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Acclimatization of micropropagated plantlets induces an antioxidative burst: a case study with *Ulmus minor* Mill.

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Abstract

In this article, the effects of increased light intensities on antioxidant metabolism during *ex vitro* establishment of *Ulmus minor* micropropagated plants are investigated. Three month old *in vitro* plants were acclimatized to *ex vitro* conditions in a climate chamber with two different light intensities, 200 μmol m^{-2} s^{-1} (high light, HL) and 100 μmol m^{-2} s^{-1} (low light, LL) during 40 days. Immediately after *ex vitro* transfer, the increase of both malondialdehyde (MDA) and electrolyte leakage in persistent leaves is indicative of oxidative stress. As the acclimatization continues, an upregulation of the superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) enzyme activities were also observed. Simultaneously, MDA content and membrane permeability stabilized, suggesting that the antioxidant enzymes decrease the deleterious effects of reactive oxygen species (ROS) generation. Unexpectedly, newly formed leaves presented a different pattern of antioxidative profile, with high levels of MDA and membrane leakage and low antioxidant enzyme activity. Despite these differences, both leaf types looked healthy (*e.g.* greenish, with no necrotic spots) during the whole acclimatization period. The results indicate that micropropagated *U. minor* plantlets develop an antioxidant enzyme system after *ex vitro* transfer and that, in general, LL treatment leads to lower oxidative stress. Moreover, new leaves tolerate higher levels of ROS without the need to activate the antioxidative pathway, which suggests that the environment at which leaves are exposed during its formation determinate their ability to tolerate ROS.

Additional key words: antioxidant enzymes; lipid peroxidation; micropropagation; *Ulmus minor*.

Introduction

In vitro propagation is an efficient method for large scale production of important and economically valuable species (Park 2002, Merkel and Nairn 2005). Nevertheless, micropropagation is in some cases restricted due to the low survival rate of the plantlets during the *ex vitro* acclimatization. Micropropagation protocols are, in general, designed to provide the optimal culture conditions and the minimal stress environment for plant multiplication. During *in vitro* conditions, plants grow under specific climatic conditions of high relative humidity, low CO_2 concentration and low light intensity in a culture medium with a large concentration of sugar. These special conditions result in a formation of plants with abnormal morphology, anatomy and physiology (Pospíšilová *et al.* 1999, Hazarica 2006, Estrada-Luna *et al.* 2001, Pinto *et al.* 2011). During the transfer to *ex vitro* conditions, *in vitro* plants are exposed to light intensities higher than those used under *in vitro* conditions, resulting usually in photoinhibition (Carvalho and Amâncio 2002a, Ali *et al.* 2005, Osório *et al.* 2010). In addition, the high differential vapour pressure between *in vitro* and *ex vitro* condition can induce water stress. These stresses may lead to an imbalance between light energy absorption and light energy utilization in acclimatized plants and ultimately to the formation of ROS (Ali *et al.* 2005, Batková *et al.* 2008, Faisal and Anis 2009). Plants possess multiple means to prevent/minimize the deleterious effects of excess light absorption, such as

