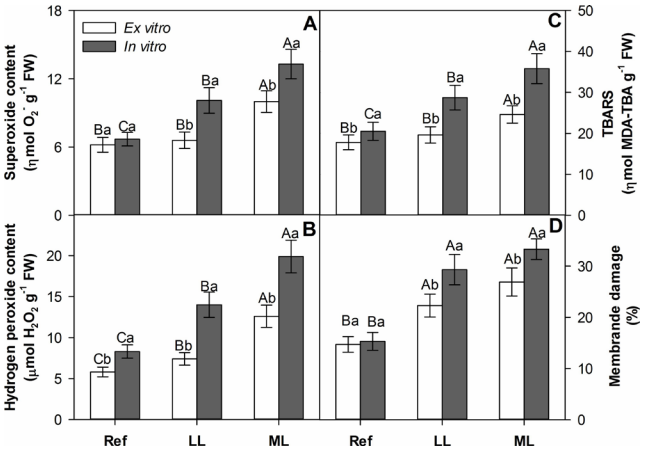

Figure 6. Quantitative indicators of oxidative stress. (A) Radical ion superoxide content, (B) hydrogen peroxide content, (C) TBARS content, and (D) membrane damage were performed in leaves from plants grown under *in vitro* and *ex vitro* conditions. Reference seedlings (A and B) consist of leaves collected immediately before the light treatments (i.e. from the previous growth conditions *in vitro* and *ex vitro* , respectively). Subsequently, plants were acclimated to low light conditions (LL; 100 μmol photons m^{-2} s^{-1}) and moderate light regimes (ML; 500 μmol photons m^{-2} s^{-1}) for 12 hours. Different capital letters mean significant difference ($p < 0.05$) among different light regimes and different lowercase letters represent significant difference between *in vitro* and *ex vitro* growth conditions.

responses exhibited by *in vitro* and *ex vitro* plants as a result of different light regime conditions, a Principal Component Analysis (PCA) was performed followed by a Pearson's correlation study and a network analysis. The PCA indicated a very high contrast between *in vitro* and *ex vitro* conditions, especially for the LL regime (Figure S4). The network analysis reinforced these results and adds evidence that *in vitro* and *ex vitro* conditions can induce a prominent adjustment in network topography (Figure 9). *Ex vitro* -related networks displayed a high contrast in the network diameter, which was 50% higher in these plantlets compared to in vitro ones. In contrast, *in vitro* -related networks displayed a greater network density, reaching values 35% higher than those exhibited by *ex vitro* -related networks. Other important parameters, such as clustering coefficient, network centrality and heterogeneity, and global conectance did not show any prominent difference in the current analysis.

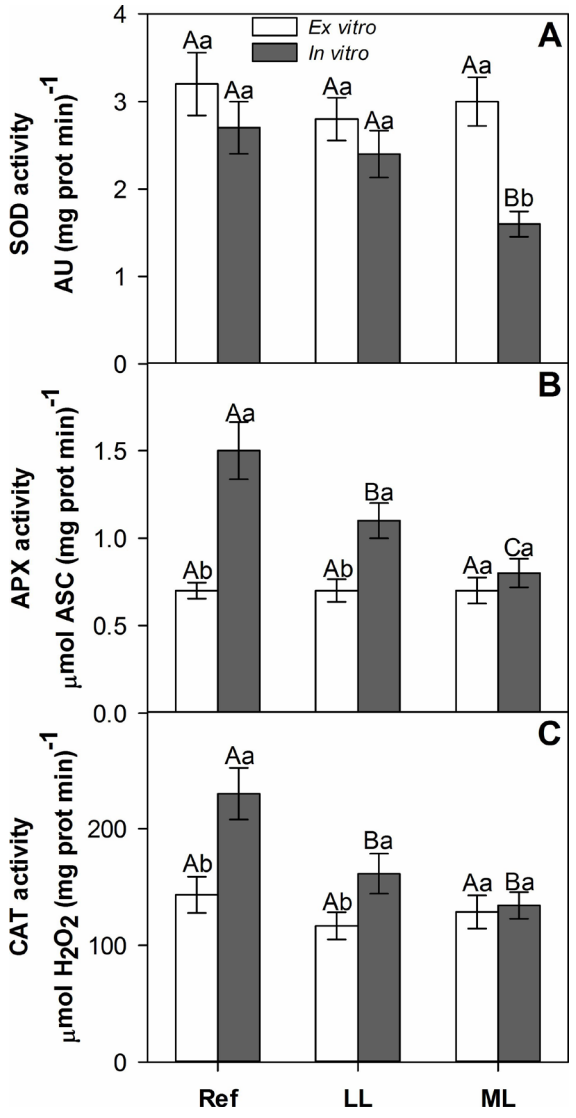

Figure 7. Enzymatic antioxidant activities. Activities of (A) superoxide dismutase, (B) ascorbate peroxidase and (C) catalase in leaves from plants grown under *in vitro* and *ex vitro* conditions. Reference seedlings consist of leaves collected immediately before the light treatments (i.e. from the previous growth conditions *in vitro* and *ex vitro* , respectively). Subsequently, plants were acclimated to low light conditions (LL; 100 μmol photons m^{-2} s^{-1}) and moderate light regimes (ML; 500 μmol photons m^{-2} s^{-1}) for 12 hours. Different capital letters mean significant difference ($p < 0.05$) among different light regimes and different lowercase letters represent significant difference between *in vitro* and *ex vitro* growth conditions.

