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Structural effects on *Cattleya xanthina* leaves cultivated *in vitro* and acclimatized *ex vitro*

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Abstract

In vitro orchid micropropagation is efficient biotechnological strategy for conservation and commercial plantlet production. However, micropropagated plantlets generally need to adapt to survive severe changes in humidity, irradiance, and growing medium that accompany the transfer to *ex vitro* conditions. Such adaptive cellular changes would give insights into the phenotypic plasticity of the model plant *Cattleya xanthina* **(**L.) Van den Berg. Therefore, we aimed to evaluate structural changes in the leaves of *C. xanthina* cultivated *in vitro* and acclimatized *ex vitro* using qualitative and quantitative analyses. During acclimatization, we observed a higher accumulation of dry mass, a greater convexity of the outer surface of epidermal cells, an increased deposition of epicuticular waxes, a greater elongation of mesophyll parenchymatic cells, and finally, the presence of chloroplasts with organized thylakoids and well-developed grana. Stomatal density was not changed. Furthermore, a gradual acclimatization allows this species the best adaptation to a new environment.

Additional key words: chlorophyll, chloroplast, epidermis, mesophyll, micropropagation, stomata, succulence.

Introduction

Orchids produce large numbers of seeds; however, less than 5 % of them germinate under natural conditions because they are small, lack endosperm, have small embryos, as well as non-differentiated cotyledons (Corrie and Tandon 1993). These unfavorable characteristics make survival of many orchids, including potential ornamentals, improbable in environments disrupted by human activity. However, *in vitro* propagation allows production of high-quality plantlets by providing nutrients to seeds through tissue culture media without the need of symbiosis for germination and growth (Arditti *et al.* 1990). *C. xanthina* is neotropical orchid with a potential ornamental value, but the species is at high risk of extinction in the near future.

During micropropagation, successful plantlet transfer from *in vitro* to *ex vitro* conditions is difficult because of significant environmental differences (Hazarika 2006, Chandra *et al*. 2010). Pospíšilová *et al.* (1999, 2007) reported that plants cultivated *in vitro* are exposed to special growing conditions, such as diminished gas exchange, high air humidity, low irradiance, and use of sugar as energy source, which could cause inhibition of photosynthesis, abnormal stomatal structure, and generally could lead to high plantlet mortality during acclimatization. Therefore, acclimatization to new environmental conditions, such as increased irradiance and low air humidity, requires that plants undergo

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Abbreviations: AC - acclimatization; DM - dry mass; FS - foliar succulence; FM - fresh mass; PAC - pre-acclimatization; LM - light microscope; SEM - scanning electron microscope; TEM - transmission electron microscope.
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physical and anatomical changes (Hazarika 2006), in turn necessitating the development of strategies to control transpiration, decrease cellular water loss, and increase photosynthesis in a CO_2-rich atmosphere (Van Telgen *et al*. 1992, Diaz-Perez *et al*. 1995, Pospíšilová *et al*. 1999). Thus, it is necessary to evaluate the physiology and morphology of *C. xanthina*, both before and after acclimatization, in order to understand the structural plasticity of its tissues in the context of plant survival.

Materials and methods

Cattleya xanthina (L.) Van den Berg plantlets were obtained from a living *in vitro* collection at the Botanical Institute of São Paulo, Brazil. The plantlets were cultured in jars containing a phytohormone-free half-strenth Murashige and Skoog (1962) medium with Morel vitamins (Morel and Wetmore 1951), 20 g dm^{-3} sucrose, 1.5 g dm^{-3} activated charcoal, and solidified with 0.7 % (m/v) agar. The cultures were incubated at a temperature of 25 ± 2 ºC, a 16-h photoperiod, and an irradiance of 50 - 60 µmol(photon) m^{-2} s^{-1} (Fig. 1*A*). *Ex vitro* acclimatization followed two steps: pre-acclimatization (PAC) and acclimatization (AC). During PAC, the plants were transplanted into trays containing an orchid substrate (*Forth Brazil*®, Brazil) and maintained in a growth chamber at a temperature of 25 ± 2 ºC, a 16-h photoperiod, an irradiance of 150 µmol m^{-2} s^{-1}, and an 80 % air humidity for 60 d (Fig. 1*B*,*C*). During the next step (AC), the plantlets were transferred to a coconut fiber and peat moss substrate in a vegetation house at an automatic daily irrigation for 5 min in the morning and night, a 70 % shadow causing an irradiance of about 200 µmol m^{-2} s^{-1}, and grown for 90 d (Fig. 1*D*).

