



RESEARCH PAPER

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



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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 CO₂ assimilation (P_N), decreased actual quantum efficiency of photosystem II (Φ PSII) associated with non-induction of non-photochemical quenching (NPQ). In contrast, acclimated *ex vitro* plants showed strong stimulation in P_N whereas Φ PSII 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

Abbreviations: APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; C_i , intercellular CO₂ partial pressure; DAB, 3,3'-diaminobenzidine; DHA, dehydroascorbate; E , leaf transpiration; EDTA, ethylenediamine tetraacetic acid; ETR, actual electron transport rate from PSII; F_m , maximum fluorescence in dark-adapted leaves; F_o , 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 , CO₂ assimilation rate (net photosynthesis); PPF, photosynthetic photon flux density; PSII, photosystem II; Ref, reference; R_n , 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; Φ PSII, effective quantum yield of PSII.

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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.

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, Wolfart, Fermino, & Santos, 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, Bandopadhyay, Kumar, & Chandra, 2010; Sáez, Bravo, Latsague, Sánchez, & Ríos, 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, Javed, Anis, & Aref, 2013). *Ex vitro* phase encompasses several favorable morphogenetic changes in photosynthetic apparatus, involving photochemical, biochemical and stomatal components (Monja-Mio, Pool, Herrera, & Martín, 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, Bertrand, Vasquez, & Etienne, 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, Medeiros, dos Anjos, & Yoshida, 2017). In parallel to stomatal organization, the photosynthetic efficiency is gradually improved to face with

gradual increase of light intensity (Alvarez, Sáez, Sáez, Sánchez-Olate, & Ríos, 2012; Monja-Mio et al., 2015; Swain, Tripathy, Mohapatra, & Chand, 2010). Moreover, the effective quantum yield of PSII (Φ PSII) 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, Vilela, Vidigal, & Mullineaux, 2006; Guan, Guo, Sui, Li, & Zhang, 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, Neukermans, Queval, Noctor, & Harbinson, 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, Wakao, Fischer, & Niyogi, 2009; Gollan, Lima-Melo, Tiwari, Tikkanen, & Aro, 2017; Lima-Melo, Gollan, & Tikkanen, 2019). 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 (Lando et al., 2016; Perveen, Anis, & Aref, 2013).

The reducing power that is not totally utilized by CO₂ 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, H₂O₂ is believed to be capable of inactivating photosystem II (PSII) reaction center through inhibition of D1 protein *de novo* synthesis (Jimbo, Yutthanasirikul, Nagano, & Hisabori, 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, Muller, Cohu, & Demmig-Adams, 2013; Ruban, 2015). However, H₂O₂ 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, Pinto, & Santos, 2011; Perveen et al., 2013a). 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, Bravo, Latsague, & Toneatti, 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-h photoperiod, external light intensity of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ 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 and chlorophyll *a* fluorescence parameters

CO₂ 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 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$, $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$, and $\text{mmol CO}_2 \text{ mol air}^{-1}$, respectively. The internal parameters in the IRGA chamber during gas exchange measurements were 1.0 ± 0.2 kPa vapor-pressure deficit and 38 Pa CO₂, at 28 °C. Actinic light employed for photosynthetic determinations was similar to those in which plants were previously grown for 12 h, *i.e.*, 100, 400, 500, 600, 800 and $\mu\text{mol m}^{-2} \text{ s}^{-1}$. A PPFD of 50 and 200 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ 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, Ribas-Carbó, Diaz-Espejo, Galmés, & Medrano, 2008). The P_N -PPFD fitting curves were determined according to models proposed by Lieth and 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, Bilger, & Neubauer, 1995) in leaves exposed to light and dark-adapted (30 min). The intensity and duration of the saturation light pulse were 8000 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ and 0.7 s, respectively. Maximum quantum yield of photosystem II (PSII) [$F_v/F_m = (F_m - F_o)/F_m$], effective quantum yield of PSII [$\Phi_{PSII} = (F_m' - F_s)/F_m'$] and non-photochemical quenching [NPQ = $(F_m - F_m')/F_m'$] were calculated according to Genty, Briantais, and Baker (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 and 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 $MD = L1/L2 \times 100$ (Blum & Ebercon, 1981). Lipid peroxidation was measured based on the formation of thiobarbituric acid-reactive substances (TBARS) in accordance with Cakmak and Horst (1991). The concentration of TBARS was calculated using its absorption coefficient ($155 \text{ mM}^{-1} \text{ cm}^{-1}$) and the results were expressed as $\eta\text{mol MDA-TBA g FW}^{-1}$ (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, Zhang, Wei, & Collinge, 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, Diwu, Panchuk-Voloshina, and Haugland (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 nm, 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 $\mu\text{mol H}_2\text{O}_2 \text{ g}^{-1} \text{ FW}$.