Discussion

Overall, the results obtained in this study corroborate other works evidencing that tobacco and some native species grown under *in vitro* environment present a malformed photosynthetic apparatus (Pospíšilová et al. 2009; Zobayed et al., 2001; Rival et al., 1997; Georgieva et al., 1996). These morpho-physiological alterations involve several organization levels such as malformation in stomata anatomy, photochemical activity and CO_2 assimilation reactions by Calvin-Benson cycle (Rival et al. 1997; Sáez et al. 2012; Semorádová et al., 2002). As a consequence, *in vitro* plantlets are not able to cope directly with moderate light intensity over a 12-h photoperiod. In this circumstance, it is important to highlight the fast and drastic decrease in CO_2 assimilation associated with no induction in NPQ, which represents an effective photoprotective component. This adverse condition is aggravated by an inefficient antioxidant system, as indicated by over-accumulation of ROS in leaves even when plantlets were exposed to a relative low light intensity like 200 μmol photons m^{-2} s^{-1} (König et al., 2018). Interestingly, the CO_2 assimilation impairment observed in *in vitro* plantlets was

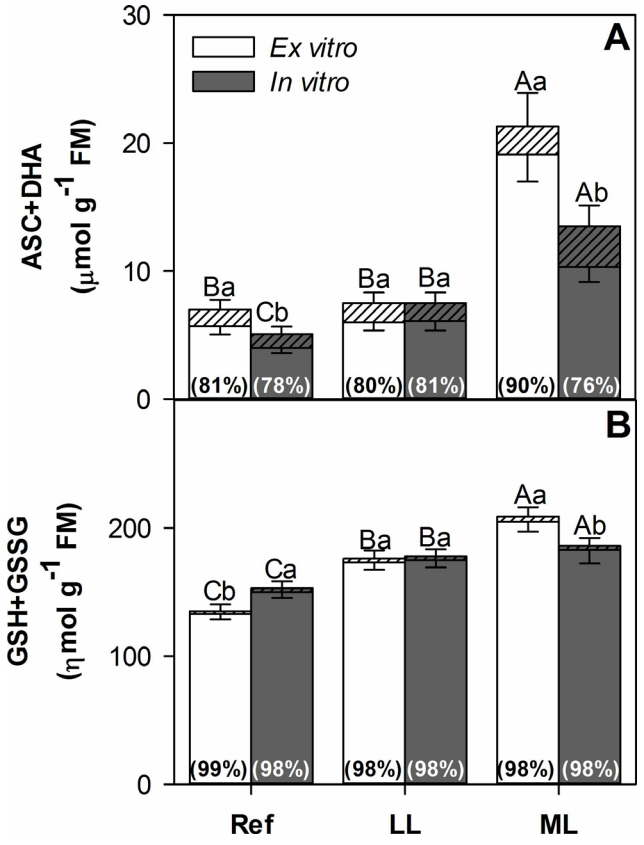

Figure 8. Non-enzymatic antioxidant contents. Contents and redox balance of (A) ascorbate and (B) glutathione were evaluated in leaves from plants grown under *in vitro* and *ex vitro* conditions. Reference seedlings consist of leaves collected immediately before the light treatments (i.e. from the previous growth conditions *in vitro* and *ex vitro* , respectively). Subsequently, plants were acclimated to low light conditions (LL; 100 μmol photons m^{-2} s^{-1}) and moderate light regimes (ML; 500 μmol photons m^{-2} s^{-1}) for 12 hours. Hatched bars represent the oxidized form of ascorbate and glutathione (DHA and GSSG) and non-hatched bars mean the reduced form of these antioxidants (ASC and GSH). Different capital letters mean significant difference ($p < 0.05$) among different light regimes and different lowercase letters represent significant difference between *in vitro* and *ex vitro* growth conditions.

associated with biochemical limitations, and not with lower CO_2 availability, since the internal concentration of CO_2 and the stomatal conductance were aberrantly higher in these plantlets compared the ones grown in *ex vitro* conditions.

Our results evidence that *ex vitro* plants experienced effective and favorable morpho-physiological changes in their photosynthetic apparatus capable to induce acclimation to increasing light intensity. These positive adjustments were clearly observed when these plantlets were compared with seed-grown plants in a greenhouse (data not shown). Nevertheless, these data indicate that the artificial conditions commonly employed by *in vitro* micropropagation, especially represented by very low light intensities associated with other unusual growth factors, are able to delay acclimatization to oscillating environmental stimuli such as those that are widespread in field (Sáez et al. 2013; Cardoso et al., 2013; Sáez et al. 2012; Kadleček et al., 2001). Indeed, besides displaying an incipient redox protective system, *in vitro* grown plants exhibit an embryonic photosynthetic apparatus, as evidenced by both gas exchange and photochemistry data (Figures 1, 2 and 3) which is inefficient in supporting proper photosynthesis and in avoiding water loss as a consequence of a malformed stomatal system (Apóstolo et al., 2005). These results suggest that *ex vitro* acclimation in order to reach maximum photosynthetic efficiency is a complex, gradual and slow process.

Importantly, this study reveals that progressive increment in light intensity *in planta* (from 200 to 1,000 μmol photons m^{-2} s^{-1}), for five consecutive days, is able to improve photosynthetic acclimation in *ex vitro* plants, favoring gas exchange as well as photochemical activity. Indeed, CO_2 assimilation and NPQ of *ex vitro* plantlets were remarkably enhanced during the increasing light conditions, which probably provided protection against photoinhibition, as indicated by unchanged Fv/Fm values. Moreover, the greatly stimulated NPQ in *ex vitro* plants probably contributed to avoid ROS accumulation (Carvalho et al., 2015; Foyer et al., 2017). In opposition, as *in vitro* plantlets were previously acclimated to very low light conditions and as the molecular machinery related to NPQ induction (such as ΔpH generation, PsbS protein expression and xanthophyll cycle components) under such conditions can be undersized, this imbalance could explain the reduced capacity of NPQ induction observed during the illumination phase (Ruban 2015).