Twenty-two expanded leaves were collected from the plantlets at the end of the *in vitro* culture, PAC, and AC for structural analysis. Samples from the middle region of a leaf blade were fixed in 2.5 % (m/v) glutaraldehyde in a 0.1 M sodium phosphate buffer (pH 7.2) and dehydrated in an ethanol series (Karnovsky 1965). The samples were infiltrated with hydroxyethyl methacrylate (*Leica Historesin*, Heidelberg, Germany) for light microscope (LM) analysis (Gerrits and Smid 1983). Lipophilic substances (cuticle and epicuticular wax) were identified with Sudan III (Johansen 1940). For qualitative analysis in LM, scanning electron microscope (SEM), and transmission electron microscope (TEM), four leaves were used for each environmental condition. Light microscope analyses were made using a *Leica DM2500 LED* microscope (*Leica*, Wetzlar, Germany) equipped with a *DFC 295* camera (*Leica*, Heerbrugg, Switzerland) and software (*Leica Application Suite 2.7.0*, Heerbrugg, Switzerland). Samples for SEM were fixed, dehydrated, critical point dried using CO_2 in *EM-CDP-030* (*Leica*, Heidelberg, Germany), metalized using an *EM SCD 500* high vacuum sputter coater (*Leica*, Vienna, Austria), and then analyzed using a *Jeol XL30* (Tokyo, Japan) SEM. Fixed samples for TEM analysis were post-fixed with osmium tetroxide, dehydrated in an increasing series of acetone aqueous solutions, and then embedded in Spurr's resin. Ultrathin sections were obtained using a *Leica EM UC7 Ultracut* ultramictrotome (*Leica*, Vienna, Austria) and stained with uranyl acetate and lead citrate. Four grids for each treatment were then examined with a *JEM 1011* (*Jeol*, Tokyo, Japan) TEM at 80 kV.

Leaf thickness (mesophyll, epidermis, and cuticle), stomatal density, and stomatal size (polar and equatorial diameter) were determined on transverse leaf sections using the *Leica Application Suite 3.7.0* software and a *Leica DM2500 LED* microscope. Nine replicates were used for each treatment, and each replicate consisted of 12 measurements. Stomatal density and diameters were analyzed using paradermal sections from fresh samples kept in glycerinated gelatin (Gerlack 1984); nine replicates per treatment were used with five microscopic fields per replicate.

To determine foliar succulence (FS), 10 leaves per treatment were placed in moistened plastic bags and maintained at 4 °C for 12 h to evaluate fresh mass (FM). Afterwards, the leaves were placed in a drying oven (60 °C, 48 h) to obtain dry mass (DM). Leaf area (A) was evaluated with an *AM350* portable leaf area meter (*ADC BioScientific*, Hoddsdon, UK). Foliar succulence was calculated using the following formula: FS = (FM - DM)/A (Mantovani 1999).

For determination of chlorophyll content according to Hiscox and Israelstam (1979) and Welburn (1994), leaf samples (100 mg; five repetitions) were incubated in a 65 °C water bath with 7 cm^3 of dimethylsulfoxide for two hours. The samples were filtered and absorbance at 480, 649, and 665 nm was measured using a *S2000 Minature* fiber optic spectrometer (*Bel Photonics*, São Paulo, Brazil).

Data normality was evaluated using the Shapiro-Wilk test. Data were then analyzed using one-way analysis of variance (*ANOVA*), followed by Tukey's post-hoc test ($P < 0.05$) for parametric data and Kruskal-Wallis analysis with Dunn's post-hoc test for nonparametric data, using the *Assistat 7.7* statistical program (Campina Grande, Brazil).

Results

Epidermal cells on the adaxial surface of the *in vitro* leaves showed periclinal, slightly convex walls with striated epicuticular structures (Fig. 1*E*). In both *ex vitro* conditions, cell wall convexity and epicuticular wax

deposition were more prominent (Fig. 1*F*,*G*). Stomata were altered after transfer from the *in vitro* to *ex vitro* conditions. The stomata from *in vitro* leaves were elliptical (Fig. 1*H*), becoming more spherical *ex vitro* (Fig 1*I*,*J*) by a higher increase in equatorial diameter than in polar diameter (Table 1). Under the PAC *ex vitro* conditions, the stomatal pore was large and had no cuticular border (Fig. 1*I*), but the stomatal pores under the later AC *ex vitro* conditions became partially blocked with cuticle (Fig. 1*J*). No evidence of stomatal density change was found during transfer to the *ex vitro* conditions (Table 1).