Superoxide *in vitro* quantification was performed according to Elstner and Heupel (1976). The methodology is based on nitrite formation from hydroxylamine in the presence of $\text{O}_2^{\cdot-}$. 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 1500 g for 5 min, the absorbance was measured spectrophotometrically at 540 nm of wavelength (Elstner & Heupel, 1976). The $\text{O}_2^{\cdot-}$ content was calculated from standard curves using NO_2^- and the results were expressed as $\eta\text{mol of O}_2^{\cdot-} \text{ g}^{-1} \text{ FW}$.

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 nm (Nakano & Asadam, 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 nm 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, Chen, & Asada, 1994). Net APX activity was calculated by the difference $A - B$ and expressed as $\text{mmol H}_2\text{O}_2 \text{ mg protein}^{-1} \text{ min}^{-1}$.

CAT activity (E.C. 1.11.1.6) was measured following the oxidation of H_2O_2 at 240 nm. 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 nm over 300 s (Havir & McHale, 1987). CAT activity was calculated according to H_2O_2 molar extinction coefficient ($36 \text{ mM}^{-1} \text{ cm}^{-1}$) and expressed as $\text{mmol H}_2\text{O}_2 \text{ mg protein}^{-1} \text{ min}^{-1}$.

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 nm (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 $\text{U mg protein}^{-1} \text{ min}^{-1}$ (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

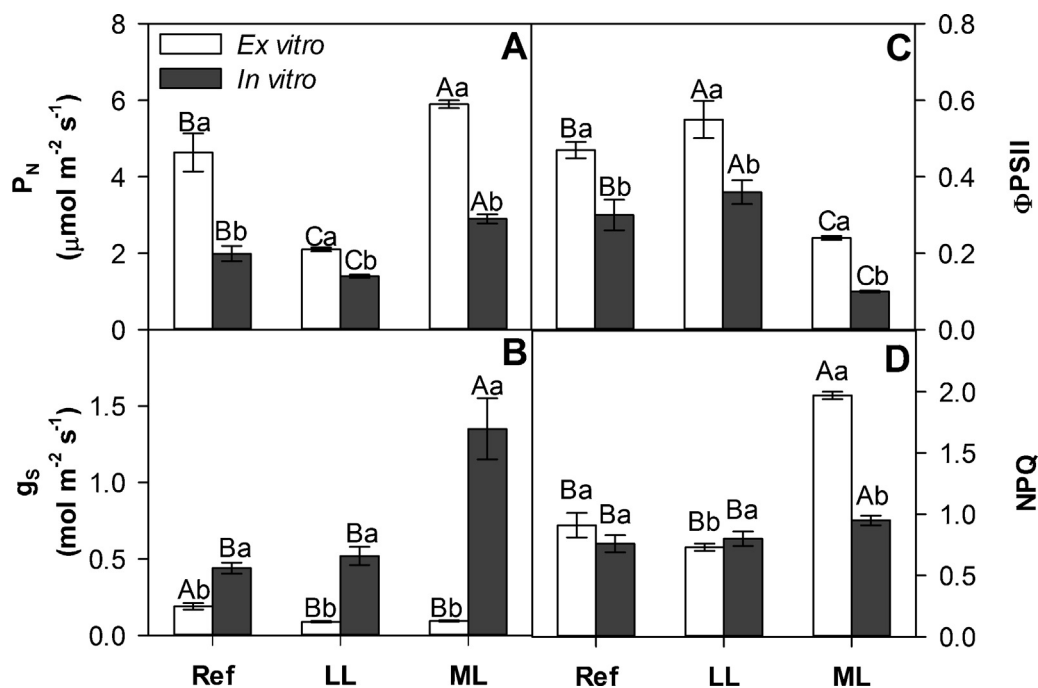


Figure 1 Photosynthetic parameters. (A) Net photosynthesis, (B) stomatal conductance, (C) quantum efficiency of PSII and (D) non-photochemical quenching in leaves from plants grown under *in vitro* and *ex vitro* conditions and acclimated to different light conditions. Reference seedlings consist of leaves collected immediately from the previous growth conditions, *i.e.*, *in vitro* and *ex vitro*, respectively. Subsequently, plants were acclimated to low light conditions ($100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and moderate light regimes ($500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 12 h. 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.

