Structural Adaptation and Antioxidant Response of *Guarianthe bowringiana* (O'Brien) Dressler & W. E. Higgins (Orchidaceae Juss.) Seedlings During Ex Vitro Acclimatization

Lyudmyla Buyun [1*], Roman Ivannikov [1],
Lyudmila Batsmanova [2], Nataliya Taran [2], Lyudmyla Kovalska[1],
Ivan Gurnenko[1], Myroslava Maryniuk[1]

[1] M. M. Gryshko National Botanic Garden, National Academy of Sciences of Ukraine, Timiryazevska Str. 1, Kyiv 01014, Ukraine
[2] Educational and Scientific Center "Institute of Biology and Medicine," Taras Shevchenko National University of Kyiv, Volodymyrska Str. 64, Kyiv 01601, Ukraine

[*] To whom correspondence should be addressed. Email: orchids.lyuda@gmail.com

Abstract

Guarianthe bowringiana is one of the oldest samples cultivated at NBG's orchid unit glasshouses since 1970s. An efficient protocol for asymbiotic in vitro seed germination of *G. bowringiana* has previously been established. Given that acclimatization is a crucial step in micropropagation, this study assesses the structural adaptation and antioxidant response of *G. bowringiana* seedlings during ex vitro acclimatization to ex vitro conditions.

The leaf surface micromorphology of the *G. bowringiana* juvenile plants propagated in vitro from seeds as well as the leaves of adult plants cultivated in glasshouse were analyzed using scanning electron microscopy. The levels of lipid peroxidation (TBARS level), superoxide dismutase (SOD) activity, and the photosynthetic activity were monitored for seven days from the transfer of seedlings from the in vitro cultivation vessels as they are markers indicating the response of the leaves of in vitro propagated *G. bowringiana* plants to oxidative stress during the early stages of acclimatization to ex vitro conditions. During the initial 2 days of the monitored acclimatization period (0–7 days), the level of photosynthetic pigments (chlorophyll *a*, *b*, and carotenoid content) increased, followed by an insignificant increase during the successive period (by the seventh day) of acclimatization. At the same time, the level of the tested antioxidant enzyme (SOD) exhibited an increasing trend throughout the acclimatization period. The SOD activities in the leaves of *G. bowringiana* seedlings were significantly affected when they were transferred from in vitro to ex vitro conditions due to drought stress.

Thus, it was revealed that in the early stages of acclimatizing to the altered environments, *G. bowringiana* seedlings exhibited a rapid increase in photosynthetic pigments, superoxide dismutase activity, and lipid peroxidation levels after being transferred to ex vitro conditions.

Comparison of the leaf micromorphologies of *G. bowringiana* plants grown under in vitro and those grown under ex vitro conditions revealed that leaf development had undergone significant changes during acclimatization to the altered conditions. In vitro to ex vitro transfer leads to a transient decrease in photosynthetic parameters.

1. Introduction

The family Orchidaceae is not only one of the most numerous, ecologically and morphologically diverse families of flowering plants, but also one of the most endangered plant taxa (Zhang et al., 2016). Orchids are one of the most popular ornamental plants worldwide. Moreover, within Orchidaceae family, epiphytes or litophytes with beautiful flowers are one of the most threatened groups because high demand leads to their overharvesting from wild populations (Hinsley et al., 2017; Mondragón, 2009).

Valued for their beautiful flowers, many wild epiphytic/lithophytic orchids, belonging to the genus *Guarianthe* Dressler & W. E. Higgins [*G. aurantiaca* (Bateman ex Lindl.) Dressler & W. E. Higgins, *G. bowringiana* (O'Brien) Dressler & W. E. Higgins, and *G. skinneri* (Bateman) Dressler & W. E. Higgins], are harvested from their natural habitats and sold as ornamental plants (Mondragón, 2009; Ticktin et al., 2020). Within its natural range, *Guarianthe* plants have been traditionally cultivated in home gardens. Additionally, the natural source of these plants has been significantly reduced due to their collection as medicinal plants, global climate changes, habitat loss, specificity of life history strategies, including specialized pollination syndromes and association with mycorrhizal fungi, and specific substrata (Gravendeel et al., 2004; Hinsley et al., 2017).

As the model organism of the study, we chose one of the *Guarianthe* species, *G. bowringiana* (O'Brien) Dressler & W. E. Higgins ("*Guarianthe bowringiana*," 2021), which belongs to the Laeliinae subtribe (Epidendroideae, Orchidaceae), encompassing 1,500 species and 50 genera distributed within Neotropics (Dressler et al., 2005). In 2003, the *Guarianthe* genus was separated from *Cattleya* Lindl. based on several structural features of the plants and was supported by a recent molecular DNA analysis (Dressler & Higgins, 2003). Therefore, before the paper of R. Dressler and W. Higgins was published, *G. bowringiana* was known as *Cattleya bowringiana* Veitch (van den Berg et al., 2000), which is now considered as a synonym of *G. bowringiana* (Dressler et al., 2005; "*Guarianthe bowringiana*," 2021).

Guarianthe bowringiana is native to Guatemala and Belize. These plants grow on rocky cliffs and on slopes of ravines near high-speed streams, where the air is saturated with moisture. In general, they are found on granite and limestone rock formations at heights of 100–1,000 m, but sometimes they are found on trees of mixed, pine-oak forests (Dressler et al., 2005).

Under glasshouse conditions, these plants reach more than 50 cm in height with fusiform pseudobulbs bearing two or three leaves. The leaves are elliptic to oblong-lanceolate. During cultivation, flowering occurs mainly in November and December (Figure 1A). The representatives of Laeliinae subtribe have been heavily harvested for horticultural purposes in recent years (Mondragón, 2009; Ticktin et al., 2020). *Guarianthe bowringiana* is listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II (*Convention on International Trade in Endangered Species of Wild Fauna and Flora*, 2020; International Union for Conservation of Nature, 2017).

Therefore, in order to conserve orchid plants in the wild and to meet the demands for ornamental and medicinal plant materials, developing new technologies for in vitro plant propagation is urgently needed. In vitro propagation of orchids to produce plants for commercial production and to re-establish the declining populations in the wild is an alternative that might help in decreasing the pressure on natural populations (Avila-Diaz et al., 2009; Coutiño-Cortés et al., 2017). To date, many native orchids species from South America and South-East Asia were propagated at the NBG through asymbiotic seed germination and tissue culture procedures (Buyun et al., 2004; Cherevchenko et al., 2007; Ivannikov, 2012).

Literature searching has revealed that the efforts of scientists are focused mainly on the optimization of various stages of in vitro propagation of tropical orchids (Astarini et al., 2015; Buyun et al., 2004; Coutiño-Cortés et al., 2017; Díaz-Álvarez et al., 2015). Moreover, various approaches towards efficient ex vitro establishment of the in vitro propagated orchids have been employed, such as using different in vitro
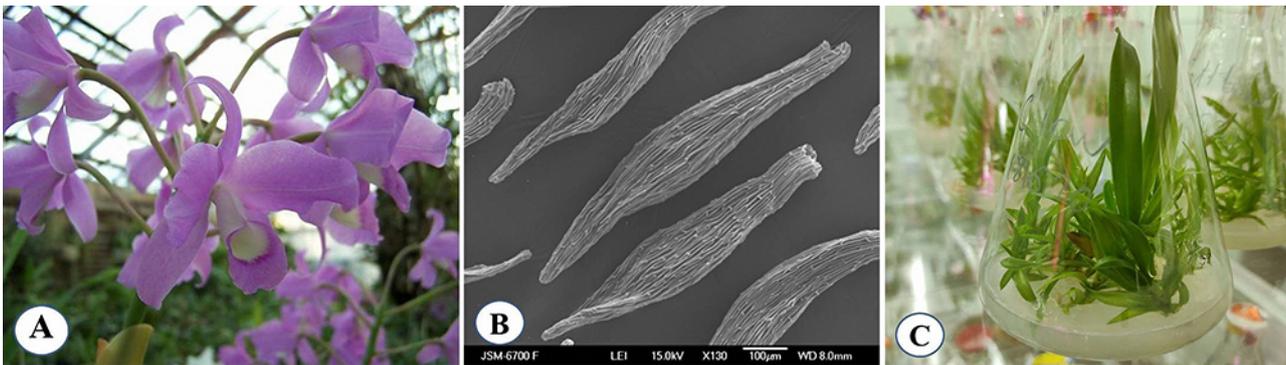
Figure 1 General view of *Guarianthe bowringiana* flower (**A**), seeds (**B**), and successfully propagated in vitro seedlings, maintained at M. M. Gryshko National Botanic Garden (Kyiv, Ukraine) (**C**).

culture media and various substrata (Ávila-Díaz et al., 2009; Pitoyo et al., 2015; da Silva et al., 2013).