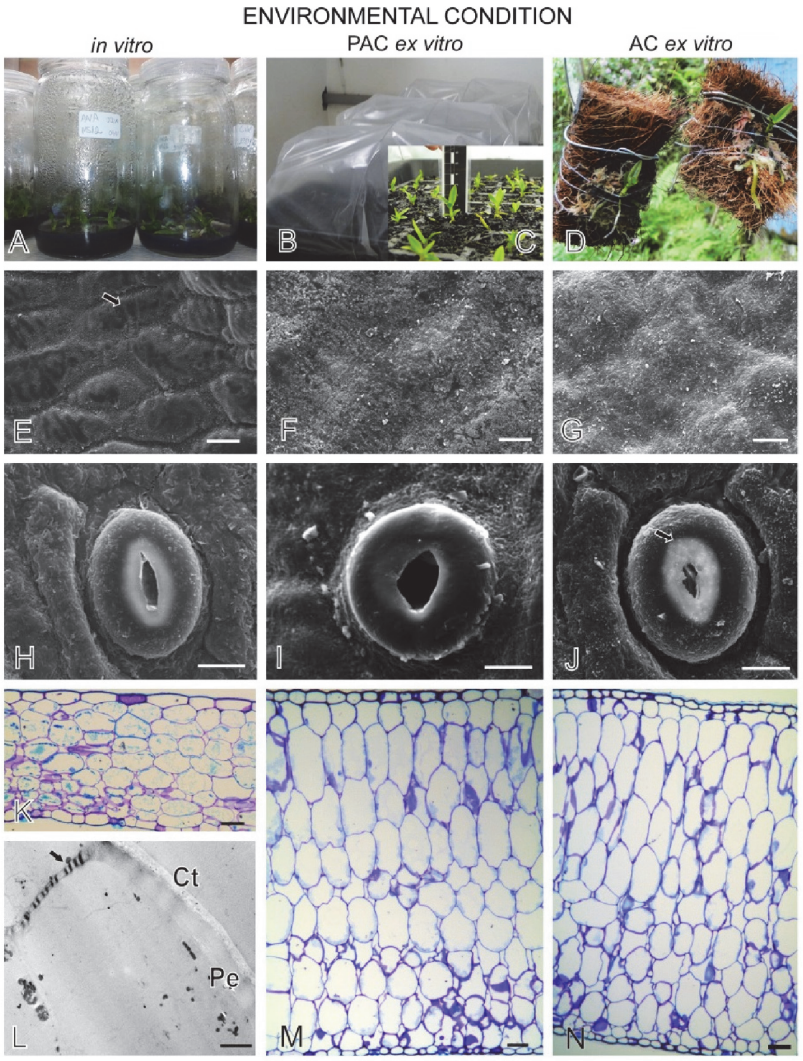

Fig. 1. *Cattleya xanthina* submitted to different environmental conditions: *in vitro,* in half-strength Murashige and Skoog culture media (*A*), pre-acclimatization *ex vitro* in a *Fitotron* with an 80 % humidity (*B,C*), acclimatization *ex vitro* in a vegetation house with 70 % shading **(***D*). General aspects of the *C. xanthina* adaxial leaf surface in a scanning electron microscope: periclinal, slightly convex external cell walls with epicuticular structures (the *arrow*) (*E*), convexity and deposition of epicuticual wax (*F*,*G*); *bars* = 20 µm. Stomata elliptical (*H*), spherical with a large stomatal pore without a cuticular border (*I*), and with an epicuticular border maintaining the pore partially covered (the *arrow*) (*J*); *bars* = 10 µm. Mesophyll bounded by epidermis tending toward cellular homogeneity *in vitro* (*K*) and with elongated cells in the adaxial side under *ex vitro* conditions (*K,M,N*); *bar* = 50 µm.

Both the abaxial side and the adaxial side of leaves showed unstratified epidermis (Fig. 1 *K,M,N*) and cells with a periclinal external wall and a thick cuticle (Fig. 1*L*). Many plasmodesmata were observed between neighboring epidermal cells (Fig. 1*L*). *In vitro* mesophyll cells were homogeneous with chlorophyllous parenchyma cells (Fig. 1*K*), but they were heterogeneous under the *ex vitro* conditions (Fig. 1*M*,*N*). Under the PAC and AC conditions, parenchyma cells were more elongated on the adaxial side with ample intercellular spaces on the abaxial side.

Leaf thickness increased during the transition from the *in vitro* to *ex vitro* conditions (Table 2), mainly as result of increasing mesophyll thickness. Epidermal thickness on the adaxial surface and cuticle on the abaxial surface did not differ with the change in environmental

Table 1. Stomatal size and density in leaves of *C. xanthina* cultured *in vitro* and acclimatized *ex vitro.* Means ± SDs, $n = 9$. Means followed by different letters differ significantly by Tukey's test ($P < 0.05$).