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) (Tables S1 and S2). 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 and 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 (C_g) 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 C_g was calculated as the average of z-values formerly obtained (Souza, Ribeiro, & Prado, 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*; Fig. 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 (Fig. 1A). This response was especially prominent at reference and moderate light conditions (ML; $500 \mu\text{mol m}^{-2} \text{s}^{-1}$), where the *ex vitro* P_N was approximately twice that exhibited by *in vitro* plantlets. Under low light conditions (LL; $200 \mu\text{mol m}^{-2} \text{s}^{-1}$), 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 (Fig. 1A).

The *in vitro* plantlets presented very high and atypical stomatal conductance (g_s) (maximum value near to $1.5 \text{ mol m}^{-2} \text{s}^{-1}$) values, which were positively modulated by light (Fig. 1B). In contrast, *ex vitro* plantlets showed very low g_s , reaching maximum values near to $0.16 \text{ mol m}^{-2} \text{s}^{-1}$. Differently from *in vitro*, the *ex vitro* plantlets exhibited effective stomatal closure in response to increased light (Fig. 1B). These contrasts in g_s were well-correlated

with stomatal opening and density (Fig. 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 $9.5 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) compared to the *ex vitro* ones ($1.5 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$). PSII activity, represented by the effective quantum yield of PSII (Φ_{PSII}), showed a similar trend to CO_2 assimilation, in which *ex vitro* tobacco plants displayed higher values than *in vitro* plantlets (Fig. 1C). The non-photochemical quenching (NPQ) also exhibited tendencies virtually equal to CO_2 assimilation over the studied photoperiod (Fig. 1D).

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 $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Fig. S3). On the other hand, light compensation point was much lower in *ex vitro* ($15.1 \mu\text{mol m}^{-2} \text{ s}^{-1}$) compared to *in vitro* plantlets ($68.0 \mu\text{mol m}^{-2} \text{ s}^{-1}$), as well as maximum light CO_2 assimilation, which were 2.81 and $6.85 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in *in vitro* and *ex vitro* plantlets, respectively (Fig. S3B and 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* (Fig. 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 (Φ_{PSII}) dynamics in response to light compared to *in vitro* plantlets (Fig. 2A). These responses were accompanied by a very limited capability of NPQ induction in response to light presented by *in vitro* plantlets (Fig. 2B). Interestingly, no significant differences in maximum fluorescence in dark-adapted leaves (F_m) were noted for *ex vitro* and *in vitro* plantlets (Fig. 2D), which should reject the hypothesis of underestimated NPQ due to PSII down-regulation. On the other hand, minimum fluorescence in dark-adapted leaves (F_0) was significantly lower in *in vitro*, as compared to *ex vitro* plantlets (Fig. 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 gradually increased from approximately 2.2 – $5.9 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ within the three first days of experiment (which corresponded to 200 , 400 , and $600 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, respectively), maintaining the same rate on the fourth day ($800 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) (Fig. 3A). A similar trend was observed for *in vitro* plantlets, but with lower P_N values, which increased from approximately 1 – $3.2 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$. The P_N values decreased in both groups on the fifth day of experiment ($1000 \mu\text{mol}$

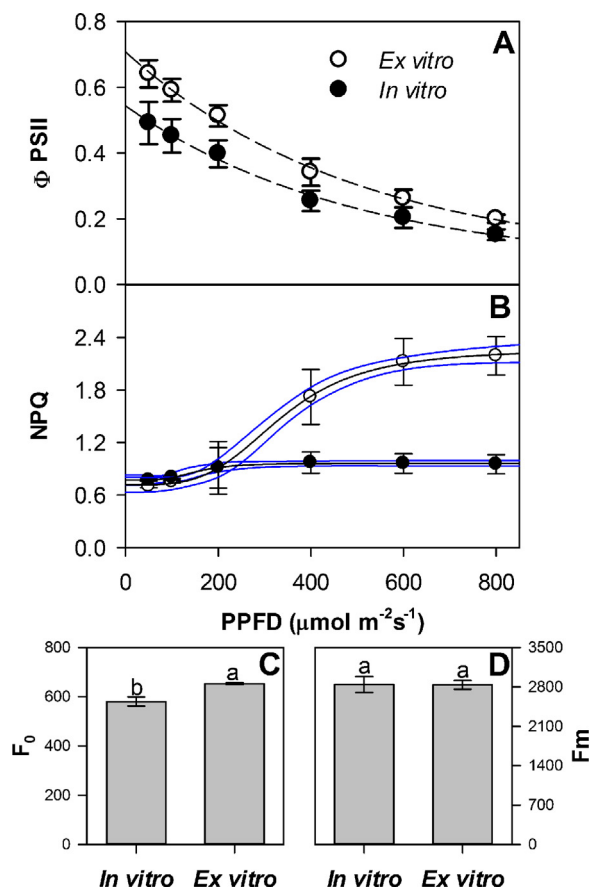


Figure 2 Photochemical responses under increasing light intensity. (A) Quantum efficiency of PSII, (B) non-photochemical quenching, (C) minimum dark fluorescence of PSII, and (D) maximum fluorescence of PSII in the dark in leaves from plants grown under *in vitro* and *ex vitro* conditions. Different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

$\text{photons m}^{-2} \text{ s}^{-1}$), probably as a consequence of some level of photoinhibition (Fig. 3A). During the whole experiment, the g_s values were much higher in *ex vitro* plants than in *in vitro* plantlets (Fig. 3C). This resulted in the observed responses for E (Fig. 3B), which were also higher in *ex vitro* plants than in *in vitro* plantlets.