Nevertheless, the adaptation of juvenile plants passing through in vitro ex vitro stress barrier, including structural adaptation and antioxidant system response was studied on a narrow range of objects, as evidenced by the few references in the scientific literature (Buiun, 2013; Gudiño et al., 2015; Torres et al., 2006; Torres & Sanabria, 2011).

Change of environment from in vitro to ex vitro conditions is considered as one of the strongest environmental stresses, which greatly reduces the success of any biotechnology project (Hazarika, 2006; Pospíšilová et al., 2007). It is believed that the main cause of plant mortality during acclimatization is water stress (Hazarika, 2003; Teixeira da Silva et al., 2017).

Therefore, to study the adaptive capacity of the seedlings obtained in vitro, it is necessary to take into account the changes in the anatomical and morphological structure and features of the antioxidant system (Batsmanova et al., 2008; Hasanuzzaman et al., 2020). Moreover, to the best of our knowledge, no studies have been carried out to assess the structural adaptation of *G. bowringiana* seedlings to abiotic stress during ex vitro acclimatization from early stage to developmental stages of generative individuals.

In view of the ornamental and conservational importance of *G. bowringiana*, this study was designed to (***i***) assess changes in the antioxidant enzyme activities, photosynthetic capacity, and lipid peroxidation levels during the early stages of acclimatization periods and to (***ii***) conduct a comparative anatomical analysis of juvenile leaves of *G. bowringiana* plants and adult plants cultivated in the conditions of greenhouse culture to find out directions of structural adaptation of plants as it transitions from in vitro to ex vitro environment.

This investigation was conducted as part of a conservation research program focusing on preventing the extinction of rare and endangered orchid species (Buyun et al., 2020).

2. Material and Methods

2.1. Collection of Plant Material

The leaves of juvenile *Guarianthe bowringiana* seedlings grown in vitro and mature plants cultivated under glasshouse conditions were sampled at M. M. Gryshko National Botanic Garden (NBG, Kyiv, Ukraine).

2.2. Acclimatization of In Vitro Propagated Seedlings

An efficient protocol for asymbiotic in vitro seed germination of *G. bowringiana* has previously been established (Buyun et al., 2004; Cherevchenko et al., 2007)

(Figure 1B,C). Seeds and young seedlings were grown on Knudson C medium modified in NBG by the addition of 2 g L^{-1} peptone, 50 mg L^{-1} potassium hummate, and 1 g L^{-1} activated charcoal. For proliferation of protocorms the Murashige and Scoog (MS) (1962) medium supplemented by 5 mg L^{-1} BAP and 2 mg L^{-1} NAA was used. The culture flasks were kept at 25 ± 2 °C, under a 16-hr photoperiod and a photosynthetic photon flux density (PPFD) of 45 µmol m^{-2} s^{-1}.

After 42 weeks, the seedlings (5.5–6.0 cm long) with five leaves and four roots were washed gently with tap water, and directly transferred to a glasshouse with 25 ± 2 °C ambient temperature and 75% ± 5% relative humidity. The seedlings were transplanted into square perforated plastic pots containing *Sphagnum* moss, covered with a plastic film to maintain high humidity, and maintained in the greenhouse under 60% shade. During acclimatization stage, pure *Sphagnum* moss was used as the sole potting medium.

To monitor the changes in the antioxidant enzyme activities, photosynthetic capacity, and lipid peroxidation levels, the leaves were sampled on Day 1, 2, and 7. Leaf surface micromorphology was evaluated from generative individuals raised from in vitro seedlings , cultivated under glasshouse conditions.

2.3. Scanning Electron Microscopy (SEM) Investigation of Leaf Surface Micromorphology

Samples for examination by SEM were kept in 100% tert-butyl alcohol for 1 hr at a temperature of −10 °C. Metal boxes with frozen samples were placed on a table cooled to −60 °C in the working volume of the vacuum universal post (VUP-5M). When vacuuming the working volume, the samples were dried due to the sublimation of frozen tert-butyl alcohol. The dried leaf samples were sputter coated with carbon in vacuum universal post (VUP-5M) and platinum in a JEOL JFC-1600 Auto Fine Coater. Visualization of leaf surface micromorphology of *Guarianthe bowringiana* was undertaken with JEOL JSM-6700F scanning electron microscope at 15 kV acceleration voltage in the high vacuum mode.

2.4. Photosynthetic Pigments Evaluation

Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll (TC), and total carotenoids (Cx + c) concentrations were assessed following the methods described by Porra et al. (1989) using spectrophotometric assays. One hundred milligrams of leaf samples were collected. The sampled leaf material was placed in a 25 mL glass vial, along with 10 mL of 95.5% acetone, and blended using a homogenizer. The glass vials were sealed with parafilm to prevent evaporation and then stored for 48 hr. Samples were kept in darkness at 4 °C to avoid pigment degradation. The Chl *a* and Chl *b* concentrations were measured using a spectrophotometer (Shimadzu UV-1500) at 662 and 644 nm wavelengths. The Cx + c concentration was also measured by a spectrophotometer at 470 nm. A solution of 95.5% acetone was used as blank.

Concentration of pigments (in mg g^{-1} fresh weight of tissue) was calculated using the following equations (Wettstein, 1957):

Chlorophyll a concentration (*Ca*) = 9.784 × *D662* − 0.99 × *D664*,

Chlorophyll b concentration (*Cb*) = 21.426 × *D644* − 4.650 × *D662*,

Total chlorophyll concentration (*Ca* + *Cb*) = 5.134 × *D662* + 20.436 × *D644*,

Total carotenoids concentration (*Cx* + c) = 4.695 × *D470* − 0.268 × (*Ca* + *Cb*),

where *D644*, *D662*, and *D470* are values of optical densities of the solution at wavelengths 644, 662, and 470 nm, respectively.

Statistical analysis of the data was done using the program Statistica 6.0. Probability of the difference between the arithmetic mean of indicators was established using the Student's *t* test. The differences are considered to be significant at a value of *p* < 0.05.

Table 1 Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll (TC), and total carotenoid (Cx + c) content in *Guarianthe bowringiana* seedlings' leaves during the early stage of ex vitro acclimation.

Treatment	RH (%)	Chl *a* (mg g^{-1} FW)	Chl *b* (mg g^{-1} FW)	TC (mg g^{-1} FW)	Cx + c (mg g^{-1} FW)
In vitro	85	0.47 ± 0.01a	0.15 ± 0.01a	0.61 ± 0.06a	0.14 ± 0.01ab
1-day exposure to water stress	70	0.57 ± 0.02b	0.18 ± 0.01b	0.77 ± 0.03b	0.16 ± 0.01a
2-day exposure to water stress	70	0.54 ± 0.02b	0.14 ± 0.01a	0.68 ± 0.03a	0.16 ± 0.01a
7-day exposure to water stress	70	0.37 ± 0.01c	0.14 ± 0.01a	0.51 ± 0.02c	0.13 ± 0.01b

Values are expressed as mean ± *SE*. The values with different superscript letters in a column are significantly different at ($p < 0.05$). RH – relative humidity; FW – fresh weight.