Environmental conditions	Equatorial diameter [µm]	Polar diameter [µm]	Stomatal density [mm^{-2}]
In Vitro	28.4 ± 0.8 ^{b}	29.8 ± 1.9 ^{a}	76.2 ± 11.9 ^{a}
PAC *ex vitro*	31.5 ± 1.7 ^{a}	30.2 ± 1.4 ^{a}	67.8 ± 14.4 ^{a}
AC *ex vitro*	31.2 ± 1.2 ^{a}	30.5 ± 1.7 ^{a}	62.6 ± 8.8 ^{a}

Table 2. Thickness of epidermis, cuticle, mesophyll [µm] and a whole leaf [mm] of *C. xanthina* cultured *in vitro* and acclimatized *ex vitro.* Means ± SDs, $n = 9$. Different letters in rows indicate significant differences between the means by Tukey's test (parametric) or Dunn's test* (non-parametric) ($P < 0.05$).

Parameters	*In Vitro*	PAC *ex vitro*	AC *ex vitro*
Adaxial epidermis*	32.12±5.94^{a}	38.17±2.11^{a}	36.80±2.64^{a}
Abaxial epidermis	27.41±5.03^{b}	32.61±1.65^{a}	29.33±1.96^{ab}
Adaxial cuticle	3.78±0.98^{a}	2.60±0.51^{b}	3.78±0.91^{a}
Abaxial cuticle	4.32±1.54^{a}	4.32±1.03^{a}	4.50±1.09^{a}
Mesophyll	281.80±109.3^{b}	709.04±227.7^{a}	841.76±307.4^{a}
Whole leaf	0.79±0.19^{b}	1.19±0.22^{a}	1.31±0.22^{a}

conditions. Cuticle on adaxial surface showed less thickness in the PAC conditions.

C. xanthina leaves showed a greater DM as plants became acclimatized (Fig. 2*A*). Values of FS showed differences only between the *in vitro* and PAC plants (Fig. 2*B*). The content of chlorophyll *a,* chlorophyll *b* and carotenoids per fresh mass unit (Fig. 3) was different in *in vitro* and PAC conditions, but between the condition *in vitro* and AC it showed no difference. The total chlorophyll in *in vitro* conditions was higher than in *ex vitro* conditions (PAC and AC) (Fig. 3).

In vitro C. xanthina chloroplasts were curved (Fig. 4*A*,*B*) with thylakoids arranged irregularly to form underdeveloped grana (Fig. 4*C*). Under the PAC conditions, subepidermal parenchyma cells contained many amyloplasts (Fig 4*D*). Mitochondria were observed among amyloplasts (Fig. 4*E*). The thylakoids were pressed between starch grains and the periphery of the plastid (Fig. 4*F*). Under the AC conditions, chloroplasts contained starch granules (Fig 4*G*) but fewer and smaller in size than under the PAC conditions. Peroxisomes and many mitochondria were seen close to the chloroplasts (Fig. 4*H*). Chloroplasts also showed well-organized thylakoids with developed grana (Fig 4*H*,*I*).

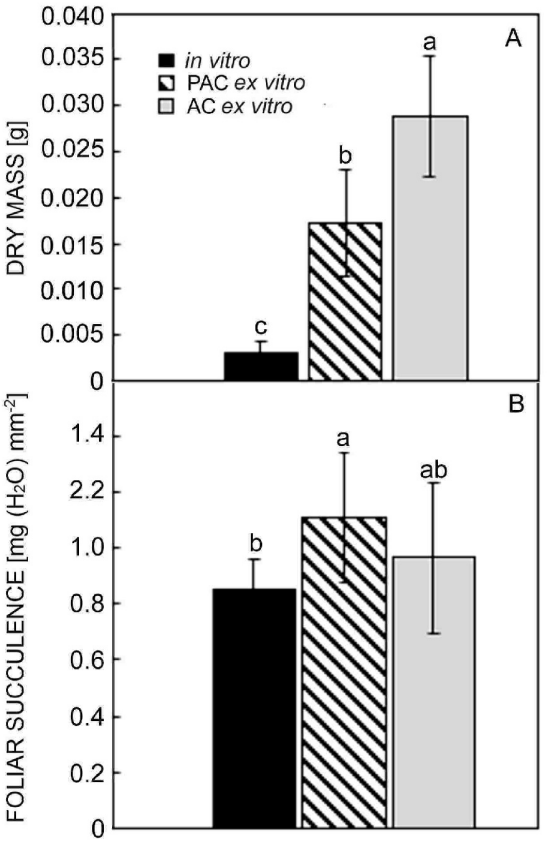

Fig. 2. Leaf dry mass (*A*) and foliar succulence (*B*) *in vitro* and *ex vitro*. Means ± SDs, $n = 10$. Different letters indicate significant differences among treatments ($P < 0.05$).