Photochemical reactions have a great potential to generate excess energy in chloroplasts, particularly if they are not closely coordinated with the demand of photosynthetic electron sink processes, mainly represented by CO_2 assimilation and photorespiration (Gollan et al., 2017; Sousa et al., 2019; Lima-Melo et al., 2019b). Our data show that *ex vitro* and *in vitro* plants displayed different levels of oxidative stress among the treatments. Although acclimated to a light intensity higher than 100 μmol photons m^{-2} s^{-1}, *ex vitro* plants displayed some level of oxidative stress also after the low light treatment, showing that acclimation clearly has a cost even if adjusting to lower light intensities. This result was unexpected considering that photooxidative stress is usually associated to conditions of excessive light intensities (Li et al., 2018; Roach & Krieger-Liszkay, 2019). However, several works have shown that short-term low light can also induce oxidative stress, as measured by different indicators (Yu et al., 2016; Li et al., 2017; Zhu et al., 2017), although the molecular mechanisms involved in this specific condition is not as well understood as for high light. Our data also show that *in vitro* leaves displayed much higher ROS accumulation than *ex vitro* leaves for all treatments, and this response was correlated with progressive decrease in SOD, CAT and APX activities. Possibly, these responses were due to low capacity to up-regulate gene expression for these targeted proteins under a stressful condition like excess light. Despite *ex vitro* plants did not display strong oxidative stress even in response to ML, they also did not present increased activity of the evaluated antioxidant enzymes.

These results suggest that the decrease in the antioxidant activities were crucial for ROS over-accumulation in *in vitro* plants while both low production of ROS from photochemical reactions and the antioxidant systems were important

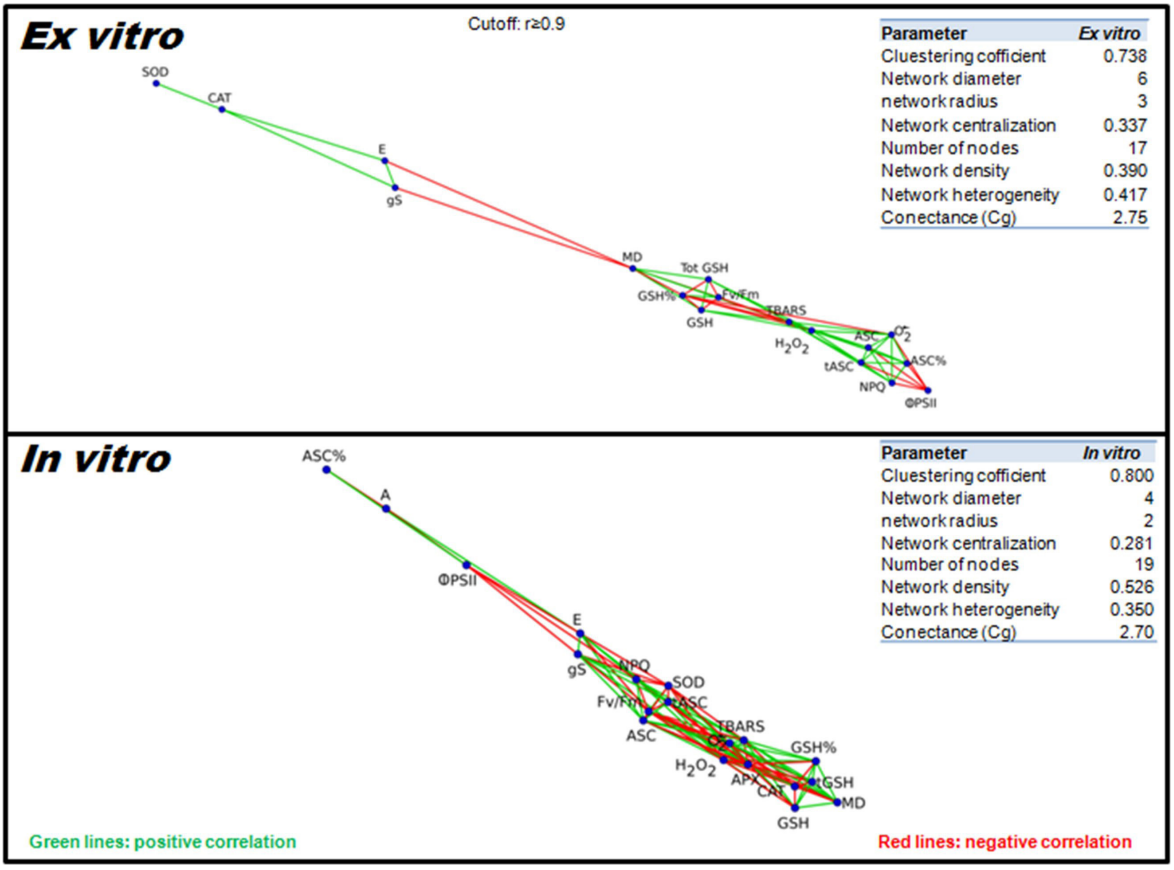

Figure 9. Correlation network analysis. Pearson's correlation network of photosynthetic and redox metabolism parameters measured in plants grown under *in vitro* conditions for 30 days and plants grown in *ex vitro* conditions. All the parameters were first normalized according to respective reference treatments. Red lines represent negative correlation with r < -0.9 and green lines represent correlation with r > 0.0 according to Pearson´s correlation coefficient (r). The network analysis parameters are described in the box.

for *ex vitro* acclimatization. In addition to the apparent importance of the antioxidant enzymes, the ascorbate synthesis and the maintenance of its high redox state were also important during *ex vitro* acclimation to high light. Interestingly, the glutathione pool was apparently less important to both *in vitro* and *ex vitro* acclimatization despite some reports employing tobacco seed-grown plants have evidenced that this antioxidant is important for oxidative protection against abiotic stress (König et al., 2018). Thus, the avoidance of oxidative stress during *ex vitro* acclimation could require integrate mechanisms involving high photosynthetic efficiency associated with effective heat dissipation via NPQ (Sáez et al., 2013), high CO_2 assimilation rates (Kapchina-Toteva et al. 2014) and other energy dissipative sinks such as water-water cycle (Perveen et al., 2013) and possibly photorespiration.