2.5. Assay of 2-Thiobarbituric Acid Reactive Substances (TBARS)

To assess the stage of stress development, the lipid peroxidation process was evaluated by estimating the content of thiobarbituric acid (TBA) reactive substances, such as malondialdehyde (MDA) equivalents (Kumar & Knowles, 1993). Plant tissues (200 mg) were homogenized with 3 mL of 0.1 M Tris-HCl buffer (pH 7.6), and 1 mL of 0.67% TBA solution and 2 mL of 20% trichloroacetic acid was added to the homogenate. The reaction mixture was boiled in a water bath (95 °C) for 30 min. After cooling for 30 min, it was centrifuged at 1,500 *g* for 10 min at 25 °C. The absorption was estimated at 533 nm wavelength using the Shimadzu spectrophotometer. The content of TBA-reactive substances was estimated using the molar extinction coefficient of MAD with the following equation:

$$MDA\ concentration = D533\varepsilon^{-1}\ l^{-1},$$

where D = optical density and ε (the coefficient of molar extinction for malondialdehyde) = 156 mM^{-1} cm^{-1}, and l = the path length of cuvette (1 cm). The results were expressed as nmol MDA equivalents per gram of fresh weight.

2.6. Measurement of Antioxidant Enzyme Activities

To determine the activity of antioxidant enzymes, plant tissues were homogenized in liquid nitrogen after adding 50 mM potassium-phosphate buffer (pH 7.8). The obtained homogenate was centrifuged at 12,000 *g* for 15 min at 4 °C. The activity of superoxide dismutase (SOD, EC 1.15.1.1) was measured according to Giannopolitis and Ries (1977). For this purpose, 50 mL of the extract was added to the reaction mixture containing 1 mL of 0.0015% riboflavin, 1 mL of 5.82% methionine, and 1 mM of 0.154% of nitroblue tetrazolium. The absorption of the solution was estimated at 560 nm after incubation of the reaction mixture in light for 15 min. One unit of SOD activity was estimated as the amount of the enzyme inhibiting the rate of formazan formation by 50%, and expressed as unit U mg^{-1} protein.

3. Results

3.1. The Photosynthetic Pigments in Juvenile *Guarianthe bowringiana* Plants Change After Transplantation to Ex Vitro Environment

Considering that photosynthetic system is the most sensitive indicator of the plant environment determining the plasticity of the species, we have investigated the changes in the content of the major photosynthetic pigments in the leaves of the in vitro grown seedlings transferred to ex vitro conditions. The obtained data is given in Table 1.

The first day after the transfer of *G. bowringiana* seedlings to the glasshouse, the content of Chl *a* increased by 21% and that of Chl *b* by 20%, compared with those
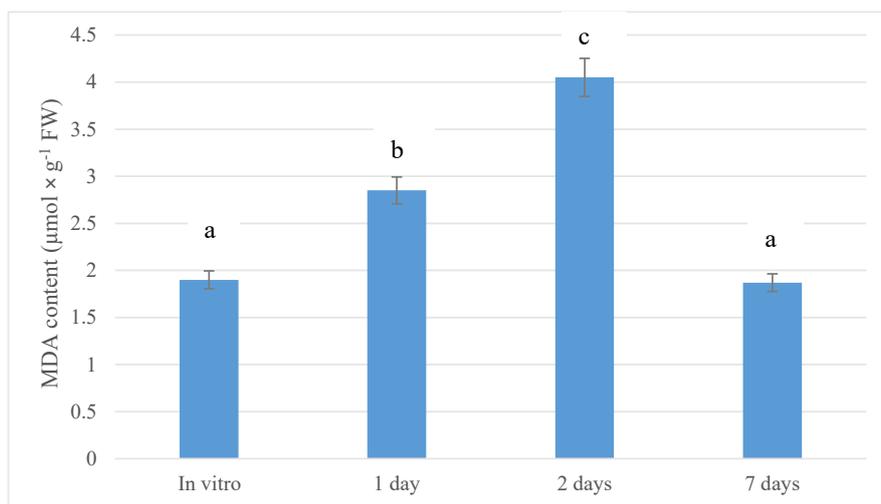
Figure 2 Changes in malondialdehyde (MDA) content during acclimatization of *Guarianthe bowringiana* seedlings after transferring from in vitro to ex vitro conditions. Bars represent the mean ± *SE*; a, b, c – different letters indicate significant differences at *p* < 0.05.

grown in vitro (Table 1). On the second day of exposure, the content of Chl *a* and Chl *b* decreased insignificantly, but by the seventh day of exposure, the content of Chl *a* decreased, while Chl *b* content remained at the same level.

Additionally, the seedlings transferred to the glasshouse also exhibited an increased level of carotenoids (12%) after 2 days of acclimatization. An increase in carotenoid levels specifies that plants sustained the light stress during the early stage of acclimatization. Moreover, such an increase in carotenoids reflects the functional response of photosynthetic apparatus to the altered light environment, since the photoprotective role of carotenoids against photooxidative damage is well documented (Choudhury & Behera, 2001). Nevertheless, by the seventh day, the carotenoid levels decreased significantly (1.2-fold; *p* < 0.05) and were comparable to the baseline. It could be explained by the destruction of carotenoids due to photoinhibition. Furthermore, photoinhibition of photosystem 2 (PS2) in vivo is often considered as a photoprotective strategy rather than a damaging process (Choudhury & Behera, 2001).

3.2. Lipid Peroxidation Levels in the Leaves of *Guarianthe bowringiana* Seedlings as a Marker of Stress Development

MDA is the principal and most intensively studied product of polyunsaturated fatty acid peroxidation which can indicate the increase in oxidative stress (Batsmanova et al., 2008; Del Rio et al., 2005). Therefore, in order to assess the stage of stress development, the lipid peroxidation process was evaluated by estimating TBARS. Lipid peroxidation levels in the leaves of *G. bowringiana* seedlings transferred from cultivation vessels to glasshouses were estimated by determining the concentration of MDA, as presented in Figure 2. The lowest MDA value was detected in the nonstressed plants (in vitro). MDA content increased significantly after transferring to ex vitro conditions. After one day of exposure to glasshouse conditions, MDA levels increased about 1.5-fold (*p* < 0.05). Furthermore, a significant increase of MDA was found after 2 days (2.13 times compared to the in vitro conditions; *p* < 0.05). On the seventh day, this indicator returned to baseline, which may indicate a successful adaptation to altered growing conditions. Analysis of the obtained results of changes in the TBARS content in the leaves of *G. bowringiana*, showed that the transfer of plants propagated in in vitro culture to ex vitro conditions is a certain stress for them (Figure 2).

According to some authors (Ayala et al., 2014; Prasad et al., 1994; Sharma et al., 2012), the activation of lipid peroxidation (LPO) is a universal result of the impact of various stressors on the living system as the result of an enhanced oxidative
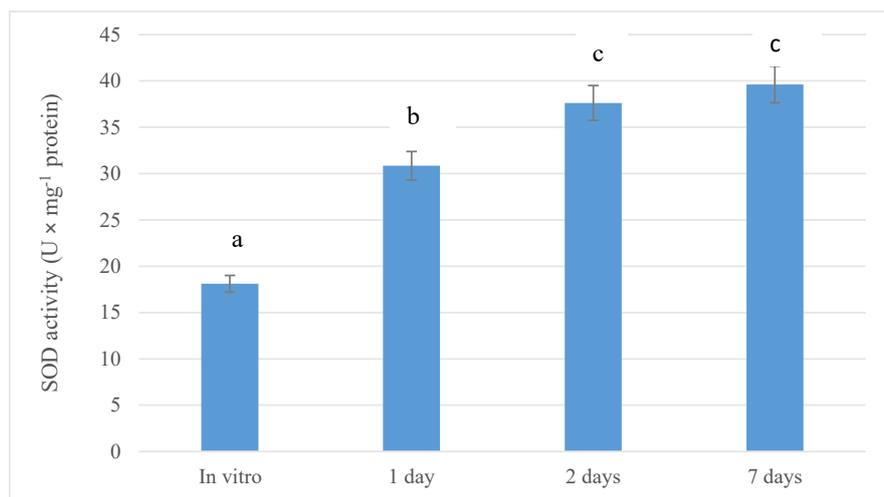
Figure 3 Changes of superoxide dismutase (SOD) activity (U mg^{-1} protein) in the leaves of *Guarianthe bowringiana* seedlings after transferring from in vitro to ex vitro conditions. Bars represent the mean ± *SE*; a, b, c – different letters indicate significant differences at *p* < 0.05.

catabolism of complex organic compounds. The products of this reaction act as the primary mediators of stress because the primary activation of lipid peroxidation is observed in the first stage of stress ("anxiety stage"), and is typical for both plants and animals, so the assessment of LPO intensity by accumulation of secondary products, namely TBARS, can be one of the criteria for the early diagnosis of stress (Del Rio et al., 2005).