Discussion

Plants cultivated *in vitro* are subjected to low irradiance, high humidity, and availability of sugars from culture media. *In vitro*-grown plants may not have structural adaptations necessary to survive a direct transfer to field conditions, possibly resulting in a high plant mortality (Hazarika 2003). Because of this, acclimatization is necessary for a progressive adaptation to the new environment. Irradiation is one of the most critical factors during acclimatization (Osório *et al*. 2010) by its direct influence on leaf structure and composition (Oguchi *et al*. 2003, Silva *et al*. 2010). For example, the periclinal curvature of epidermal cells aids in radiation capture and distribution across photosynthetic tissue (Vogelmann and Martin 1993, Brodersen and Vogelmann 2007). The external periclinal walls on the adaxial surface of the *ex vitro C. xanthina* leaves were more convex, which favored AC because it enhanced radiation capture and transfer to chlorenchyma. In addition, epicuticular wax deposits aid in reflecting excess radiation, reducing water loss due to transpiration, and exerting control over the amount of radiation entering the leaf (Gutschick 1999, Dickison 2000). Contrary to expectations, *C. xanthina*

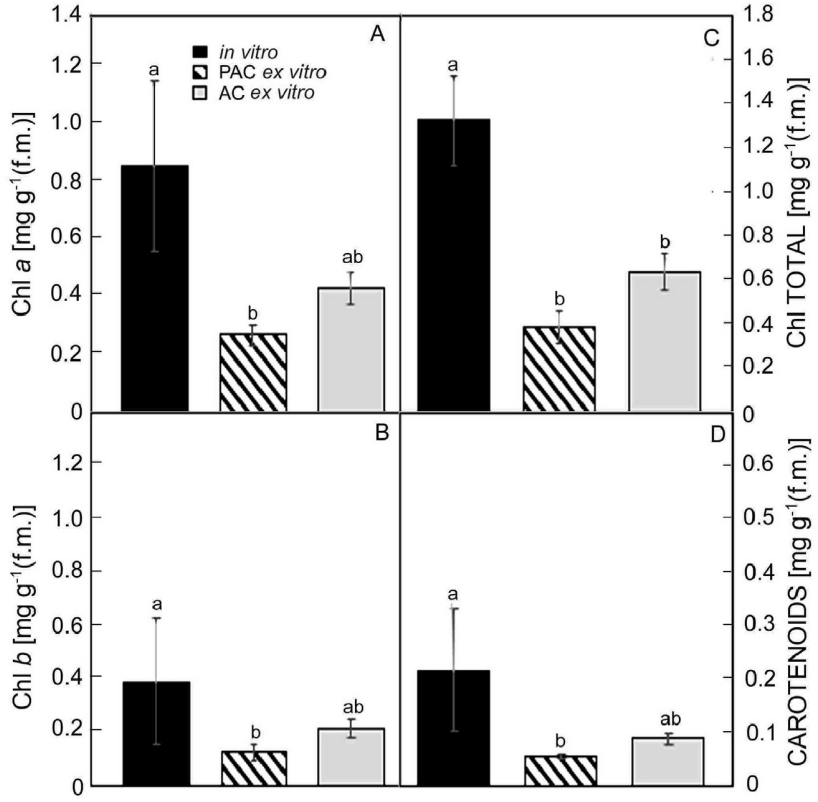

Fig. 3. Content of chlorophyll (Chl) *a* (*A*), Chl *b* (*B*), total Chl (*C*), and carotenoids (*D*) *in vitro* and *ex vitro*. Means ± SDs, $n = 5$. Different letters indicate significant differences among treatments ($P < 0.05$).