Generation of transgenic plants as research models and for genetic engineering for agricultural biotechnology essentially employs *in vitro* micropropagated plants, including transformation to enhance photosynthesis by improvement of energy transference from NPQ to CO_2 assimilation as has been reported for tobacco plants (Kromdijk et al. 2016). Several alterations in gene expression probably occur during *in vitro* and *ex vitro* acclimatization (Li et al., 2019; Wickramasuriya & Dunwell, 2015). Thus, the establishment of suitable techniques for a faster and full photosynthetic acclimatization is crucial to carry out confident transgenic transformations and to avoid unexpected side-effects related to acclimatization process. Our data reinforce that is plausible to handle light acclimatization focusing increasing light intensities by monitoring of photosynthetic and redox metabolism in order to induce an effective plant endurance.

The network analyses contributed for the understanding of some aspects involved with photosynthetic efficiency and redox protection during *in vitro* and *ex vitro* acclimatization. In fact, *ex vitro* pre-acclimation has induced plants to trigger various photoprotective mechanisms, especially related to NPQ induction, PSII quantum efficiency and stomatal regulation. Accordingly, the correlation networks revealed large topographic contrasts between these different plantlets. The parameters "diameter" and "density" of two contrasting nets suggest that *ex vitro* plants show less strict correlations between the parameters analyzed, despite the average correlation (conectance) have not differed between the networks. This may reflect the fact that *ex vitro* plantlets have a more robust photosynthetic machinery, probably shaping their phenotypic plasticity for an effective homeostasis maintenance under increasing light conditions, which can lead to lower ROS accumulation and, thus, not requiring the induction of a complex antioxidant system.

On the other hand, the similarity between important parameters, such as "clustering coefficient" and "network

centrality" might indicate no increase in the number of hubs in response to contrasting *in vitro* and *ex vitro* conditions. The centrality indicates the intensity of occurrence of relevant hubs, which may reflect an importance degree for a node. The absence of changes in this parameter may indicate that both *in vitro* and *ex vitro* plantlets have adjusted their systems to specific environments without privileging any particular parameter. Obviously, the network studied in the present work consists of only 23 nodes, which allow a number of missing variables, not considered here. Therefore, the use of integrative analyzes such as this presented here but with higher amount of data has a great potential to answer complex questions such as how *ex vitro* plantlets become better acclimated to field conditions.

In conclusion, acclimation of *in vitro* tobacco leaves and their shift to *ex vitro* conditions involves several morphogenetic, molecular and physiological changes associated with photosynthesis improvement and oxidative protection. In parallel, CO_2 assimilation is improved by improvements in biochemical CO_2 assimilation cycle reactions together with regulation of quantum efficiency of PSII associated with effective excess energy dissipation via NPQ. These features, in parallel with gene expression of antioxidant components represented by SOD, APX, and CAT, as well as the maintenance of the ascorbate redox state, are crucial to avoid oxidative stress, protecting chloroplast components and allowing high photosynthetic activity during acclimation period. Together, these mechanisms might allow a virtuous cycle, favoring *ex vitro* acclimatization of tobacco plants.

Author's contributions

CFV performed all the experiments, data analyses and was involved in manuscript writing. FELC contributed in data interpretation and manuscript writing. YLM contributed in manuscript writing and figure edition. CPSC collaborated to experimental design and supervised micropropagation experiments. MCLN collaborated to experimental conduction and chlorophyll *a* data analysis. MOM cooperated with gas exchange determinations and photosynthesis data analysis. JAGS designed all the experiments, supervised the experimental conduction, contributed in data interpretation and was involved in manuscript writing.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES), INCT Plant Stress Biotech (Conselho de Desenvolvimento Científico e Tecnológico) Proc. 465480/2014-4 and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) for funding. FELC is supported by FUNCAP/CAPES (Bolsista CAPES/BRASIL – Proc. 88887.162856/2018-00).

Abbreviations

APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; Ci, intercellular CO_2 partial pressure; DAB, 3,3'-diaminobenzidine; DHA, dehydroascorbate; *E*, leaf transpiration; EDTA, ethylenediamine tetraacetic acid; ETR, actual electron transport rate from PSII; Fm, maximum fluorescence in dark-adapted leaves; Fo, minimum fluorescence in dark-adapted leaves; F_v/F_m, maximum quantum yield of PSII; FW, fresh weight; g_s, stomatal conductance; GSH, glutathione; GSSG, glutathione disulfide; IRGA, infrared gas analyzer; LL, low light; MD, membrane damage; MDA, malondialdehyde; ML, moderate light; NBT, nitroblue tetrazolium; NPQ, non-photochemical quenching; PCA, principal component analysis; P_N max, maximum net photosynthesis; P_N, CO_2 assimilation rate (net photosynthesis); PPFD, photosynthetic photon flux density; PSII, photosystem II; Ref, reference; Rn, dark respiration; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; α, photosynthetic quantum efficiency; Γ*, light compensation point; ΦPSII, effective quantum yield of PSII.

References

Adams 3rd, W. W., Muller, O., Cohu, C. M., & Demmig-Adams, B. (2013). May photoinhibition be a consequence, rather than a cause, of limited plant productivity? *Photosynthesis Research*, *117*(1-3), 31-44. http://dx.doi.org/10.1007/s11120-013-9849-7. PMid:23695654.

Alvarez, C., Sáez, P., Sáez, K., Sánchez-Olate, M., & Ríos, D. (2012). Effects of light and ventilation on physiological parameters during in vitro acclimatization of *Gevuina avellana* mol. *Plant Cell, Tissue and Organ Culture*, *110*(1), 93-101. http://dx.doi.org/10.1007/s11240-012-0133-x.