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Abbreviations: APX – ascorbate peroxidase; CAT – catalase; DTT – dithiothreitol; FM – fresh mass; GR – glutathione reductase; HL – high-light treatment; IBA – idolbutiric acid; L_0 – electrical conductivity after autoclaving; L_t – electrical conductivity before autoclaving; LL – low-light treatment; MDA – malondialdehyde; NAA – naftalenoacetic acid; NBT – nitroblue tetrazolium chloride; PMSF – phenylmethylsulfonyl fluoride; PPFD – photosynthetic photon flux density; ROS – reactive oxygen species; SOD – superoxide dismutase; TBA – thiobarbituric acid; TCA – trichloroacetic acid; ε – extinction coefficient.
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an integrated array of antioxidant enzymes and metabolites that detoxify ROS and a mechanism that safely dissipates excess absorbed light (thermal energy dissipation) preventing from ROS formation. However, if ROS are formed, they must be removed immediately since they can damage biomolecules such as lipids, proteins, pigments, and nucleic acids (Schuetzenduebel and Polle 2002). As a protection against ROS, plants cells develop several antioxidant metabolites (*e.g.* ascorbate, glutathione, α-tocopherol) and antioxidant enzymes that can neutralize free radicals and reduce the potential damage (Asada 2006). Superoxide dismutase is an antioxidant enzyme that converts the $O_2^{\bullet -}$ radical into H_2O_2 and water. The accumulation of H_2O_2 is prevented in the cell by its reduction to water through the actions of either CAT, guaiacol peroxidase or APX (Apel and Hirt 2004). Several enzymes are involved in the reduction and oxidation of antioxidant biomolecules, such as ascorbate and glutathione. Among these enzymes, APX catalyses ascorbate oxidation to dehydroascorbate while GR reduces glutathione with help of NAD(P)H (Smirnoff 2000).

Ulmus spp. is among the Europe´s Noble Hardwoods (FAO 1999) important both as ornamental and as a timber supplier. However, elm population faces a drastic decline since the beginning of the 20th century due to Dutch Elm Disease (Dunn 2000). Efforts on germplasm preservation and breeding programs involving biotechnological strategies have been encouraged (Harvengt 2004). Research dealing with *in vitro* micropropagation/acclimatization of *U. minor* has been successfully conducted (Conde *et al.* 2004, Conde *et al.* 2008, Dias *et al*. 2010). However, up to the knowledge of the authors, information concerning the role of the antioxidant enzymatic system in plant protection against the deleterious effect of ROS formation after *ex vitro* acclimatization is unknown for this species. A sufficient pool of antioxidant metabolites and a well developed antioxidant enzyme system in *in vitro* grow plants is decisive for the success of the acclimatization process since it influences directly plant survival and plant performance (Batková *et al.* 2008). Moreover, no less important are also those changes after *ex vitro* transfer. However, little information is available on the influence of critical factors, such as light intensity during *ex vitro* establishment of micropropagated plant on the status of these protective enzymatic systems (van Huylenbroeck *et al.* 2000, Carvalho and Amâncio 2002b, Ali *et al.* 2005, Guan *et al.* 2008, Faisal and Anis 2009). Understanding the effect of these critical factors would allow to optimize environmental conditions during acclimatization and, consequently, reduce plant loss and improve plant growth and development. In the particular case of this work, the goal is to investigate the effect of different light intensities during the *ex vitro* acclimatization of micropropagated *U. minor* plantlets on the protective antioxidant enzyme system for ROS scavenging by measuring the antioxidant enzyme activities (SOD, CAT, GR and APX), electrolyte leakage, lipid peroxidation, and H_2O_2 formation.

Materials and methods

Plant material, culture conditions, and acclimatization treatments: Plant material used in this study was obtained from micropropagated *in vitro* culture collection of *Ulmus minor* Mill. at the Laboratory of Biotechnology and Cytomics (University of Aveiro) previously established from mature trees of a natural population of elms from a National Park (Mata Nacional do Choupal, Coimbra, Portugal) as described by Conde *et al.* (2004). *U. minor* plantlets were micropropagated according to Conde *et al.* (2004), at two-month intervals, on Driver and Kuniyuki (DKW) culture medium supplemented with 0.05 μM of IBA and 4.4 μM of NAA. The *in vitro* rooting assay was performed using 5 cm long shoots obtained from the stock cultures on DKW culture medium without growth regulators. Cultures were maintained in a growth chamber with a temperature of 24ºC and a 16/8-h (day/night) photoperiod with light provided by fluorescent lamps (*OSRAM L36 W/10*) at a photosynthetic photon flux density (PPFD) of 50 μmol m^{-2} s^{-1}. After two months, rooted plantlets were transferred to *ex vitro* acclimatization.