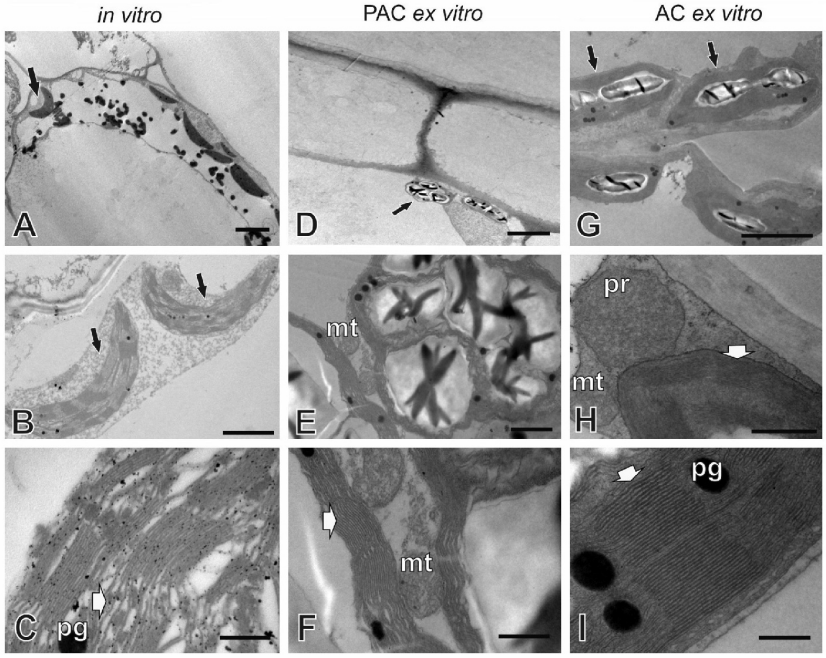

Fig. 4. A transmission electron micrograph of *C. xanthina* leaf transversal cross sections: *In vitro* cultivation (*A*,*B*,*C*): curved chloroplasts (the *black arrows*) and disorganized thylakoids (the *arrowhead*) with a poorly developed granum. Pre-acclimatized *ex vitro* (*D*,*E*,*F*): amyloplast (the *black arrow*) in parenchyma tissue with large starch grains and thylakoids pressed between them and the periphery of the plastid (the *arrowhead*). Acclimatization *ex vitro* (*G*,*H*,*I*): chloroplasts with few starch grains (the *black arrow*s) and organized thylakoids in a granum (the *arrowheads*). Mitochondria (mt), plastoglobules (pg), peroxisomes (pr), *bars* *A*,*D*,*G* = 5 μm, *B*,*E*,*H* =1 μm, and *C*,*F*,*I* = 0.5 μm.

leaves showed a reduced cuticle thickness on the adaxial surface when transferred to the PAC conditions; however, the plants made a greater investment in epicuticular wax during the AC. Therefore, structural plasticity of the epidermis favored water control and radiation capture to enable survival and plant development during AC.

The cuticle border in *C. xanthina* stomatal pores observed in the *ex vitro* AC conditions constitutes another strategy to reduce excessive water loss. This border increases resistance of the passage of water molecules from stomata to the atmosphere. Therefore, the stomata are open which enable gas exchange (Dickison 2000, Lambers *et al*. 2008).

The *C. xanthina* stomata were elliptical in shape when cultured *in vitro*, but they became rounded as result of an increased equatorial diameter of stomata after transfer to the *ex vitro* conditions. This is contrary to previous reports which found that multiple species have rounded stomata when grown *in vitro* and developed elliptical stomata after AC to *ex vitro* conditions (Wetzstein and Sommer 1983, Brutti *et al*. 2002, Khan *et al*. 2003, Ziv and Chen 2008). Despite differences in stomatal structure, the *C. xanthina* plantlet AC was successful, thus suggesting that growing conditions may lead to different structural changes in individual species. Changes in stomatal equatorial diameter in *ex vitro* conditions may favor better carbon dioxide uptake because it increases the total area for gas exchange, increasing the flow of CO_2 into the leaf. Thus, the increase of CO_2 may favor the photosynthesis when the plants were transferred to the environment that required a strictly autotrophic metabolism in comparison to the freely available sucrose in the *in vitro* conditions.

The greater irradiance in the *ex vitro* PAC and AC conditions stimulated chlorophyllous parenchyma cell elongation, enhancing the appearance of palisade cells in the mesophyll. An increased palisade parenchyma thickness is largely based on the formation of longer cells which increased the inner surface of the cells for a larger number of chloroplasts per unit of leaf area (Lambers *et al*. 2008). In chlorenchyma cells, a central tubular vacuole presses protoplasts to the periphery, thus defining positioning chloroplasts (Oguchi *et al.* 2003, 2005). Additionally, mesophyll cell extension results in a greater proximity of chloroplasts to the plasma membrane, which leads to an increased carbon dioxide diffusion (Oguchi *et al.* 2005, Flexas *et al*. 2012). Such cellular changes show the plasticity of *C. xanthina* when transferred from *in vitro* to *ex vitro* conditions. This is reinforced by the increased starch in mesophyll cells, leaf thickness, and dry mass under *ex vitro* conditions. An increase in leaf thickness results in a greater dry mass and accumulation of photosynthates per unit of leaf area (Niinemts 1999).

Water storage capacity is important factor during AC, especially for *C. xanthina* and other epiphytic species. The plants increased FS during the PAC to the *ex vitro* conditions. This indicates an adaptive strategy to increase leaf water storage to protect the plant from sudden dehydration and leaf wilting (Bacelar *et al.* 2004).