The photochemical data show that F_v/F_m values were similar for both growth conditions during the whole experiment, except for the last day, on which the $1000 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ treatment induced a strong decrease in this parameter only for the *in vitro* plantlets (Fig. 3D). Although the Φ_{PSII} 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 (Fig. 3E). The Φ_{PSII} 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 (Fig. 3E). The increasing light intensity of $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ from the first to the

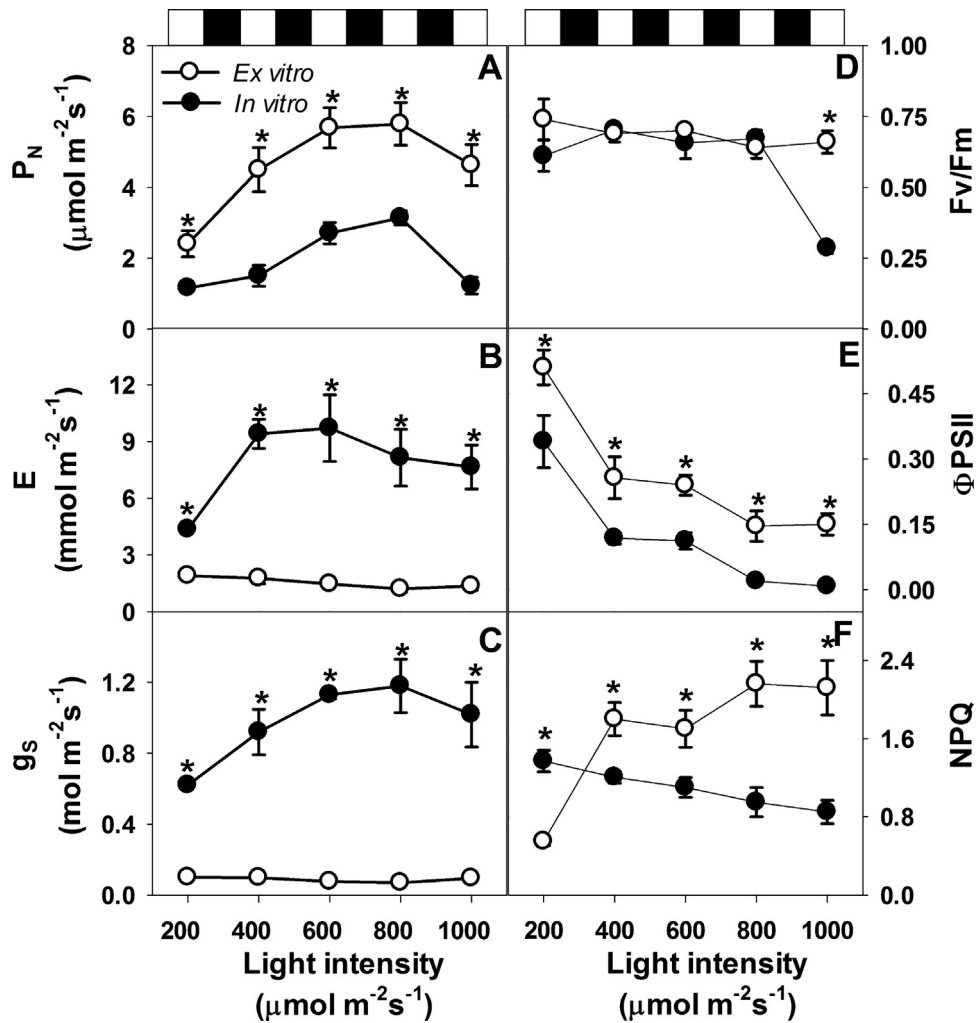


Figure 3 Photosynthetic parameters in tobacco leaves grown under increased light conditions. (A) CO_2 assimilation, (B) stomatal conductance, (C) leaf transpiration, (D) maximum photosynthetic quantum potential, (E) effective photosynthetic quantum yield and (F) non-photochemical quenching in plants 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 $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). The light intensities were supplied for 12 h followed by 12 h 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.