3.3. Antioxidant Response of *Guarianthe bowringiana* Seedlings During the Early Stage of Ex Vitro Acclimatization

As summarized in Figure 3, the plants of *G. bowringiana* activated their antioxidative system in response to the altered conditions. In particular, just after 1 day of acclimation (drought stress), SOD activity increased significantly (1.7-fold; *p* < 0.05), compared to the values measured in control (in vitro) plants. The same trend was observed after 2 days (2.0-fold; *p* < 0.05) and seven days (2.2-fold; *p* < 0.05) (Figure 3). In general, the activity of the enzyme increased linearly during the experiment. Thus, it is indicated that the function of the defense enzymes in seedlings was intensified under altered conditions. After 7 days of exposure, this parameter was not monitored.

3.4. Comparative Micromorphology of the Leaves of Juvenile Plants Propagated In Vitro and of Adult Plants Cultivated Under Glasshouse Conditions

The leaf surface micromorphology of *G. bowringiana* juvenile plants propagated in vitro from seeds as well as that of adult plants cultivated in glasshouse was analyzed by SEM. SEM analysis of the leaf micromorphology of *G. bowringiana* plants under various cultivation conditions (in vitro and ex vitro) demonstrated that they all had the same basic structural characteristics (Figure 4), which are common for the species of Laeliinae subtribe in general and have been described previously (Stern & Carlsward, 2009). In vitro propagated *G. bowringiana* plants showed the presence of irregular epidermal cells with curved walls of varying sizes on both the adaxial and abaxial surfaces (Figure 4A,B). Stomata were located only on the lower surface of the leaf (hypostomatous leaves). The type of the stomata is mostly tetracytic which is characterized by two guard cells surrounded by four epidermal cells. Stomata are distributed regularly throughout the leaf surface. Stomatal distribution patterns on the leaves of both juvenile and mature plants grown under glasshouse conditions were the same. Stomata are usually scattered or distributed in small groups.
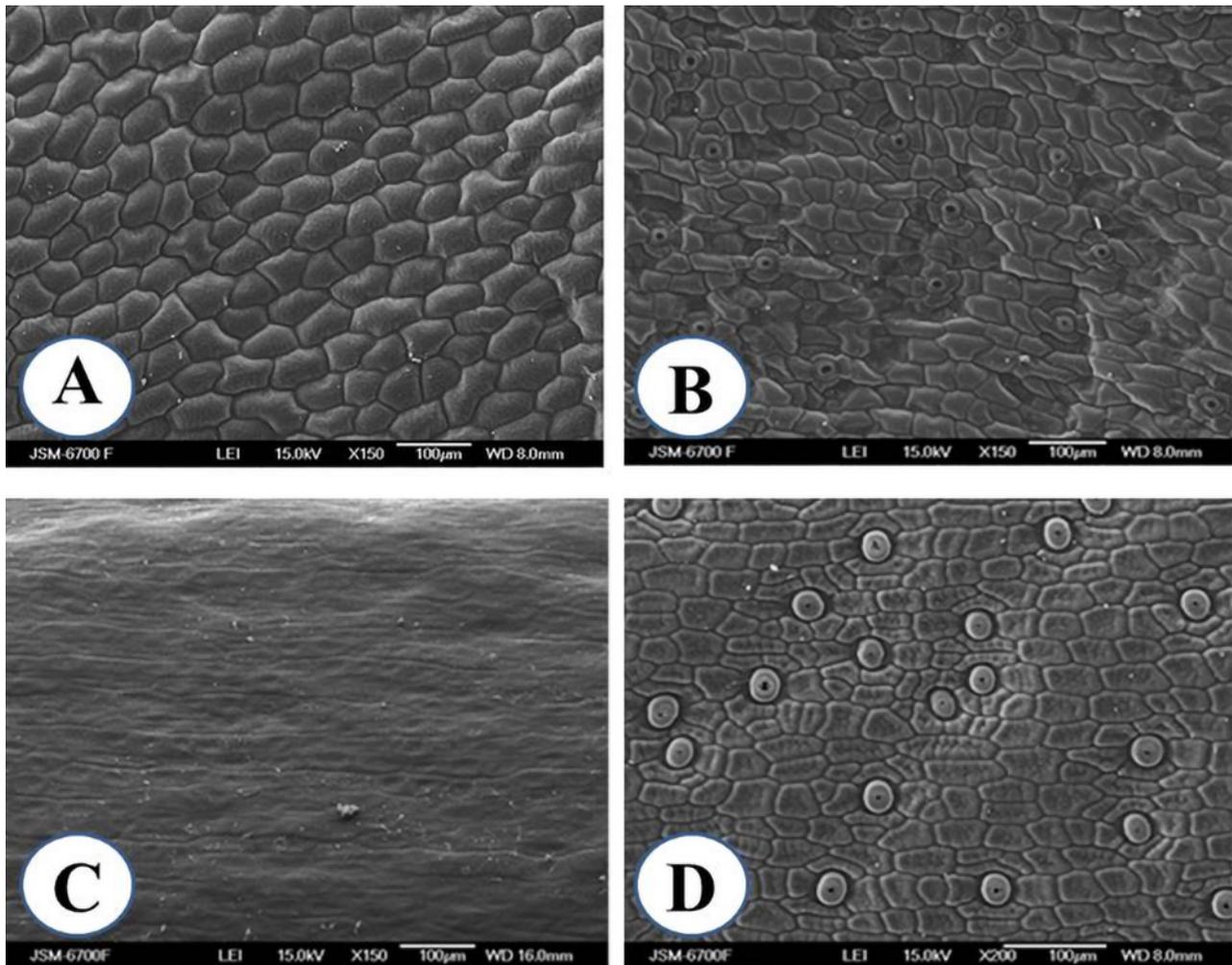
Figure 4 Scanning electron microscopy micrographs of leaf surfaces of *Guarianthe bowringiana* juvenile in vitro seedlings (**A,B**); adult plants grown ex vitro (**C,D**); adaxial surface (**A,C**); abaxial surface (**B,D**).

We observed a single stomata on the adaxial surface of the leaves in juvenile plants from in vitro culture (amphistomaty), apparently, induced by in vitro conditions (Buiun, 2013; Pospíšilová et al., 2007). It should be noted that the adaxial leaf surface view revealed the presence of stomata 6 months after the transfer of juvenile plants from the greenhouse to ex vitro conditions.

Nevertheless, on the leaves newly formed in *G. bowringiana* seedlings after the plants were transferred to glasshouse, stomata were not observed.

The adaxial leaf surface of adult plants is moderately undulate and pavement cells in the adaxial epidermis are difficult to recognize due to well-developed cuticle. Nevertheless, epicuticular wax structures on the adaxial surface have not been revealed. The cells of the abaxial epidermis of the leaves of adult plants are mostly rectangular or irregularly shaped, the walls are straight or curved, and the angles within adjacent boundaries are straight or pointed (Figure 4D).