Plantlets with approximately 8 cm of height and with at least 2 roots of 5–10 cm were transplanted to 400 ml pots containing an autoclaved mixture of peat and perlite (3:2, v:v) and transferred to a growth chamber (*Model Microclima 1000, ASL-Snijders*). The acclimatization took place in a growth chamber with a day/night temperature of 22 ± 2/ 20 ± 2ºC and a 16-h photoperiod. Two different PPFD were applied: (*1*) a light intensity of approx. 200 ± 20 μmol m^{-2} s^{-1}, called as high-light treatment (HL), and (*2*) a light intensity of 100 ± 20 μmol m^{-2} s^{-1}, called as low-light treatment (LL). These light intensities were based on the light saturation of photosynthesis for *U. minor* at *in vitro* conditions (approx. at 200 μmol m^{-2} s^{-1}, data not shown). Therefore, the irradiance of 200 μmol m^{-2} s^{-1} and a lower one, 100 μmol m^{-2} s^{-1} (which is below the light saturation of photosynthesis but still two times higher than *in vitro* light conditions) were used in this study in order to promote plant autotrophy under *ex vitro* conditions. A higher light intensity was not applied to avoid photoinhibitory effects.

The relative humidity (%RH) inside the growth chamber was maintained at 98% during the first 7 days of acclimatization and then decreased 5% every week until RH attained the 65% value.

Leaf samples were collected at day zero (under *in vitro* conditions) and after 7, 14, 25, and 40 days of acclimatization in persistent leaves (leaves formed under *in vitro* conditions that remained attached to the plant during the experiment) and at days 25 and 40 in the first

new expanded leaf formed after *ex vitro* transfer.

Cell membrane permeability: Electrolyte leakage was used to assess cell membrane permeability as described by Lutts *et al.* (1996). Leaf segments of *U. minor* were detached, washed with deionized water, placed in closed vials containing 20 ml of deionized water and incubated over night at 25ºC, on a rotary shaker. Electrical conductivity of the bathing solution (L_t) was determined after 24 h. Samples were then autoclaved at 120ºC for 20 min and a last conductivity reading (L_0) was obtained upon equilibration at 25ºC. The electrolyte leakage was defined as L_t/ L_0 and expressed as percentage.

Lipid peroxidation on leaves was obtained by measuring malondialdehyde (MDA) production (Dhindsa *et al*. 1981). Approximately 0.5 g of leaves were homogenized with 5 ml of 0.1% TCA (w/v) and centrifuged at 10,000 × *g* for 10 min at 4ºC. After centrifugation, 1 ml of supernatant was mixed with 4 ml 20% TCA (w/v) in 0.5% TBA (w/v) and incubated for 30 min at 95ºC. The extract was then cooled immediately on ice to stop the reaction and centrifuged at 10,000 × *g* for 10 min at 4ºC. MDA concentration was estimated by subtracting the nonspecific absorption at 600 nm from the absorption at 532 nm using an absorbance coefficient of extinction (ε), 155 mM^{-1} cm^{-1}.

Antioxidant enzymes activity and soluble protein content: For the determination of antioxidant enzyme activities, leaves (0.5 g) were homogenized in 5 ml of extraction buffer in a prechilled mortar and pestle by liquid nitrogen. The extraction buffer contained 0.1 M potassium phosphate buffer (pH 7.5), 0.5 mM Na_2EDTA, 1% PVP (m/v), PMSF 1mM, 0.2% Triton X-100 (v/v) and 2 mM DTT. The homogenate was filtered through four layers of cheesecloth and centrifuged at 8,000 × *g* for 20 min at 4ºC. The supernatant was used for enzyme assays (APX, CAT, SOD and GR) and total soluble protein quantification. Protein concentration was determined according to the method of Bradford (1976) using the *Total Protein Kit, Micro* (*Sigma*, Germany).