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 (Fig. 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 $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 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 closing control, impaired CO_2 assimilation and PSII activity, combined with inability for NPQ induction by light (Fig. 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; $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and moderate (ML; $500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) light for 12 h of continuous illumination. In presence of LL, *in vitro* plantlets displayed intense accumulation of H_2O_2 (Fig. 4) and radical ion superoxide (Fig. 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 $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ for *in vitro* and for *ex vitro* pots consisting of an oscillating light with a maximum of

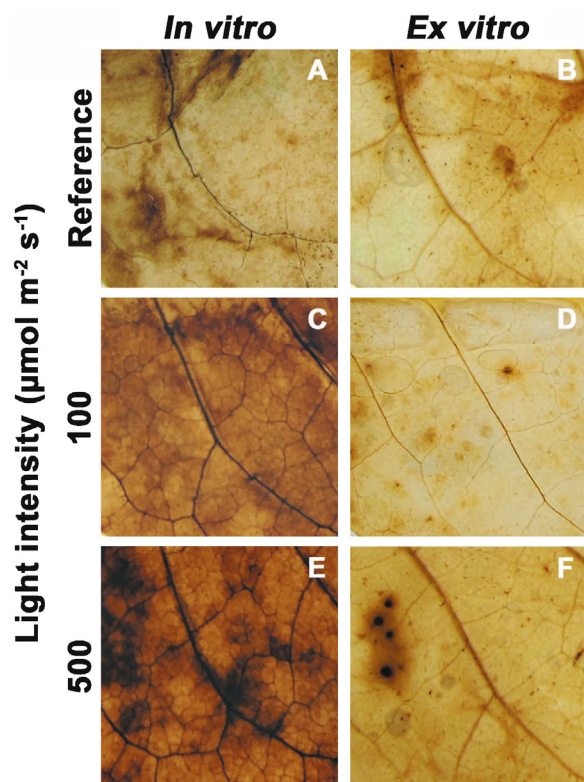


Figure 4 *In situ* hydrogen peroxide DAB staining in leaves. Assays were performed in leaves from plants grown under *in vitro* and *ex vitro* conditions. Reference seedlings (A and B) consist of leaves collected immediately before the light treatments (*i.e.* from the previous growth conditions *in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

$230 \mu\text{mol m}^{-2} \text{s}^{-1}$ at noon of a typical day, accumulate low ROS levels (Figs. 4 and 5). These qualitative results were corroborated by chemical analysis in both light intensities (Fig. 6A and B) and closely correlated with lipid peroxidation (TBARS levels; Fig. 6C) and membrane damage (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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%) (Fig. 8A). Similarly, total glutathione accumulation was also stimulated in response to light intensity, reaching slightly higher values in *ex vitro* plantlets ($200 \eta\text{mol g}^{-1} \text{FW}$) compared to

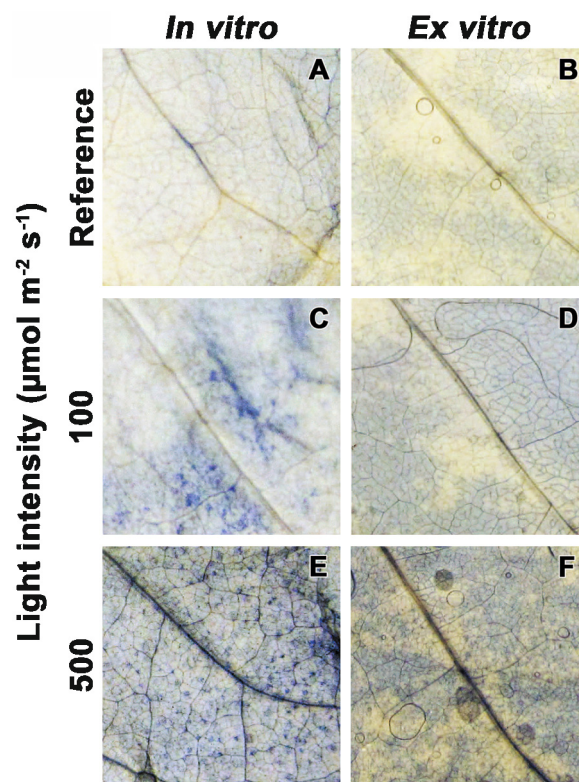


Figure 5 *In situ* radical superoxide NBT staining in leaves. Assays were performed in leaves from plants grown under *in vitro* conditions for 30 days and plants grown in *ex vitro* conditions. Reference seedlings (A and B) consist of leaves collected immediately before the light treatments (*i.e.*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

in vitro plantlets (Fig. 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 (Fig. 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 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 (Fig. S4). The network analysis reinforced these results and adds evidence that *in vitro* and *ex vitro* conditions can induce a prominent adjustment in network