Total chlorophyll content in the *C. xanthina* leaves was higher in the plantlets *in vitro*; however, thylakoids did not form grana suggesting that photosynthetic rate was low. Chlorophyll *a* chlorophyll *b*, and carotenoids content were reduced after transfer from the *in vitro* to PAC conditions, possibly reflecting the effect of transition from the culture medium to the new environmental conditions. However, the gradual AC resulted in recovery of chlorophylls *a*, *b*, and carotenoids during the AC phase along with the formation of grana in the chloroplasts. The *Cassia angustifolia* plantlets also showed a decreasing content of chlorophyll *a*, chlorophyll *b*, and carotenoids during initial days after the transfer from *in vitro* to *ex vitro* conditions, but later occurred the linear increase (Parveen and Shahzad 2014). In *Cassia occidentalis*, Naz *et al*. (2015) observed a higher increase in the photosynthetic pigments during acclimatization *ex vitro* under higher irradiance than under lower irradiance.

The ultrastructure analysis of the *in vitro* leaves shows that inner membranes in chloroplasts were arranged irregularly with less developed grana and absence of starch grains. However, after transfer to the *ex vitro* conditions and the corresponding increase in irradiance, the chloroplasts presented a structured granum and starch deposits in agreement with the findings of Wetztein and Sommer (1982, 1983) and Kapchina-Toteva *et al.* (2014). The AC process in *C. xanthina* was efficient with the PAC period; the gradual adjustment to a decreased humidity and increased irradiance favored plant structure modifications necessary for plantlet survival in the natural environmental conditions. These results demonstrate that a gradual process of acclimatization will promote expression of phenotypic plasticity and plant survival.

References

Arditti, J., Ernst, R., Yam, T.W., Glabe, C.: The contributions of orchid mycorhizae fungi to seed germination: a speculative review. - Lindleyana **5**: 249-255, 1990.

Bacelar, E.A., Correia, C.M., Moutinho-Pereira, J.M., Gonçalves, B.C., Lopes, J.I., Torres Pereira, J.M.S.G.: Sclerophylly and leaf anatomical traits of five field-grown olive cultivares growing under drought conditions. - Tree Physiol. **24**: 233-239, 2004.

Brodersen, C.R., Vogelmann, T.C.: Do epidermal lens cells facilitate the absorptance of diffuse light? - Amer. J. Bot. **94**: 1061-1066, 2007.

Brutti, C., Rubio, E., Llorente, B., Apóstolo, N.: Artichoke leaf morphology and surface features in different micropropagation stages. - Biol. Plant **45**: 197-204, 2002.

Chandra, S., Bandopadhyay, R., Kumar, V., Chandra, R.: Acclimatization of tissue cultured plantlets: from laboratory to land. - Biotechnol. Lett **32**: 1199-1205, 2010.

Corrie, S., Tandon, P.: Propagation of *Cymbidium giganteum*

Wall. through high frequency conversion of encapsulated protocorms under *in vivo* and *in vitro* conditions. - Indian J. exp. Biol **31**: 61-64, 1993.

Díaz-Pérez, J.C., Sutter, E.G., Shacke, K.A.: Acclimatization and subsequent gas-exchange, water relations, survival and growth of microcultured apple plantlets after transplanting them in soil. - Physiol. Plant. **95**: 225-232, 1995.

Dickison, W.C.: Integrative Plant Anatomy. - Harcourt Academic Press, San Diego 2000.

Flexas, J., Barbour, M.M., Brendel, O., Cabrera, H.M., Carriquí, M., Díaz-Espejo, A., Douthe, C., Dreyer, E., Ferrio, J.P., Gago, J., Gallé, A., Galmés, J., Kodama, N., Medrano, H., Niinemets, Ü., Peguero-Pina, J.J., Pou, A., Ribas-Carbó, M., Tomás, M., Tosens, T., Warren, C.R.: Mesophyll diffusion conductance to CO_2: an unappreciated central player in photosynthesis. - Plant. Sci. **193**: 70- 84, 2012.

Gerlach, D.: Botanische Mikrotechnik. [Botanical Micro-technique.] - Georg Thieme Verlag, Stuttgart 1984. [In German]

Gerrits, P.O., Smid, L.: A new, less toxic polymerization system for the embedding of soft tissues in glycol methacrylate and subsequent preparing of serial sections. - J. Microscopy **132**: 81-85, 1983.

Gutschick, V.P.: Biotic and abiotic consequences of differences in leaf structure. - New Phytol. **143**: 3-18, 1999.

Hazarika, B.N.: Acclimatization of tissue-cultured plants. - Curr. Sci. **85**: 1704-1712, 2003.

Hazarika, B.N.: Morphophysiological disorders *in vitro* culture of plants. - Sci. Hort. **108**: 105-120, 2006.

Hiscox, J.D., Israelstam, G.F.: A method for the extraction of chlorophyll from leaf tissue without maceration. - Can. J. Bot. **57**: 1332-1334, 1979.

Johansen, D.A.: Plant Microtechnique. - McGraw-Hill Book Company, New York 1940.