SOD activity (EC 1.15.1.1) was assayed at 25ºC by monitoring the decrease of absorbance at 560 nm generated by the inhibition of the reduction of NBT (nitroblue tetrazolium chloride) following the method of Agarwal *et al.* (2005). The reaction mixture contained 13.3 mM methionine, 63 µM NBT, 0.1 mM EDTA, 50 mM potassium phosphate buffer (pH 7.8), 50 mM Na_2CO_3, and an aliquot of extract. Reaction was started by adding 2 µM riboflavin and placing the tubes under a 15 W fluorescent lamp for 15 min. A reaction mixture without the extract was used for control. To stop the reaction, the light was switched off and the tubes were maintained in the dark. For enzymatic and control assays a nonirradiated correspondent reaction mixture, with or without extract respectively, was used as blank. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT in comparison with control.

CAT (EC 1.11.1.6) activity was assayed at 25ºC as described by Beers and Sizer (1953). Assay mixture contained 0.1 M potassium phosphate buffer (pH 7.0) and enzyme extract. To start the reaction, 20 mM H_2O_2 (ε = 39.4 mM^{-1} cm^{-1}) was added and the decrease of absorbance at 240 nm was recorded.

GR (EC 1.6.4.2) activity was determined at 30ºC according to Sgherri *et al.* (1994). The reaction mixture contained 0.2 M potassium phosphate (pH 7.5), 0.2 mM Na_2EDTA, 1.5 mM $MgCl_2$, 0.25 mM of oxidized glutathione (GSSG), and enzyme extract. The reaction was started with 25 µM NADPH, and the decrease in absorbance at 340 nm was monitored. The GR activity was calculated using the extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}).

APX (EC 1.11.1.11) activity was determined at 25ºC by recording the decrease in absorbance at 290 nm due to ascorbic acid (ε = 2.8 mM^{-1} cm^{-1}) oxidation to dehydroascorbate by H_2O_2, according to the method of Nakano and Asada (1981). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, and an aliquot of enzyme extract, and 0.5 mM H_2O_2 was added to start the reaction. The expression of one unit of APX activity was mmol of ascorbic acid oxidized per minute, calculated using its extinction coefficient (ε) 2.8 mM^{-1} cm^{-1}.

H_2O_2 concentration was measured according to Zhou *et al.* (2006). Briefly, half gram of a leaf tissue was ground in 5 ml precooled 5% TCA (w/v) and activated charcoal. After centrifugation, extracts were adjusted to pH 8.4 with 17 M ammonia, and H_2O_2 was spectrophotometrically quantified following its reaction with 4-aminoantipyrine and phenol to form a stable red product in the presence of 150 U mg^{-1} peroxidase. Blanks containing 8 µg catalase were run for each sample as well as for the calibration with H_2O_2 standards, which were added to the extraction medium in parallel to the samples.

Data analysis: Antioxidant enzyme activities data are averages of samples collected from 6 plantlets per experiment. H_2O_2, MDA, and membrane permeability data are averages of individual measurements in 4 plantlets. The results were analyzed by t-test or one-way analysis of variance (*ANOVA*) using *Sigma Stat for Windows, version 3.1*, depending on the context. Comparisons between means were evaluated by Post Hoc Test (*Holm-Sidak* Test, Multiple Comparison Test) at a significant level set to 0.05. When necessary, data were transformed to achieve normality and equality of variances. When these criteria were not verified, the nonparametric test, *Kruskal-Wallis* one-way *ANOVA* by ranks, was performed.

Results

General morphological aspect during acclimatization: *U. minor* plantlets from both treatments were morphologically similar, with well developed leaves and without any symptoms of necrosis or chlorosis. Plantlets acclimatized under HL conditions showed a higher survival rate and plant growth rate than plantlets under LL. In both light treatments, the new leaves appeared after the second week of acclimatization and reached full expansion after the third week of acclimatization.