During the acclimation of in vitro propagated plants to glasshouse conditions, the following changes in the leaf surface micromorphology were observed: (*i*) configuration of epidermal cells changed; (*ii*) dimensions of typical epidermal cells reduced; (*iii*) stomata density significantly decreased ($p < 0.05$), while their dimensions significantly increased ($p < 0.05$).

These data suggest that structural changes, can probably be regarded as an adaptation to avoid excessive rate of transpiration during a period of acclimation of the *G. bowringiana* juvenile plants to the glasshouse conditions. Furthermore,
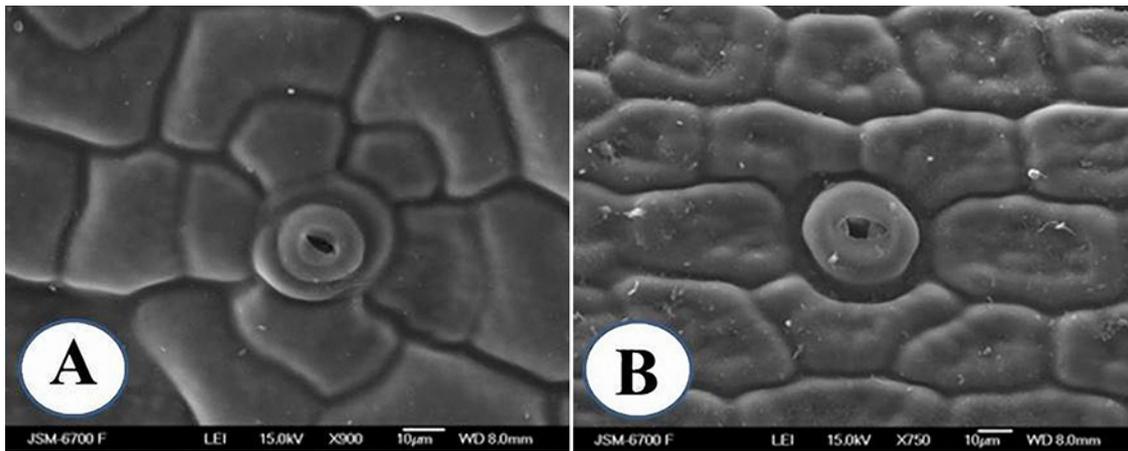
Figure 5 Scanning electron microscopy micrographs of stomatal apparatus of *Guarianthe bowringiana* leaves under different conditions: (**A**) in vitro; (**B**) ex vitro.

acclimation to CO_2 enrichment often involves changes in leaf morphology, stomatal patterning, and structure of the leaf epidermis (Woodward & Williams, 1987).

Investigation of leaf surfaces of both in vitro propagated seedlings and mature plants cultivated at glasshouse conditions has revealed that the number of epidermal cells in adult plants, derived from glasshouse conditions, significantly exceeded the number of cells in juvenile plants, 953.8 ± 47.1 and 781.5 ± 38.4, respectively ($p < 0.05$).

The stomatal density of in vitro and ex vitro transferred plants was 77.01 ± 12.97 and 39.16 ± 6.74, respectively ($p < 0.05$). In vitro propagated plants showed leaves with stomata that had raised, rounded guard cells (Figure 5A), compared with the normal elliptical, sunken guard cells (Figure 5B). The length of the stomata (guard cell length) was significantly shorter in in vitro propagated plants than in adult plants cultivated under glasshouse conditions, 24.3 ± 0.8 μm and 38.1 ± 0.7 μm, respectively ($p < 0.05$). In juvenile plants, the stomata protruded above the surface of the leaves (Figure 5A), while in adult plants they were located in shallow crypts (Figure 5B).

It is believed that a more elliptical shape is characteristic of a functional stomata, while a more spherical shape is frequently considered to be associated with a less functional stomata (Hassankhah et al., 2014).

4. Discussion

4.1. Micromorphological Leaf Changes and Antioxidant Defense System of In Vitro Propagated Plants During Ex Vitro Acclimation

The conservation of endangered orchid species threatened with extinction in the wild can be aided by in vitro propagation, a procedure that enables massive production of plants for ex situ conservation, reintroduction or commercial purposes, thus reducing the pressure on natural populations (Ávila-Díaz et al., 2009; Lando et al., 2016; Lesar et al., 2012; Ortega-Loeza et al., 2011).

Environmental conditions for ex vitro growth are quite different from those used for in vitro cultivation (Hazarika, 2003, 2006; Kozai et al., 1991). Therefore, the advantages of the in vitro orchid propagation method can be fully realized only if the plants propagated in the sterile culture successfully adapt to ex vitro conditions. The altered leaf micromorphology, anatomy, and physiology of orchid plants under in vitro culture environment and during acclimation stage are rarely reviewed and predominantly restricted to plants of commercial importance like *Dendrobium* and *Phalaenopsis* (Cha-um et al., 2009; de Fario et al., 2004; da Silva et al., 2013; Teixeira da Silva et al., 2017).

An important functional and reliable diagnostic marker of the adaptation process in plants is the content and quantitative composition of pigments. It was revealed that plantlets cultured in vitro often show a low rate of photosynthesis and incomplete

autotrophy, and these may be the reasons for low survival rates of plants during the acclimatization stage (de Fario et al., 2004; Jeon et al., 2006; Teixeira da Silva et al., 2017). Thus, the ability to photosynthesize, to maintain water balance, and respond to stress during and after in vitro culture determines the final performance of regenerants. It was revealed that the photosynthetic pigment contents of acclimatized *Phalaenopsis* plantlets decreased significantly after transplantation to in vivo environments (Cha-um et al., 2009). Our results are quite similar to a previous study on photosynthetic pigment stabilization in seedlings of *Calanthe cardioglossa* and *Paphiopedilum appletonianum* subjected to short-term cold stress after transferring from in vitro to ex vitro environment (Batsmanova et al., 2008).

On the contrary, the photosynthetic pigment content in the leaves of in vitro propagated *G. bowringiana* seedlings during the initial days was high. However, chlorophyll *a*/*b* ratio remained unchanged during the initial 7 days, while the total chlorophyll/carotenoids ratio increased. Thus, our findings suggest that photosynthetic capacity of in vitro propagated seedlings was not greatly affected by transferring to ex vitro environment. Consequently, we believe that by 300 days of in vitro cultivation of *G. bowringiana* seedlings, having 5.0 ± 0.24 leaves and 4.0 ± 0.19 roots, we will be able to ensure fully autotrophic growth. The survival rate of propagated in vitro seedlings was about 95%.

Recently, there has been a growing interest in the study of reactive oxygen species (ROS) and the antioxidant defense mechanisms during shoot organogenesis, embryogenesis, and ex vitro acclimatization of in vitro propagated plants (Eslami Fard et al., 2020; Gupta & Datta, 2003).

Under in vitro conditions, plants experience significant stress when exposed to extreme culture conditions (low light intensity, high relative humidity, limited gaseous exchange, higher sucrose or nitrogen levels, and exogenous growth hormones) resulting in morphological and physiological disorders (Ahmed & Anis, 2014).

Because plants are sessile organisms and cannot easily avoid stressful conditions, they have evolved numerous mechanisms for accommodating changes arising in their fluctuating growth conditions to enable functional adaptability under the influence of environmental factors without affecting cellular and developmental physiological processes (Arnold et al., 2019; Monforte, 2020).

Conditions in cultivation vessels under in vitro conditions (high humidity (over 95%), low light ($30-80$ µmol m^{-2} s^{-1}), high sugar content in the culture medium, etc., cause the formation of juvenile plants with abnormalities in the anatomical structure of the leaf (malformed stomata, amphistomaty), which leads to certain changes in water status and even 100% plant death in greenhouse or field conditions (Hazarika, 2003, 2006; Pospišilová et al., 2007).