Amako, K., Chen, G.-X., & Asada, K. (1994). Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. *Plant & Cell Physiology*, *35*, 497-504.

Apóstolo, N. M., Brutti, C. B., & Llorente, B. E. (2005). Leaf anatomy of *Cynara scolymus* L. in succesive micropropagation stages. *In vitro Cellular & Developmental Biology. Plant*, *41*(3), 307-313. http://dx.doi.org/10.1079/IVP2004606.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology*, *141*(2), 391-396. http://dx.doi.org/10.1104/pp.106.082040. PMid:16760493.

Barry-Etienne, D., Bertrand, B., Vasquez, N., & Etienne, H. (2002). Comparison of somatic embryogenesis-derived coffee (*Coffea arabica* L.) plantlets regenerated *in vitro* or *ex vitro* : Morphological, mineral and water characteristics. *Annals of Botany*, *90*(1), 77-85. http://dx.doi.org/10.1093/aob/mcf149. PMid:12125775.

Beauchamp, C., & Fridovich, I. (1971). Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry*, *44*(1), 276-287. http://dx.doi.org/10.1016/0003-2697(71)90370-8. PMid:4943714.

Blum, A., & Ebercon, A. (1981). Cell membrane stability as a measure of drought and heat tolerance in wheat. *Crop Science*, *21*(1), 43-47. http://dx.doi.org/10.2135/cropsci1981.0011183X002100010013x.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1-2), 248-254. http://dx.doi.org/10.1016/0003-2697(76)90527-3. PMid:942051.

Cakmak, I., & Horst, W. J. (1991). Effect of aluminium on net efflux of nitrate and potassium from root tips of soybean (*Glycine max* L.). *Journal of Plant Physiology*, *138*(4), 400-403. http://dx.doi.org/10.1016/S0176-1617(11)80513-4.

Cardoso, J. C., Rossi, M. L., Rosalem, I. B., & Teixeira da Silva, J. A. (2013). Pre-acclimatization in the greenhouse: An alternative to optimizing the micropropagation of gerbera. *Scientia Horticulturae*, *164*, 616-624. http://dx.doi.org/10.1016/j.scienta.2013.10.022.

Carvalho, F. E. L., Ware, M. A., & Ruban, A. V. (2015). Quantifying the dynamics of light tolerance in arabidopsis plants during Ontogenesis. *Plant, Cell & Environment*, *38*(12), 2603-2617. http://dx.doi.org/10.1111/pce.12574. PMid:26012511.

Carvalho, L. C., Vilela, B. J., Vidigal, P., Mullineaux, P. M., & Amâncio, S. (2006). Activation of the ascorbate-glutathione cycle is an early response of micropropagated *Vitis vinifera* L. explants transferred to ex vitro . *International Journal of Plant*, *167*(4), 759-770. http://dx.doi.org/10.1086/503919.

Chandra, S., Bandopadhyay, R., Kumar, V., & Chandra, R. (2010). Acclimatization of tissue cultured plantlets: From laboratory to land. *Biotechnology Letters*, *32*(9), 1199-1205. http://dx.doi.org/10.1007/s10529-010-0290-0. PMid:20455074.

Daloso, D. M., Medeiros, D. B., Anjos, L., Yoshida, T., Araújo, W. L., & Fernie, A. R. (2017). Metabolism within the specialized guard cells of plants. *The New Phytologist*, *216*(4), 1018-1033. http://dx.doi.org/10.1111/nph.14823. PMid:28984366.

Dias, M. C., Pinto, G., & Santos, C. (2011). Acclimatization of micropropagated plantlets induces an antioxidative burst: A case study with *Ulmus minor* Mill. *Photosynthetica*, *49*(2), 259-266. http://dx.doi.org/10.1007/s11099-011-0028-9.

Dietz, K.-J. (2014). Redox regulation of transcription factors in plant stress acclimation and development. *Antioxidants & Redox Signaling*, *21*(9), 1356-1372. http://dx.doi.org/10.1089/ars.2013.5672. PMid:24182193.

Elstner, E. F., & Heupel, A. (1976). Inhibition of nitrite formation from hydroxylammoniumchloride: A simple assay for superoxide dismutase. *Analytical Biochemistry*, *70*(2), 616-620. http://dx.doi.org/10.1016/0003-2697(76)90488-7. PMid:817618.

Exposito-Rodriguez, M., Laissue, P. P., Yvon-Durocher, G., Smirnoff, N., & Mullineaux, P. M. (2017). Photosynthesis-dependent H2O2 transfer from chloroplasts to nuclei provides a high-light signalling mechanism. *Nature Communications*, *8*(1), 49. http://dx.doi.org/10.1038/s41467-017-00074-w. PMid:28663550.

Flexas, J., Ribas-Carbó, M., Diaz-Espejo, A., Galmés, J., & Medrano, H. (2008). Mesophyll conductance to CO2: Current knowledge and future prospects. *Plant, Cell & Environment*, *31*(5), 602-621. http://dx.doi.org/10.1111/j.1365-3040.2007.01757.x. PMid:17996013.

Foyer, C. H. (2018). Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. *Environmental and Experimental Botany*, *154*, 134-142. http://dx.doi.org/10.1016/j.envexpbot.2018.05.003. PMid:30283160.

Foyer, C. H., Neukermans, J., Queval, G., Noctor, G., & Harbinson, J. (2012). Photosynthetic control of electron transport and the regulation of gene expression. *Journal of Experimental Botany*, *63*(4), 1637-1661. http://dx.doi.org/10.1093/jxb/ers013. PMid:22371324.

Foyer, C. H., Ruban, A. V., & Noctor, G. (2017). Viewing oxidative stress through the lens of oxidative signalling rather than damage. *The Biochemical Journal*, *474*(6), 877-883. http://dx.doi.org/10.1042/BCJ20160814. PMid:28270560.