Cell membrane stability and lipid peroxidation: Electrolyte leakage increased significantly, in the persistent leaves of both treatments, during the first week after *ex vitro* transfer and stabilized thereafter (Fig. 1*A*). In general, persistent leaves acclimatized under HL showed higher electrolyte leakage than those under LL. The new leaves formed under HL and LL showed similar electrolyte leakage (Fig. 1*A*). The persistent leaves acclimatized under LL conditions presented a significantly lower electrolyte leakage values than the persistent leaves under HL and the new leaves under both light intensities.

MDA concentration showed a similar profile to electrolyte leakage (Fig. 1*A,B*). Independently of the light treatments, MDA content in the persistent leaves increased significantly during the first week of acclimatization. At the end of the experiment, MDA was found to be similar in the persistent leaves under HL [10.1 nmol g^{-1}(FM)] and LL [11.1nmol g^{-1}(FM)]. MDA content was significantly higher in the newly formed leaves under HL than under LL (Fig. 1*B*). Only the new leaves formed under HL presented a significantly higher MDA content than the persistent leaves.

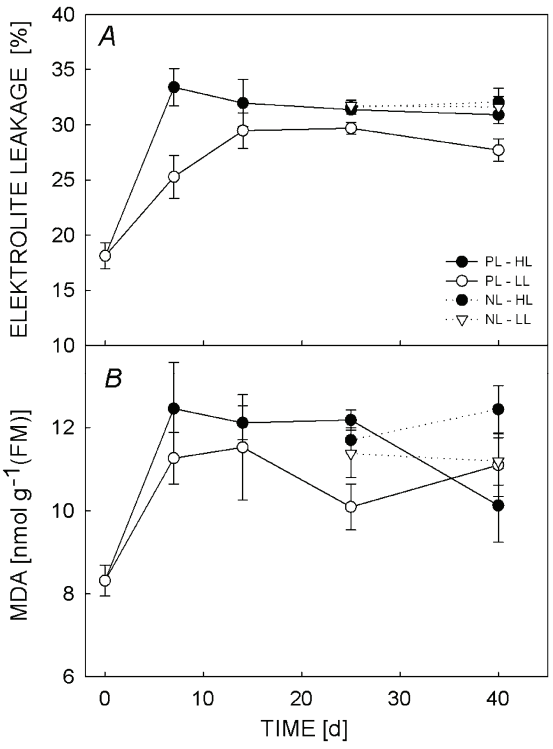

Fig. 1. Changes in electrolyte leakage (*A*) and MDA concentration (*B*) in persistent leaves (PL) and new leaves (NL) of *Ulmus minor* plantlets during acclimatization at 200 μmol m^{-2} s^{-1} (HL) and 100 μmol m^{-2} s^{-1} (LL). Values are means ± SD (n = 4).

Antioxidant enzymes: The effect of PPFD on antioxidant enzymes activities are presented in Fig. 2. The activity of SOD increased significantly in persistent leaves immediately after *ex vitro* transfer to HL conditions (Fig. 2*A*). After the second week of acclimatization, SOD activity in these leaves decreased significantly and stabilized in the last days of the experiment. In contrast, SOD activity in the persistent leaves under LL conditions showed an increasing trend along the acclimatization treatment, though not significant. The activity of SOD was significantly higher under HL than under LL during the acclimatization and only at the end of the experiment, both treatments showed similar activities. The leaves formed after *ex vitro* transfer showed always significantly lower SOD activities than the persistent leaves (Fig. 2*A*). At the last day of the experiment, the new leaves of both light treatments had similar SOD activities.

Catalase activity increased significantly in persistent leaves during the first week of acclimatization under LL and stabilized thereafter (Fig. 2*B*). Under HL conditions, a similar increase in CAT activity was observed in the first week. However, after a significant increase observed from day 14 to day 25, CAT activity decreased significantly during the last days of the experiment, reaching values similar to the *in vitro* plantlets (day zero). Independently of the light treatment, the persistent leaves showed always significantly higher CAT activity than that of the new leaves. Additionally, new leaves formed under LL had significantly higher CAT activity than the new leaves formed at HL at the last day of the experiment (Fig. 2*B*).