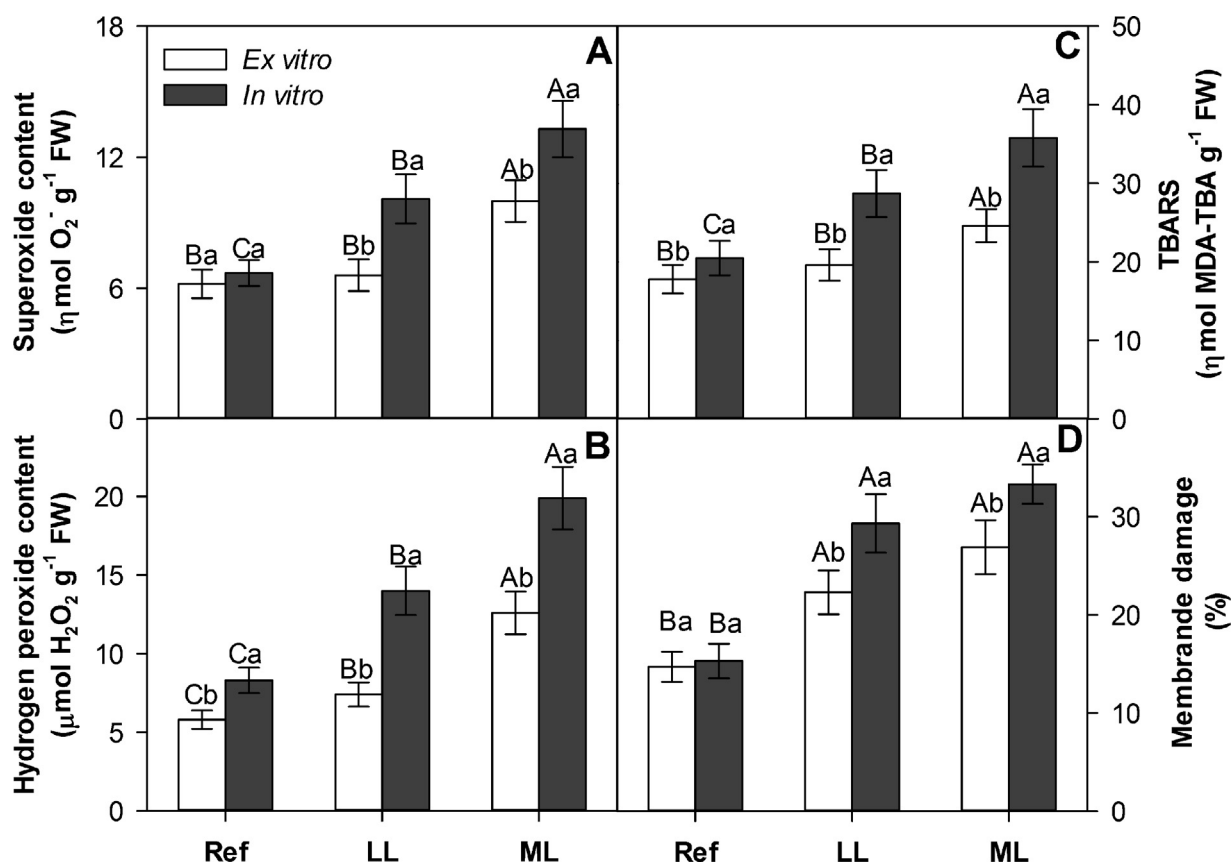


Figure 6 Quantitative indicators of oxidative stress. (A) Radical ion superoxide content, (B) hydrogen peroxide content, (C) TBARS content, and (D) membrane damage were performed in leaves from plants grown under *in vitro* and *ex vitro* conditions. Reference seedlings (A and B) consist of leaves collected 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; $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and moderate light regimes (ML; $500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) for 12 h. 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.

topography (Fig. 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 connectance did not show any prominent difference in the current analysis.