In vitro acclimatization is one of the key factors in producing healthy plantlets before they are transplanted to ex vitro conditions (Hazarika, 2003, 2006; Pospíšilová et al., 2007).

It is generally suggested that maintaining homeostasis in plants depends on the functions of regulatory systems on the various organizational levels. The increase in ROS levels resulting from many types of impact leads to the induction of antioxidant activities as part of the general adaptation syndrome, first described in medicine for human stress (Selye, 1950). Antioxidant enzymes are an essential part of the defense response.

It is believed, that in vitro orchid micropropagation is an efficient biotechnological strategy for conservation and commercial plantlet production. However, micropropagated plantlets generally need to adapt in order to survive the severe changes in humidity, irradiance, and growth medium that accompany the transfer to ex vitro conditions (Hazarika, 2006; Pospíšilová et al., 2007). The structural changes in the leaves of in vitro cultivated and ex vitro acclimatized plants using qualitative and quantitative analyses have been evaluated in *Cattleya xanthina* (L.) Van den Berg (Lando et al., 2016). It was observed that during acclimatization, a higher accumulation of dry mass, a greater convexity of the outer surface of epidermal cells, an increased deposition of epicuticular waxes, and a greater elongation of mesophyll

parenchymatic cells occurred. Nevertheless, stomatal density was not changed. Furthermore, a need of gradual acclimatization, allowing the species to adapt its best to the new environment, has been emphasized (Lando et al., 2016).

Comparative vegetative anatomy of representatives of Laeliinae subtribe in vivo was studied by Stern and Carlsward (2009). In this subtribe, the exterior surface of the leaf becomes coated with a waterproof layer of cuticle and an extended ledge or lip forms around each stomatal pore, which is known as the outer cuticular ledge (OCL). It should be noted that in our study, the outer stomatal ledge was observed on the leaves' surface of both juvenile and adult plants. The exact functions of this cuticular ledge are unknown, but it has been suggested this structure serves to prevent water loss by sealing the pore when the stomate is closed, and to prevent water droplets from entering when the pore is open (Pautov et al., 2017).

Comparison of the leaf micromorphology of in vitro and ex vitro grown *G. bowringiana* plants did not reveal malformed stomatal apparatus in the former. Moreover, the stomatal apparatus of in vitro seedlings was normal, although the stomatal density was increased, which indicates that these plantlets were more likely to be able to adapt to environmental conditions of the glasshouse.

The decreased stomatal density in adult plants cultivated under glasshouse/field conditions probably could be explained considering the fact that elevation of atmospheric carbon dioxide concentration often results in low stomatal density (Woodward & Williams, 1987). In addition, the higher stomatal density under in vitro conditions may also be influenced by growth hormones as reported in *Leonurus cardiaca* by Stefanova and Koleva (2014).

Alterations in the shape of the stomata directly affects its functionality. It is believed that a more elliptical shape is characteristic of functional stomata, while a more spherical shape is frequently associated with less functional stomata (Hassankhah et al., 2014).

Our findings are in line with data of Torres and Sanabria (2011), who observed that in *Cattleya jenmanii* Rolfe and *C. lueddemanniana* Rchb. f. (Orchidaceae) the adjustment to ex vitro conditions were not completed during eight weeks of acclimatization.

MDA, a lipoxidation end product of polyunsaturated fatty acids, is very often used as a suitable biomarker for lipid peroxidation to measure the level of oxidative stress (Bailly et al., 1996).

The response of plants to the action of stressors and the preservation of homeostasis is the basis of adaptability (Arnold et al., 2019; Monforte, 2020). When there is a change in the environment, seedlings primarily react by transforming the physiological and biochemical processes in order to adapt to altered new environment.

The accumulation of MDA, a byproduct of oxidative damage of membrane lipids, in *G. bowringiana* seedlings under altered (water stress) conditions is indicative of increased lipid peroxidation. These findings are in agreement with the observations reported for other orchid plant species. For example, Batsmanova et al. (2008) showed increased MDA contents in cold-stressed (15 °C, 7 days) in vitro propagated seedlings of orchid species, namely *Calanthe cardioglossa*, *Guarianthe bowringiana*, and *Paphiopedilum appletonianum*, during the early stage (7 days of exposure) of acclimatization to ex vitro conditions.

As a marker that indicates response to oxidative stress, superoxide dismutase, considered as the first line of antioxidant defense enzymes are usually monitored. SOD converts the superoxide anion into hydrogen peroxide. Therefore, this enzyme is used as a biomarker of both oxidative damage and antioxidant defense enzymes in plants (Bose et al., 2017).

In this study, the intensity of SOD in the seedlings exposed to ex vitro conditions was much higher than in the in vitro seedlings, which justifies the ability of the plants with developed antioxidant enzymatic defense system to cope up with oxidative stress during ex vitro transfer (Ahmed & Anis, 2014; Laxa et al., 2019). The exhibited rate of increase of SOD activity allows us to suggest that the activity of this enzyme

reached the highest value on the seventh day of acclimation stage. The further decline in the SOD activity may be correlated with the reduced oxidative stress and membrane peroxidation, which signifies adjustment of plants to the external stress during the latter phase of the acclimatization period (Bose et al., 2017).

Our findings are in accordance with the studies reported on enhanced activities of antioxidant enzymes during in vitro culture and acclimatization in micropropagated orchids such as *Cymbidium giganteum* Wall. (Kumaria et al., 1990) and *Dendrobium fimbriatum* var. *oculatum* (Kumaria & Tandon, 2000).

A similar pattern of increasing SOD activity during the acclimatization period has been previously documented in other orchid species such as *Cattlea* hybrid and *Cymbidium* hybrid (J. Li et al., 2001; Y. Li et al., 2004), *Calanthe cardioflossa* Schltr., and *Paphiopedilum appletonianum* (Gower) Rolfe (Batsmanova et al., 2008) when exposed to cold treatment (15 °C) for 7 days after transferring from in vitro culture to ex vitro conditions.

4.2. Implications for Conservation

The in vitro propagation and acclimatization of *G. bowringiana* and other orchids to produce plants of exceptional quality is an alternative that might facilitate conservation by decreasing the pressure on natural populations. Orchids, especially epiphytes with sympodial habit and inherited long life span, are well adapted for long-time cultivation under glasshouse conditions. Our findings suggest that orchids being long-lived plants are more suitable for long-term conservation than the plants from any other group. The samples of *G. bowringiana* have been maintained at NBG since 1973. Many limitations which are considered to restrict applicability of ex situ approach to conservation, such as loss of species integrity through spontaneous hybridization, are not so crucial in this case.

Since 1999, the whole collection of tropical and subtropical plants (including orchids) has had the status of National Heritage Collection of Ukraine and is supported by a State Funding. Besides, the NBG collection of tropical orchids is registered at the Administrative Organ of CITES in Ukraine (Ministry of Environment Protection, registration No. 6939/19/1-10 of June 23, 2004).

5. Conclusions

Thus, the *G. bowringiana* plants adapting to the altered conditions passed through two successive phases of stress response. During the first phase ("anxiety phase") lipoperoxidation process was activated, while the second phase was characterized by stabilization of pro-oxidant and antioxidant balance. In this case, it occurred on the seventh day of exposure to ex vitro conditions. It can be defined as a phase of resistance in which the intensity of LPO decreased due to the increase in antioxidant resources.

The new leaves formed during the ex vitro acclimatization stage (in glasshouse) exhibited the development of adaptive micromorphological features in in vitro propagated plants (decreased stomatal density and normal stomatal apparatus), which promotes the autotrophy enabling plants to survive under altered conditions (high light intensity and low relative humidity in comparison with in vitro culture conditions).

In conclusion, our findings indicate that the MDA content and SOD activity level could be used as early stress markers (the first line of defense) to assess the stress development, particularly during early acclimatization stage in orchids of different ecological groups and to estimate the tolerance limits when changing from in vitro to ex vitro conditions.