Genty, B., Briantais, J.-M. M., & Baker, N. R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta. G, General Subjects*, *990*(1), 87-92. http://dx.doi.org/10.1016/S0304-4165(89)80016-9.

Georgieva, K., Yordanov, I., & Kroumova, A. (1996). Photosynthetic characteristics of transformed tobacco plants grown in vitro after their transplantation in natural conditions. *Bulgarian Journal of Plant Physiology*, *22*, 3-13.

Giannopolitis, C. N., & Ries, S. K. (1977). Superoxide dismutases: II. purification and quantitative relationship with water-soluble protein in seedlings. *Plant Physiology*, *59*(2), 315-318. http://dx.doi.org/10.1104/pp.59.2.315. PMid:16659840.

Gollan, P. J., Lima-Melo, Y., Tiwari, A., Tikkanen, M., & Aro, E.-M. (2017). Interaction between photosynthetic electron transport and chloroplast sinks triggers protection and signalling important for plant productivity. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *372*(1730), 20160390. http://dx.doi.org/10.1098/rstb.2016.0390. PMid:28808104.

Guan, Q. Z., Guo, Y. H., Sui, X. L., Li, W., & Zhang, Z. X. (2008). Changes in photosynthetic capacity and antioxidant enzymatic systems in micropropagated *Zingiber officinale* plantlets during their acclimation. *Photosynthetica*, *46*(2), 193-201. http://dx.doi.org/10.1007/s11099-008-0031-y.

Havir, E. A., & McHale, N. A. (1987). Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. *Plant Physiology*, *84*(2), 450-455. http://dx.doi.org/10.1104/pp.84.2.450. PMid:16665461.

Jimbo, H., Yutthanasirikul, R., Nagano, T., Hisabori, T., Hihara, Y., & Nishiyama, Y. (2018). Oxidation of translation factor EF-Tu inhibits the repair of photosystem II. *Plant Physiology*, *176*(4), 2691-2699. http://dx.doi.org/10.1104/pp.18.00037. PMid:29439212.

Kadleček, P., Tichá, I., Haisel, D., Čapková, V., & Schäfer, C. (2001). Importance of *in vitro* pretreatment for *ex vitro* acclimatization and growth. *Plant Science*, *161*(4), 695-701. http://dx.doi.org/10.1016/S0168-9452(01)00456-3.

Kamada, T., & Kawai, S. (1989). An algorithm for drawing general undirected graphs. *Information Processing Letters*, *31*(1), 7-15. http://dx.doi.org/10.1016/0020-0190(89)90102-6.

Kapchina-Toteva, V., Dimitrova, M. A., Stefanova, M., Koleva, D., Kostov, K., Yordanova, ZhP., Stefanov, D., & Zhiponova, M. K. (2014). Adaptive changes in photosynthetic performance and secondary metabolites during white dead nettle micropropagation. *Journal of Plant Physiology*, *171*(15), 1344-1353. http://dx.doi.org/10.1016/j.jplph.2014.05.010. PMid:25046755.

König, K., Vaseghi, M. J., Dreyer, A., & Dietz, K.-J. (2018). The significance of glutathione and ascorbate in modulating the retrograde high light response in *Arabidopsis thaliana* leaves. *Physiologia Plantarum*, *162*(3), 262-273. http://dx.doi.org/10.1111/ppl.12644. PMid:28984358.

Kromdijk, J., Głowacka, K., Leonelli, L., Gabilly, S. T., Iwai, M., Niyogi, K. K., & Long, S. P. (2016). Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. *Science*, *354*(6314), 857-861. http://dx.doi.org/10.1126/science.aai8878. PMid:27856901.

Lando, A. P., Wolfart, M. R., Fermino, P. C. P. P., & Santos, M. (2016). Structural effects on *Cattleya xanthina* leaves cultivated in vitro and acclimatized *ex vitro* . *Biologia Plantarum*, *60*(2), 219-225. http://dx.doi.org/10.1007/s10535-016-0589-3.

Li, J., Wang, M., Li, Y., Zhang, Q., Lindsey, K., Daniell, H., Jin, S., & Zhang, X. (2019). Multi-omics analyses reveal epigenomics basis for cotton somatic embryogenesis through successive regeneration acclimation process. *Plant Biotechnology Journal*, *17*(2), 435-450. http://dx.doi.org/10.1111/pbi.12988. PMid:29999579.

Li, L., Aro, E. M., & Millar, A. H. (2018). Mechanisms of photodamage and protein turnover in photoinhibition. *Trends in Plant Science*, *23*(8), 667-676. http://dx.doi.org/10.1016/j.tplants.2018.05.004. PMid:29887276.

Li, Y., Fan, Y., Ma, Y., Zhang, Z., Yue, H., Wang, L., Li, J., & Jiao, Y. (2017). Effects of exogenous γ-aminobutyric acid (GABA) on photosynthesis and antioxidant system in pepper (*Capsicum annuum* L.) seedlings under low light stress. *Journal of Plant Growth Regulation*, *36*(2), 436-449. http://dx.doi.org/10.1007/s00344-016-9652-8.

Li, Z., Wakao, S., Fischer, B. B., & Niyogi, K. K. (2009). Sensing and responding to excess light. *Annual Review of Plant Biology*, *60*(1), 239-260. http://dx.doi.org/10.1146/annurev.arplant.58.032806.103844. PMid:19575582.

Lichtenthaler, H., & Wellburn, A. (1983). Determinations of total carotenoids and chlorophylls *b* of leaf extracts in different solvents. *Biochemical Society Transactions*, *11*(5), 591-592. http://dx.doi.org/10.1042/bst0110591.