The activity of GR in the persistent leaves under both light treatments showed a similar profile during the acclimatization experiment (Fig. 2*C*). Glutathione reductase increased significantly in the first week of acclimatization and decreased significantly thereafter. However, at the end of the experiment, the persistent leaves under HL [333.7 nmol mg(protein)$^{-1}$ min^{-1}], showed significantly higher GR activity than the persistent leaves under LL [237.0 nmol mg(protein)$^{-1}$ min^{-1}]. The new leaves formed under HL showed significantly higher GR activity than the new leaves formed at LL conditions (Fig. 2*C*). Additionally, the newly formed leaves under HL had a GR activity similar to the persistent leaves under LL.

Directly after e*x vitro* transfer, APX activity decreased significantly in the persistent leaves under LL and stabilized thereafter (Fig. 2*D*). In the persistent leaves

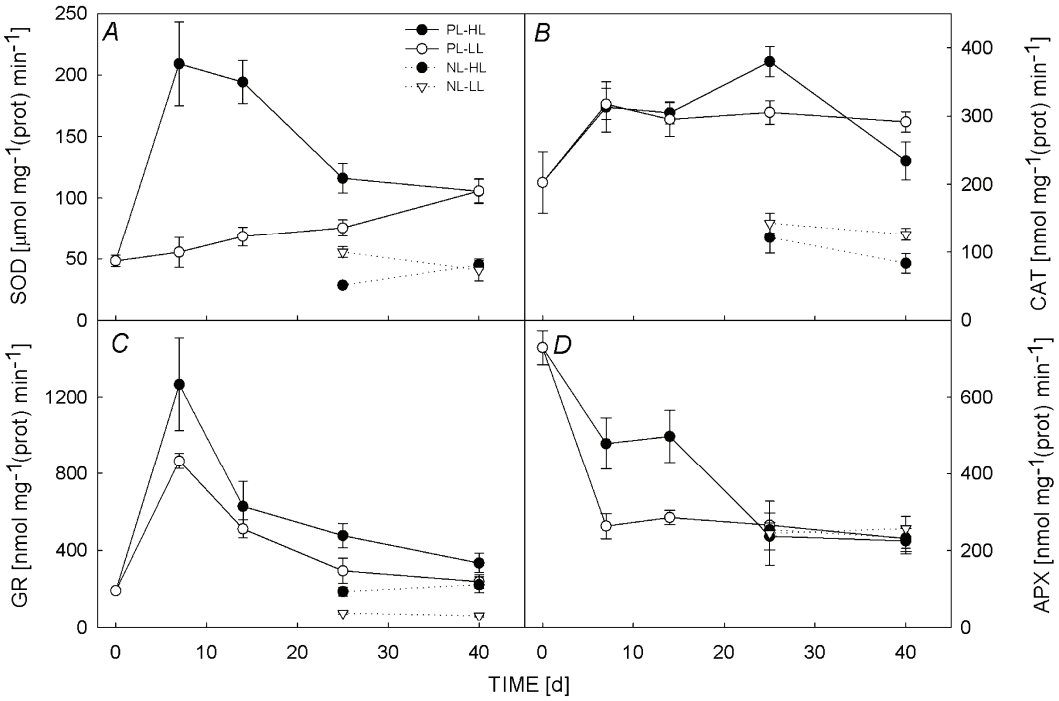

Fig. 2. Changes in the activities of SOD (*A*), CAT (*B*), GR (*C*) and APX (*D*) in persistent leaves (PL) and new leaves (NL) of *Ulmus minor* plantlets during acclimatization at 200 µmol m^{-2} s^{-1} (HL) and 100 µmol m^{-2} s^{-1} (LL) PPFD. Values are means ± standard deviation (n = 6).