The evaluation of structural characteristics and specificity of their interactions with the environment allows us to optimize the growth conditions and enhance plant resistance through induction of natural defense mechanisms.

Additionally, these findings may serve as a basis for the commercial production of this threatened species.

References

Ahmed, M. R., & Anis, M. (2014). Changes in activity of antioxidant enzymes and photosynthetic machinery during acclimatization of micropropagated *Cassia alata* L. plantlets. *In Vitro Cellular and Developmental Biology – Plant*, *50*, 601–609. https://doi.org/10.1007/s11627-014-9609-1

Arnold, P. A., Kruuk, L. E., & Nicotra, A. B. (2019). How to analyse plant phenotypic plasticity in response to a changing climate. *New Phytologist*, *222*(3), 1235–1241. https://doi.org/10.1111/nph.15656

Astarini, I. A., Claudia, V., Adi, N. K. A. P., Sudirga, S. K., & Astiti, N. P. A. (2015). In vitro propagation and acclimatization of black orchid (*Coelogyne pandurata* Lindl.). *Acta Horticulturae*, *1078*, 155–158. https://doi.org/10.17660/ActaHortic.2015.1078.21

Ávila-Díaz, I., Oyama, K., Gómez-Alonso, C., & Salgado-Garciglia, R. (2009). In vitro propagation of the endangered orchid *Laelia speciosa*. *Plant Cell, Tissue and Organ Culture*, *99*, Article 335. https://doi.org/10.1007/s11240-009-9609-8

Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity*, *2014*, Article 360438. https://doi.org/10.1155/2014/360438

Bailly, C., Benamar, A., Corbineau, F., & Dôme, D. (1996). Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seed as related to deterioration during accelerated aging. *Physiologia Plantarum*, *97*, 104–110. https://doi.org/10.1111/j.1399-3054.1996.tb00485.x

Бацманова [Batsmanova], Л. М. [L. M.], Буюн [Buiun], Л .I. [L. I.], Ковальська [Koval's'ka], Л. А. [L. A.], & Таран [Taran], Н. Ю. [N. Iu.]. (2008). Про-антиоксиданти листків деяких видів тропічних орхідних за низькотемпературної адаптації [Pro-antioxidants in leaves of some species of tropical orchids under conditions of low-temperature adaptations]. *Український ботанічний журнал* [Ukrainian Botanical Journal], *65*(6), 912–921.

Bose, B., Kumaria, S., Choudhury, H., & Tandon, P. (2017). Insights into nuclear DNA content, hydrogen peroxide and antioxidative enzyme activities during transverse thin cell layer organogenesis and ex vitro acclimatization of *Malaxis wallichii*, a threatened medicinal orchid. *Physiology and Molecular Biology of Plants*, *23*(4), 955–968. https://doi.org/10.1007/s12298-017-0474-3

Буюн [Buiun], Л. [L.]. (2013). Адаптивні зміни поверхні листка тропічної орхідеї *Cattleya gaskelliana* (N. E. Br.) D. S. Williams при зміні умов культивування (in vitro → ex vitro) [Adaptative changes of leaf surface of tropical orchid *Cattleya gaskelliana* (N. E. Br.) Williams after transferring from in vitro to ex vitro conditions]. *Modern Phytomorphology*, *3*, 235–238.

Buyun, L., Gaidarzhy, M., & Prokopiv, A. (2020). Living collections of tropical plants as national heritage collections of Ukraine. In M. D. Espírito-Santo, A. L. Soares, & M. Veloso (Eds.), *Botanic gardens, people and plants for a sustainable world* (pp. 105–113). IsaPress.

Buyun, L., Lavrentyeva, A., Kovalska, L., & Ivannikov, R. (2004). In vitro germination of seeds of some rare tropical orchids. *Acta Universitatis Latviensis, Biology*, *676*, 159–162.

Cha-um, S., Puthea, O., & Kirdmanee, C. (2009). An effective in-vitro acclimatization using uniconazole treatments and ex-vitro adaptation of *Phalaenopsis* orchid. *Scientia Horticulturae*, *121*, 468–473. https://doi.org/10.1016/j.scienta.2009.02.027

Cherevchenko, T. M., Buyun, L. I., Kovalska, L. A., & Long, V. N. (2007). Ex situ conservation of tropical orchids in Ukraine. *Lankesteriana*, *7*(1–2), 129–133. https://doi.org/10.15517/lank.v7i1-2.18451

Choudhury, N. K., & Behera, R. K. (2001). Photoinhibition of photosynthesis: Role of carotenoids in photoprotection of chloroplast constituents. *Photosynthetica*, *39*(4), 481–488. https://doi.org/10.1023/A:1015647708360

Convention on International Trade in Endangered Species of Wild Fauna and Flora Appendices I, II and III. (2020). https://cites.org/sites/default/files/eng/app/2020/E-Appendices-2020-08-28.pdf

Coutiño-Cortés, A. G., Bertolini, V., Iracheta-Donjuan, L., Ruiz-Montoya, L., & Valle-Mora, J. F. (2017). In vitro callogenesis induction of *Guarianthe skinneri* (Bateman) Dressler & W. E. Higgins (Orchidaceae). *Acta Agronomica*, *66*(2), 254–259. https://doi.org/10.15446/acag.v66n2.57982

da Silva, J. M., Jr., Rodrigues, M., de Castro, E. M., Kelly, S., Bertolucci, S. K. V., & Pasqual, M. (2013). Changes in anatomy and chlorophyll synthesis in orchids propagated in vitro in the presence of urea. *Acta Scientiarum, Agronomy*, *35*(1), 65–72. https://doi.org/10.4025/actasciagron.v35i1.15356

de Fario, R. T., Rodrigues, F. N., Oliveira, L. V. R., & Müller, C. (2004). In vitro *Dendrobium nobile* plant growth and rooting in different sucrose concentrations. *Horticultura Brasileira*, *22*(4), 780–783. https://doi.org/10.1590/S0102-05362004000400023

Del Rio, D., Stewart, A. J., & Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, Metabolism & Cardiovascular Diseases*, *15*, 316–328. https://doi.org/10.1016/j.numecd.2005.05.003

Díaz-Álvarez, E. A., Torres-Galeano, C., Rojas-Cortés, A. P., & de la Barrera, E. (2015). In-vitro germination and development of two endangered endemic Colombian orchids, *Cattleya mendelii* and *Cattleya quadricolor*. *Gayana Botánica*, *72*, 303–310. https://doi.org/g779

Dressler, R., & Higgins, W. (2003). *Guarianthe*, a generic name for the "*Cattleya*" *skinneri* complex. *Lankesteriana*, *7*(2), 37–38. https://doi.org/10.15517/lank.v3i2.23009

Dressler, R., Pridgeon, A., & Veitch, N. C. (2005). 302. *Guarianthe*. In A. M. Pridgeon, P. J. Cribb, M. W. Chase, & F. N. Rasmussen (Eds.), *Genera Orchidacearum, Vol. 4. Epidendroideae (Part 1)* (pp. 251–254). Oxford University Press.

Eslami Fard, S., Yarnia, M., Farahvash, F., Khalilvand Behrouzyar, E., & Rashidi, V. (2020). Arbuscular mycorrhizas and phosphorus fertilizer affect photosynthetic capacity and antioxidant enzyme activity in peppermint under different water conditions. *Acta Agrobotanica*, *73*(4), Article 7345. https://doi.org/10.5586/aa.7345

Giannopolitis, C. N., & Ries, S. K. (1977). Superoxide dismutases: I. Occurrence in higher plants. *Plant Physiology*, *59*(2), 309–314. https://doi.org/10.1104/pp.59.2.309

Gravendeel, B., Smithson, A., & Slik, F. J. W. (2004). Epiphytism and pollinator specialization: Drivers for orchid diversity? *Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences*, *359*(1450), 1523–1535. https://doi.org/10.1098/rstb.2004.1529

Guarianthe bowringiana *(O'Brien) Dressler & W. E. Higgins*. (2021). World Checklist of Selected Plant Families. Retrieved September 3, 2021, from https://wcsp.science.kew.org/namedetail.do?name_id=247365.