Lieth, J. H., & Reynolds, J. F. (1987). The nonrectangular hyperbola as a photosynthetic light response model: Geometrical interpretation and estimation of the parameter - θ. *Photosynthetica*, *21*, 363-366.

Lima-Melo, Y., Alencar, V. T. C. B., Lobo, A. K. M., Sousa, R. H. V., Tikkanen, M., Aro, E.-M., Silveira, J. A. G., & Gollan, P. J. (2019b). Photoinhibition of photosystem I provides oxidative protection during imbalanced photosynthetic electron transport in Arabidopsis thaliana. *Frontiers of Plant Science*, *10*, 916. http://dx.doi.org/10.3389/fpls.2019.00916. PMid:31354779.

Lima-Melo, Y., Gollan, P. J., Tikkanen, M., Silveira, J. A. G., & Aro, E. M. (2019a). Consequences of photosystem-I damage and repair on photosynthesis and carbon use in *Arabidopsis thaliana*. *The Plant Journal*, *97*(6), 1061-1072. http://dx.doi.org/10.1111/tpj.14177. PMid:30488561.

Monja-Mio, K. M., Pool, F. B., Herrera, G. H., EsquedaValle, M., & Robert, M. L. (2015). Development of the stomatal complex and leaf surface of *Agave angustifolia* haw. 'Bacanora' plantlets during the *in vitro* to *ex vitro* transition process. *Scientia Horticulturae*, *189*, 32-40. http://dx.doi.org/10.1016/j.scienta.2015.03.032.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, *15*(3), 473-497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Nakano, Y., & Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. *Plant & Cell Physiology*, *22*, 867-880.

Perveen, S., & Anis, M. (2015). Physiological and biochemical parameters influencing ex vitro establishment of the in vitro regenerants of *Albizia lebbeck* (L.) Benth.: An important soil reclaiming plantation tree. *Agroforestry Systems*, *89*(4), 721-733. http://dx.doi.org/10.1007/s10457-015-9809-7.

Perveen, S., Anis, M., & Aref, I. M. (2013a). Lipid peroxidation, H2O2 content, and antioxidants during acclimatization of *Abrus precatorius* to *ex vitro* conditions. *Biologia Plantarum*, *57*(3), 417-424. http://dx.doi.org/10.1007/s10535-013-0328-y.

Perveen, S., Javed, S. B., Anis, M., & Aref, I. M. (2013b). Rapid *in vitro* multiplication and *ex vitro* establishment of caribbean copper plant (*Euphorbia cotinifolia* L.): An important medicinal shrub. *Acta Physiologiae Plantarum*, *35*(12), 3391-3400. http://dx.doi.org/10.1007/s11738-013-1374-y.

Pospíšilová, J. H., Synková, H., Haisel, D., & Baťková, P. (2009). Effect of abscisic acid on photosynthetic parameters during *ex vitro* transfer of micropropagated tobacco plantlets. *Biologia Plantarum*, *53*(1), 11-20. http://dx.doi.org/10.1007/s10535-009-0003-5.

Rival, A., Beule, T., Lavergne, D., Nato, A., Havaux, M., & Puard, M. (1997). Development of photosynthetic characteristics in oil palm during *in vitro* micropropagation. *Journal of Plant Physiology*, *150*(5), 520-527. http://dx.doi.org/10.1016/S0176-1617(97)80314-8.

Roach, T., & Krieger-Liszkay, A. (2019). Photosynthetic regulatory mechanisms for efficiency and prevention of photo-oxidative stress. *Annual Plant Reviews*, *2*(1), 1-34. http://dx.doi.org/10.1002/9781119312994.apr0666.

Ruban, A. V. (2015). Evolution under the sun: Optimizing light harvesting in photosynthesis. *Journal of Experimental Botany*, *66*(1), 7-23. http://dx.doi.org/10.1093/jxb/eru400. PMid:25336689.

Sáez, P. L., Bravo, L. A., Latsague, M. I., Sánchez, M. E., & Ríos, D. G. (2012). Increased light intensity during *in vitro* culture improves water loss control and photosynthetic performance of *Castanea sativa* grown in ventilated vessels. *Scientia Horticulturae*, *138*, 7-16. http://dx.doi.org/10.1016/j.scienta.2012.02.005.

Sáez, P. L., Bravo, L. A., Latsague, M. I., Toneatti, M. J., Coopman, R. E., Álvarez, C. E., Sánchez-Olate, M., & Ríos, D. G. (2015). Influence of *in vitro* growth conditions on the photosynthesis and survival of *Castanea sativa* plantlets during *ex vitro* transfer. *Plant Growth Regulation*, *75*(3), 625-639. http://dx.doi.org/10.1007/s10725-014-9965-1.

Sáez, P. L., Bravo, L. A., Latsague, M. I., Toneatti, M. J., Sánchez-Olate, M., & Ríos, D. G. (2013). Light energy management in micropropagated plants of *Castanea sativa*, effects of photoinhibition. *Plant Science*, *201-202*, 12-24. http://dx.doi.org/10.1016/j.plantsci.2012.11.008. PMid:23352399.

Schreiber, U., Bilger, W., & Neubauer, C. (1995). Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of *in vivo* photosynthesis. In E. D. Schulze & M. M. Caldwell (Eds.), *Ecophysiology of Photosynthesis* (pp. 49-70). Springer. http://dx.doi.org/10.1007/978-3-642-79354-7_3.

Semorádová, Š., Synková, H., & Pospíšilová, J. (2002). Responses of tobacco plantlets to change of irradiance during transfer from *in vitro* to *ex vitro* conditions. *Photosynthetica*, *40*(4), 605-614. http://dx.doi.org/10.1023/A:1024316405903.

Silveira, J. A. G., & Carvalho, F. E. L. (2016). Proteomics, photosynthesis and salt resistance in crops: An integrative view. *Journal of Proteomics*, *143*, 24-35. http://dx.doi.org/10.1016/j.jprot.2016.03.013. PMid:26957143.