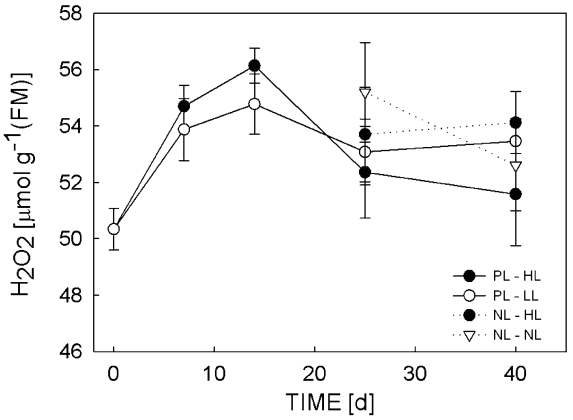

Fig. 3. Changes in H_2O_2 concentration in persistent leaves (PL) and new leaves (NL) of *Ulmus minor* plantlets during acclimatization at 200 µmol m^{-2} s^{-1} (HL) and 100 µmol m^{-2} s^{-1} (LL) PPFD. Values are means ± SD (n = 4).

acclimatized under HL, APX activity decreased significantly from day 0 to day 25 and stabilized thereafter. At the end of the experiment APX activities were similar for both light treatments. Contrarily to SOD and CAT, APX activities in the persistent and new leaves were similar independently of the time and light intensity (Fig. 2*D*).

H_2O_2 content increased significantly in the persistent leaves under LL conditions, after *ex vitro* transfer and stabilized up the second week of acclimatization (Fig. 3). The increase of H_2O_2 after *ex vitro* transfer was significantly higher under HL than under LL conditions. However, under HL, two weeks after the start of the acclimatization, H_2O_2 content decreased significantly and reached similar values to the *in vitro* conditions at the end of the experiment [day 40: 51.6 µmol g^{-1}(FM) and day zero: 50.3 µmol g^{-1}(FM)]. At this time, H_2O_2 content was similar in persistent leaves for both light conditions. At the end of the experiment, the new leaves formed under HL showed significantly higher H_2O_2 content than the persistent leaves under the same light intensity (Fig. 3). No significant differences of H_2O_2 content were observed between the new leaves under HL and those under LL conditions.

Discussion

Under natural conditions, plants are in general exposed to several environmental stresses. However, this stress exposure is particularly unavoidable when plants are transferred from a highly protected environment, such as *in vitro* growth conditions, to the new growth environment, *ex vitro* conditions. Plants are well equipped with numerous protective mechanisms involved in preventing oxidative and photoinhibitory damage. The antioxidant

system employs enzymes and nonenzymatic components that protect plants against the deleterious effects of ROS. However, such a defence system may be inadequate under certain stress conditions, especially when an overproduction of ROS occurs (Foyer *et al.* 1994, Ort and Baker 2002).

MDA is a cytotoxic product of lipid peroxidation and its formation is routinely used as a general indicator of the extent of lipid peroxidation resulting from oxidative stress. The increase in the amount of MDA observed immediately after *U. minor ex vitro* transfer is an indirect indicator of the generation of ROS, similar to those findings in *Phalaenopsis* (Ali *et al.* 2005), in *Olea maderensis* (Brito *et al.* 2009) and in *Rauvolfia tetraphylla* (Faisal and Anis 2009) during acclimatization. As expected by the observed increase in MDA content, the acclimatization of *in vitro U. minor* plantlets significantly affected electrolyte leakage, which indicates injury of biological membranes (Lutts *et al.* 1996). Given that MDA and electrolyte leakage are considered as biochemical markers for the ROS mediated injury (Bacelar *et al.* 2006), results show that acclimatization of *U. minor* persistent leaves increased the oxidative damage on cell membranes by lipid peroxidation. In particular, this effect was more pronounced in persistent leaves acclimatized under HL conditions. Moreover, the simultaneous increase of H_2O_2 levels and the changes in the antioxidant enzyme activities in *U. minor* persistent leaves after *ex vitro* transfer, suggest an activation of the antioxidant enzymatic system against oxidative stress. After the first week of acclimatization, the upregulation of SOD, CAT, and GR activities resulted in a reduction and stabilization of the H_2O_2 formation. Consequently, a stabilization of the electrolyte leakage and a decreased of the MDA content also occurred.