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 (Georgieva, Yordanov, & Kroumova, 1996; Pospíšilová, Synková, Haisel, & Bat'ková, 2009; Rival, Beule, Lavergne, Nato, & Havaux, 1997; Zobayed, Armstrong, & Armstrong, 2001). 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, Synková, & Pospíšilová, 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 $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (König, Vaseghi, Dreyer, & Dietz, 2018). Interestingly, the CO_2 assimilation impairment observed in *in vitro* plantlets was 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 to 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

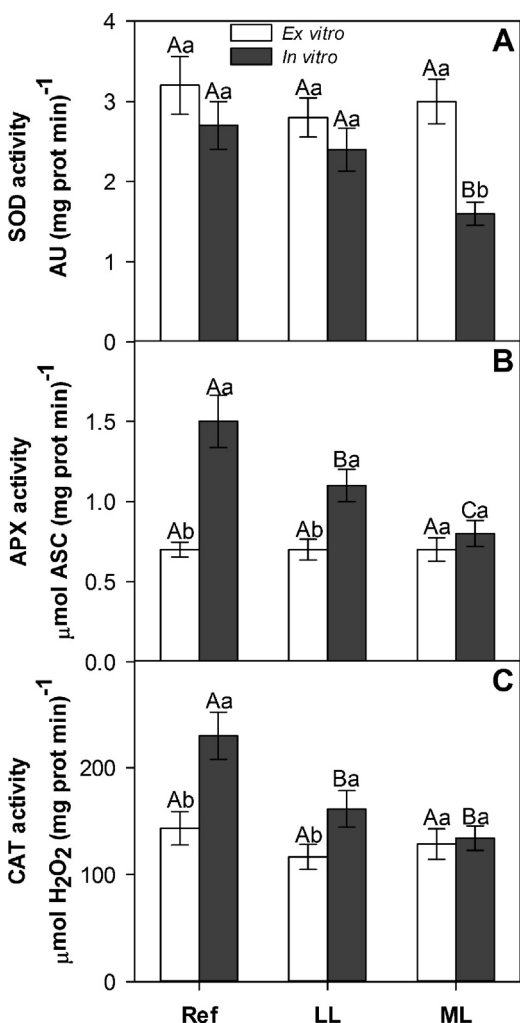


Figure 7 Enzymatic antioxidant activities. Activities of (A) superoxide dismutase, (B) ascorbate peroxidase and (C) catalase in leaves from plants grown under *in vitro* and *ex vitro* conditions. Reference seedlings consist of leaves collected immediately before the light treatments (*i.e.* from the previous growth conditions *in vitro* and *ex vitro*, respectively). Subsequently, plants were acclimated to low light conditions (LL; 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and moderate light regimes (ML; 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 12 h. 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.

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 (Cardoso, Rossi, Rosalem, & Silva, 2013; Kadleček, Tichá, Haisel, Apková, & Schäfer, 2001; Sáez et al., 2012; Sáez, Bravo, Latsague, & Toneatti, 2013). 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 (Figs. 1–3) which is inefficient in supporting proper photosynthesis and in

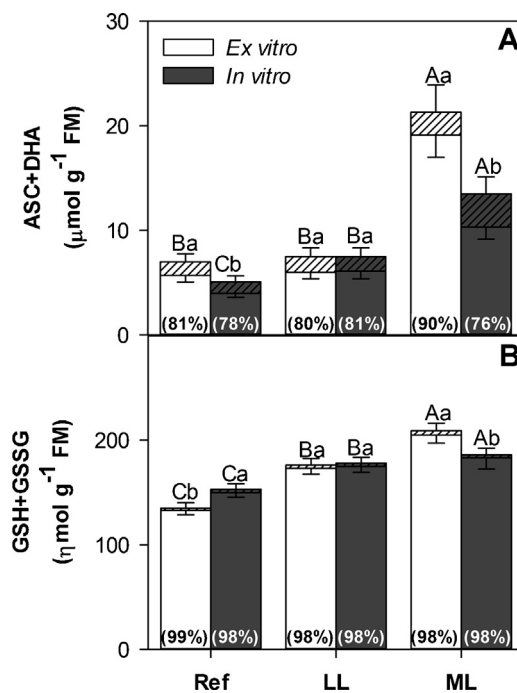


Figure 8 Non-enzymatic antioxidant contents. Contents and redox balance of (A) ascorbate and (B) glutathione were evaluated in leaves from plants grown under *in vitro* and *ex vitro* conditions. Reference seedlings consist of leaves collected immediately before the light treatments (*i.e.* from the previous growth conditions *in vitro* and *ex vitro*, respectively). Subsequently, plants were acclimated to low light conditions (LL; 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and moderate light regimes (ML; 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 12 h. 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.

avoiding water loss as a consequence of a malformed stomatal system (Apóstolo, Brutti, & Llorente, 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 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), for five consecutive days, is able to improve photosynthetic acclimation in *ex vitro* plants, favoring gas exchange as well as photochemical activity. Indeed, CO₂ 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 F_v/F_m values. Moreover, the greatly stimulated NPQ in *ex vitro* plants probably contributed to avoid ROS accumulation (Carvalho, Ware, & Ruban, 2015; Foyer, Ruban, & Noctor, 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