Sousa, R. H. V., Carvalho, F. E. L., Lima-Melo, Y., Alencar, V. T. C. B., Daloso, D. M., Margis-Pinheiro, M., Komatsu, S., & Silveira, J. A. G. (2019). Impairment of peroxisomal APX and CAT activities increases protection of photosynthesis under oxidative stress. *Journal of Experimental Botany*, *70*(2), 627-639. http://dx.doi.org/10.1093/jxb/ery354. PMid:30312463.

Souza, G. M., Ribeiro, R. V., Prado, C. H. B. A., Damineli, D. S. C., Sato, A. M., & Oliveira, M. S. (2009). Using network connectance and autonomy analyses to uncover patterns of photosynthetic responses in tropical woody species. *Ecological Complexity*, *6*(1), 15-26. http://dx.doi.org/10.1016/j.ecocom.2008.10.002.

Swain, S. S., Tripathy, T., Mohapatra, P. K., & Chand, P. K. (2010). Photosynthetic and transpiration responses of *in vitro* -regenerated *Solanum nigrum* L. plants to *ex vitro* adaptation. *In vitro Cellular & Developmental Biology - Plant*, *46*(2), 134-141. http://dx.doi.org/10.1007/s11627-009-9269-8.

Thordal-Christensen, H., Zhang, Z., Wei, Y., & Collinge, D. B. (1997). Subcellular localization of H2O2 in plants. H2O2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *The Plant Journal*, *11*(6), 1187-1194. http://dx.doi.org/10.1046/j.1365-313X.1997.11061187.x.

Varshney, A., & Anis, M. (2012). Improvement of shoot morphogenesis *in vitro* and assessment of changes of the activity of antioxidant enzymes during acclimation of micropropagated plants of desert teak. *Acta Physiologiae Plantarum*, *34*(3), 859-867. http://dx.doi.org/10.1007/s11738-011-0883-9.

Wickramasuriya, A. M., & Dunwell, J. M. (2015). Global scale transcriptome analysis of arabidopsis embryogenesis *in vitro* . *BMC Genomics*, *16*(1), 301. http://dx.doi.org/10.1186/s12864-015-1504-6. PMid:25887996.

Yu, H., Zhao, W., Wang, M., Yang, X., & Jiang, W. (2016). The exogenous application of spermidine alleviates photosynthetic inhibition and membrane lipid peroxidation under low-light stress in tomato (*Lycopersicon esculentum* Mill.) seedlings. *Plant Growth Regulation*, *78*(3), 413-420. http://dx.doi.org/10.1007/s10725-015-0102-6.

Zhou, M., Diwu, Z., Panchuk-Voloshina, N., & Haugland, R. P. (1997). A stable nonfluorescent derivative of resorufin for the fluorometric

determination of trace hydrogen peroxide: Applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Analytical Biochemistry*, *253*(2), 162-168. http://dx.doi.org/10.1006/abio.1997.2391. PMid:9367498.

Zhu, H., Li, X., Zhai, W., Liu, Y., Gao, Q., Liu, J., Ren, L., Chen, H., & Zhu, Y. (2017). Effects of low light on photosynthetic properties, antioxidant enzyme activity, and anthocyanin accumulation in purple pak-choi (*Brassica campestris* ssp. *Chinensis* Makino). *PLoS One*, *12*(6), 1-17. http://dx.doi.org/10.1371/journal.pone.0179305. PMid:28609452.

Zobayed, S. M. A., Armstrong, J., & Armstrong, W. (2001). Leaf anatomy of *in vitro* tobacco and cauliflower plantlets as affected by different types of ventilation. *Plant Science*, *161*(3), 537-548. http://dx.doi.org/10.1016/S0168-9452(01)00438-1.

Zulfugarov, I. S., Tovuu, A., Eu, Y.-J., Dogsom, B., Poudyal, R. S., Nath, K., Hall, M., Banerjee, M., Yoon, U. C., Moon, Y. H., An, G., Jansson, S., & Lee, C. H. (2014). Production of superoxide from Photosystem II in a rice (*Oryza sativa* L.) mutant lacking PsbS. *BMC Plant Biology*, *14*(1), 242. http://dx.doi.org/10.1186/s12870-014-0242-2. PMid:25342550.

Supplementary material

Supplementary material accompanies this paper.

Figure S1. Habitus from tobacco plants grown under (A) in vitro and (B) ex vitro conditions.

Figure S2. Surface micrography of tobacco leaves from plants grown under different conditions highlighting stomata distribution and morphology. Figures represent stomata structure and morphology in leaf adaxial surface. Stomata distribution and morphology are shown in (A and D) in vitro plantlets, (B and E) ex vitro plants, and (C and F) seed plants (greenhouse). Pictures are the most representative of five independent micrographies.

Figure S3. P_N-PPFD curves and related parameters. (A) P_N-PPFD fitted curves, (B) light compensation point, (C) maximum net photosynthesis, (D) photosynthetic quantum efficiency and (E) dark respiration in leaves from plants grown under in vitro or ex vitro conditions. Different letters mean significant difference ($p < 0.05$) between in vitro and ex vitro growth conditions.

Figure S4. Loading plot based on the principal component analysis (PCA) results. Data was obtained from assays performed in leaves from plants grown under in vitro conditions for 30 days and plants grown in ex vitro conditions. Plants were then acclimated to low light conditions (LL; 100 µmol photons m^{-2} s^{-1}) and moderate light (ML; 500 µmol photons m^{-2} s^{-1}) regimes for 12 hours. The PCA was performed employing normalized photosynthetic and redox metabolism parameters, based on the reference data from each in vitro and ex vitro plants, respectively. The principal components (PC) 1 and 2 explain 68.45% of the variance.

This material is available as part of the online article from http://www.biori.periodikos.com.br/