In accordance with this work, van Huylenbroeck *et al.* (2000) reported that micropropagated *Calathea* plantlets developed an antioxidant mechanism during acclimatization. Moreover, these authors suggest that the increase of SOD, APX, and GR activities reveal a protection against photooxidative stress linked to photoinhibition. Increases in SOD, CAT, and APX activities were reported after *ex vitro* transfer of *Calathea louisae* (Huylenbroeck *et al.* 2000), *R. tetraphylla* (Faisal and Anis 2009) and *Phalaenopsis* (Ali *et al.* 2005) micropropagated plantlets against oxidative stress.

The ascorbic acid-dependent antioxidants enzymes, *e.g*. APX and GR, are predominantly localized in the chloroplast, which are the major site of H_2O_2 production in leaves (Foyer *et al.* 1997). However, the activities of antioxidant enzymes in other cell compartments are also important in ROS scavenging and suggest the formation of ROS in mitochondria and peroxisomes (van Huylenbroeck *et al.* 2000, Faisal and Anis 2009). GR is considered a key enzyme responsible for maintaining the reduced form of glutathione pool (Foyer *et al.* 1997). In this study, the increase in GR activity in the early stages of acclimatization suggests that GR may play an important role in scavenging H_2O_2 that is produced in chloroplast of persistent leaves, under both light intensities, during this period. These findings also corroborate those reported for *C. louisae* and *Vitis vinifera* (van Huylenbroeck *et al.* 2000, Carvalho and Amâncio 2002b) during the acclimatization process. The decreased activity of APX in persistent leaves immediately after *ex vitro* transfer for both light conditions may be explained by an inactivation of stromal APX, thylakoid-bound APX easily or due to low concentrations of ascorbate (<20 mM) as demonstrated by Chen and Asada (1989), Miyake and Asada (1996) and Ishikawa *et al.* (1998). Reduced APX activities during an acclimatization of other micropropagated plant was also reported by van Huylenbroeck *et al.* (2000), Ali *et al.* (2005), Guan *et al.* (2008), and Faisal and Anis (2009).

Concerning the new leaves formed after *ex vitro* transfer, a different pattern of antioxidative profile was observed. New leaves have reduced antioxidant enzyme activities, but similar or even higher levels of oxidative damage as compared to persistent leaves. These results demonstrate that the antioxidative burst only occurs in *in vitro* formed leaves, and not in the *ex vitro* formed ones. Therefore, considering that these *ex vitro* formed leaves are healthy (Dias *et al.* 2010) and do not present any senescence signal, despite having high levels of H_2O_2 (as compared to those formed *in vitro*), these results suggest that the predominant environment of leaf formation (*in vitro vs. ex vitro*) determines and precondition the status of oxidative stress in leaves, and their ability to tolerate higher levels of ROS, without having the need to activate any antioxidant cascade. Thus, the results suggest that new leaves are more tolerant to the environmental stress imposed during acclimatization.

In conclusion the results demonstrate that: (*1*) *ex vitro* acclimatization affects oxidative stress metabolism; (*2*) LL conditions lead in general to low oxidative stress; (*3*) persistent leaves were exposed to an oxidative burst, mostly immediately after transfer to *ex vitro* conditions, which is then compensated by an upregulation of the antioxidative enzymes; contrarily, leaves formed under *ex vitro* conditions need to activate less the antioxidant enzyme system to achieve similar status of oxidative stress, suggesting that the ability to tolerate ROS may be preconditioned during the first days of leaf formation.

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