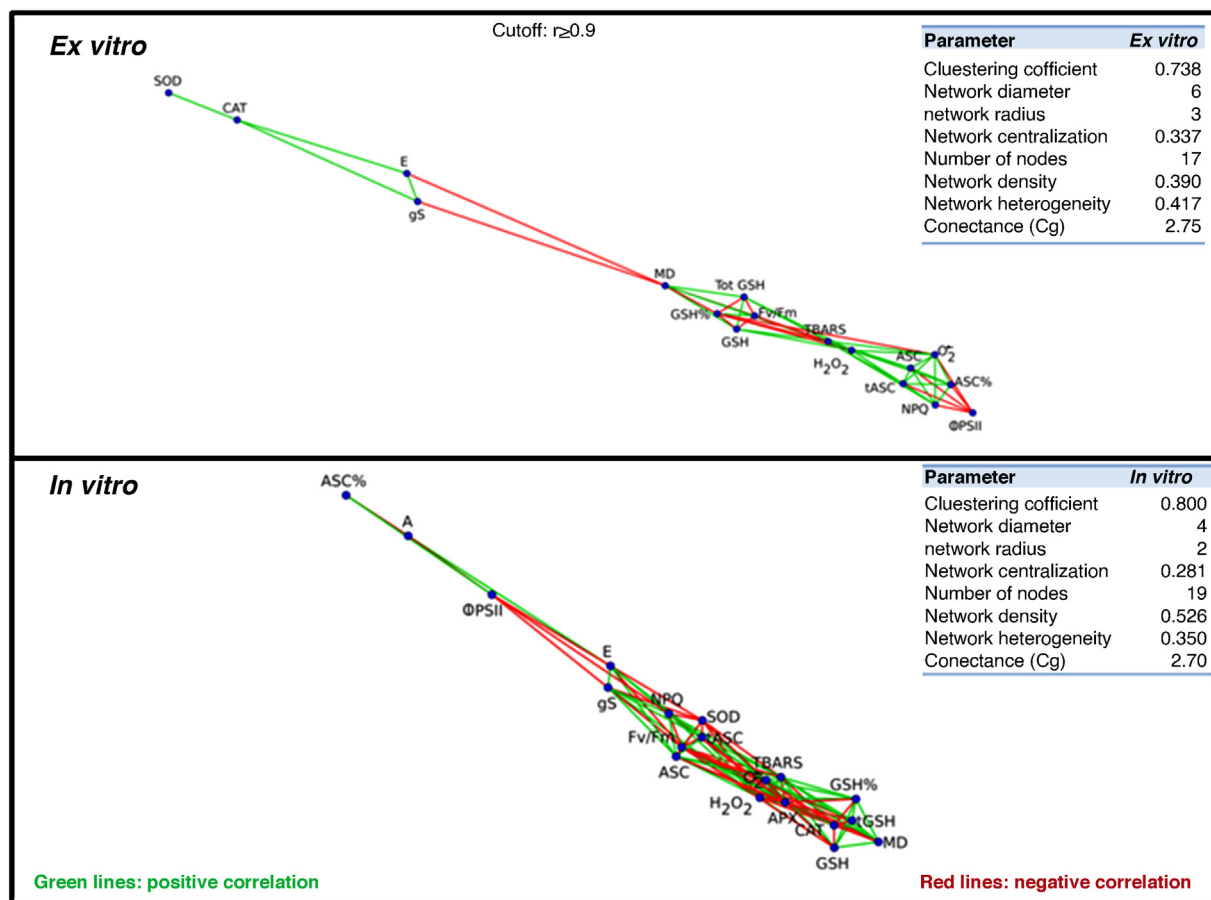


Figure 9 Correlation network analysis. Pearson's correlation network of photosynthetic and redox metabolism parameters measured in plants grown under *in vitro* conditions for 30 days and plants grown in *ex vitro* conditions. All the parameters were first normalized according to respective reference 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.

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; Lima-Melo et al., 2019b; Sousa, Carvalho, & Lima-Melo, 2019;). 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 $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, *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, Aro, & Millar, 2018; Roach & Krieger-Liszky, 2019). However, several works have shown that short-term low light can also induce oxidative stress, as measured by different indicators (Li et al., 2017; Yu, Zhao, Wang, Yang, & Jiang, 2016; 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 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 CO₂ assimilation rates (Kapchina-Toteva et al., 2014) and other energy dissipative sinks such as water-water cycle (Perveen et al., 2013a) 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 CO₂ assimilation as has been reported for tobacco plants (Kromdijk, Głowacka, Leonelli, & Gabilly, 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 (connectance) 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, CO₂ assimilation is improved by improvements in biochemical CO₂ 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 interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.biori.2020.04.001](https://doi.org/10.1016/j.biori.2020.04.001).

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