Kapchina-Toteva, V., Dimitrova, M.A., Stefanova, M., Koleva, D., Kostov, K., Yordanova, Zh.P., Stefanov, D., Zhiponova, M.K.: Adaptive changes in photosynthetic performance and secondary metabolites during White dead nettle micropropagation. - J. Plant. Physiol. **171**: 1344-1353, 2014.

Karnovsky, M.J.: A formaldehyde-glutaraldehyde fixative of high osmolality for use in eletron microscopy. - J. cell. Biol. **27**: 137-138, 1965.

Khan, S., Kozai, T., Nguyen, Q., Kubota, C., Dhawan, V.: Growth and water relations of *Paulownia fortunei* under photomixotrophic and photoautrotrophic conditions. - Biol. Plant. **46**: 161-166, 2003.

Lambers, H.,Chapin, F.S., Pons, T.L.: Plant Physiological Ecology. 2nd Ed. - Springer-Verlag, New York 2008.

Mantovani, A.: A Method to improve leaf succulence qualification. - Braz. Arch. Biol. Technol. **42**: 9-14, 1999.

Morel, G.M., Wetmore, R.H.: Tissue culture of mono-cotyledons. - Amer. J. Bot. **38**: 138-140, 1951.

Murashige, T., Skoog, F.: A revised medium for rapid growth and biossays with tobacco tissue cultures. - Physiol. Plant. **15**: 473- 497, 1962.

Naz, R., Anis, M., El Atta, H.A.: Micropropagation of *Cassia occidentalis* L. and the effect of irradiance on photosynthetic pigments and antioxidative enzymes. - Biol. Plant. **59**: 1-10, 2015.

Niinemets, U.: Components of leaf dry mass per area – thickness and density – alter leaf photosynthetic capacity in reverse directions in woody plants. - New Phytol. **144**: 35-47, 1999.

Oguchi, R., Hikosaka, K., Hirose, T.: Does the photosynthetic light acclimation need change in leaf anatomy? - Plant Cell Environ. **26**: 505-512, 2003.

Oguchi, R., Hikosaka, K., Hirose, T.: Leaf anatomy as a constraint for photosynthetic acclimation: differential responses in leaf anatomy to increasing growth irradiance among three deciduous trees. - Plant Cell Environ. **28**: 916-927, 2005.

Osório, M.L., Osório, J., Romano, A.: Chlorophyll fluorescence in micropropagated *Rhododendron ponticum* subsp. *baeticum* plants in response to different irradiances. - Biol. Plant **54**: 415-422, 2010.

Parveen, S., Shahzad, A.: Somatic embryogenesis and plantlet regeneration of *Cassia angustifolia* from immature cotyledon-derived callus. - Biol. Plant. **58**: 411-418, 2014.

Pospíšilová, J., Synková, H., Haisel, D., Semorádová, Š.: Acclimation of plantlets to *ex vitro* conditions: effects of air humidity, irradiance, CO_2 concentration and abscisic acid. - Acta Horticult. **748**: 29-38, 2007.

Pospíšilová, J., Tichá, I., Kadleček, P., Haisel, D., Plzáková, Š.: Acclimatization of micropropagated plants to *ex vitro* conditions. - Biol. Plant. **42**: 481-497, 1999.

Silva, A.S., Oliveira, J.G., Cunha, M., Vitória, A.P.: Photosynthetic performance and anatomical adaptations in *Byrsonima sericea* DC. under contrasting light conditions in a remnant of the Atlantic forest. - Braz. J. Plant. Physiol. **22**: 245-254, 2010.

Van Telgen, H.J., Van Mil, A., Kunneman, B.: Effect of propagation and rooting condition on acclimatization of micropropagated plants. - Acta. bot. nederl. **41**: 453-459, 1992.

Vogelmann, T.C., Martin, G.: The functional significance of palisade tissue: penetration of directional *versus* diffuse light. - Plant Cell Environ. **16**: 65-72, 1993.

Wellburn, A.R.: The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. - J. Plant Physiol. **144**: 307-313, 1994.

Wetzstein, H.Y., Sommer, H.E.: Leaf anatomy of tissue cultured *Liquidambar styraciflua* (*Hamamelidaceae*) during acclimatization. - Amer. J. Bot. **69**: 1579-1586, 1982.

Wetzstein, H.Y., Sommer, H.E.: Scanning electron microscopy of *in vitro* cultured *Liquidambar styraciflua* plantlets during acclimatization. - J. amer. Soc. hort. Sci. **108**: 475-480, 1983.

Ziv, M., Chen, J.: The anatomy and morphology of tissue cultured plants. - In: George, E.F., Hall, M.A., Klerk, G.J. (ed.): Plant Propagation by Tissue Culture. Vol. 1. 3rd Ed. Pp. 465-478. Springer, Dordrecht 2008.