Gudiño, W., Ávila-Díaz, I., Oyama, K., & de la Barrera, E. (2015). High-temperature tolerance by the endangered Mexican Mayflower orchid, *Laelia speciosa*. *Tropical Conservation Science*, *8*(4), 983–991. https://doi.org/10.1177/194008291500800408

Gupta, S. D., & Datta, S. (2003). Antioxidant enzyme activities during in vitro morphogenesis of gladioulus and the effect of application of antioxidants on plant regeneration. *Biologia Plantarum*, *47*(2), 179–183. https://doi.org/bjvtkg

Hasanuzzaman, M., Bhuyan, M. H. M. B., Zulfiqar, F., Raza, A., Mohsin, S. M., Mahmud, J. A., Fujita, M., & Fotopoulos, V. (2020). Reactive oxygen species and antioxidant defense in plants under abiotic stress: Revisiting the crucial role of a universal defense regulator. *Antioxidants*, *9*(8), 681. https://doi.org/10.3390/antiox9080681

Hassankhah, A., Vahdati, K., Lotfi, M., Mirmasoumi, M., Preece, J., & Assareh, M.-H. (2014). Effects of ventilation and sucrose concentrations on the growth and plantlet anatomy of micropropagated persian walnut plants. *International Journal of Horticultural Science and Technology*, *1*(2), 111–120.

Hazarika, B. N. (2003). Acclimatization of tissue-cultured plants. *Current Science*, *85*(12), 1704–1712.

Hazarika, B. N. (2006). Morpho-physiological disorders in in vitro culture of plants. *Scientia Horticulturae*, *108*(2), 105–120. https://doi.org/10.1016/j.scienta.2006.01.038

Hinsley, A., de Boer, H. J., Fay, M. F., Gale, S. W., Gardiner, L. M., Gunasekara, R. S., Kumar, P., Masters, S., Metusala, D., Roberts, D. L., Veldman, S., Wong, S., & Phelps, J. (2017). A review of the trade in orchids and its implications for conservation. *Botanical Journal of the Linnean Society*, *186*(4), 435–455. https://doi.org/10.1093/botlinnean/box083

International Union for Conservation of Nature. (2017). *Guidelines for Using the IUCN Red List Categories and Criteria, Version 13*. https://www.iucnredlist.org/resources/redlistguidelines/

Іванніков [Ivannikov], Р. В. [R. V.]. (2012). *Репродукційна біологія орхідних in vitro* [Reproductive biology of orchids in vitro] [Unpublished doctoral dissertation]. Національний ботанічний сад імені М. М. Гришка НАН України [M. M. Gryshko National Botanic Garden, NAS of Ukraine].

Jeon, M.-W., Ali, M. B., Hahn, E.-J., & Paek, K.-Y. (2006). Photosynthetic pigments, morphology and leaf gas exchange during ex vitro acclimatization of micropropagated CAM *Doritaenopsis* plantlets under relative humidity and air temperature. *Environmental and Experimental Botany*, *55*(1–2), 183–194. https://doi.org/10.1016/j.envexpbot.2004.10.014

Kozai, T., Iwabuchi, K., Watanabe, K., & Watanabe, I. (1991). Photoautotrophic and photomixotrophic growth of strawberry plantlets in vitro and changes in nutrient composition of the medium. *Plant Cell, Tissue and Organ Culture*, *25*, 107–115.

Kumar, G., & Knowles, N. R. (1993). Changes in lipid peroxidation and lipolytic and free-radical scavenging enzyme activities during aging and sprouting of potato (*Solanum tuberosum*) seed-tubers. *Plant Physiology*, *102*(1), 115–124. https://doi.org/10.1104/pp.102.1.115.30

Kumaria, S., & Tandon, P. (2000). Effect of growth regulators on peroxidase, polyphenol oxidase, and IAA-oxidase activities and phenolic contents during protocorm development of *Dendrobium fimbriatum* var. *oculatum* Hook. f. *The Journal of the Orchid Society of India*, *14*, 27–39.

Kumaria, S., Chrungoo, N. K., & Tandon, P. (1990). Activities of some oxidative enzymes in axenic cultures of protocorms of *Cymbidium giganteum* Wall. as influenced by different growth regulators. *The Journal of the Orchid Society of India*, *4*, 37–44.

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

Laxa, M., Liebthal, M., Telman, W., Chibani, K., & Dietz, K.-J. (2019). The role of the plant antioxidant system in drought tolerance. *Antioxidants (Basel)*, *8*(4), Article 94. https://doi.org/10.3390/antiox8040094

Lesar, H., Hlebec, B., Čeranič, N., Kastelec, D., & Luthar, Z. (2012). Acclimatization of terrestrial orchid *Bletilla striata* Rchb. f. (*Orchidaceae*) propagated under in vitro conditions. *Acta Agriculturae Slovenica*, *99*(1), 69–75. https://doi.org/10.2478/v10014-012-0001-8

Li, J., Matsui, S., & Onwona-Agyeman, S. (2001). Effects of cultural temperatures on antioxidative levels in leaves of *Cattlea hybrid* and *Cymbidium hybrid*. *Environmental Control in Biology*, *39*, 1–7. https://doi.org/10.2525/ecb1963.39.1

Li, Y., Imai, K., Ohno, H., & Matsui, S. (2004). Effects of acclimatization temperatures on antioxidant enzyme activities in mericlones of a *Cattleya* hybrid. *Journal of the Japanese Society for Horticultural Science*, *73*, 386–392. https://doi.org/10.2503/jjshs.73.386

Mondragón, D. (2009). Population viability analysis for *Guarianthe aurantiaca*, an ornamental epiphytic orchid harvested in Southeast Mexico. *Plant Species Biology*, *24*(1), 35–41. https://doi.org/10.1111/j.1442-1984.2009.00230.x

Monforte, A. J. (2020). Time to exploit phenotypic plasticity. *Journal of Experimental Botany*, *71*(18), 5295–5297. https://doi.org/10.1093/jxb/eraa268

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, *15*, 473–497. https://doi.org/dh6dj8

Ortega-Loeza, M. M., Salgado-Garciglia, R., Gómez-Alonso, C., & Ávila-Díaz, I. (2011). Acclimatization of the endangered Mexican epiphytic orchid, *Laelia speciosa* (H. B. K.) Schltr. *European Journal of Environmental Sciences*, *1*(2), 48–54.

Pautov, A., Bauer, S., Ivanova, O., Krylova, E., Sapach, Y., & Gussarova, G. (2017). Role of the outer stomatal ledges in the mechanics of guard cell movements. *Trees*, *31*, 125–135. https://doi.org/10.1007/s00468-016-1462-x

Pitoyo, A., Hani, M. R., & Anggarwulan, E. (2015). Application of chitosan spraying on acclimatization success of tiger orchid (*Grammatophyllum scriptum*) plantlets. *Nusantara Bioscience*, *7*, 179–185. https://doi.org/10.13057/nusbiosci/n070222

Porra, R. J., Thompson, W. A., & Kriedemann, P. E. (1989). Determination of accurate extinction coefficients and simultaneous equation for assaying chlorophyll *a* and *b* with four different solvents: Verification of the concentration of chlorophyll by atomic absorption spectroscopy. *Biochimica et Biophysica Acta*, *975*, 384–394. https://doi.org/cjrxr6

Pospíšilová, J., Synková, H., Haisel, D., & Semorádová, Š. (2007). Acclimatization of plantlets to ex vitro conditions: Effects of air humidity, irradiance, CO_2 concentration and abscisic acid (a review). *Acta Horticulturae*, *748*, 29–38. https://doi.org/10.17660/ActaHortic.2007.748.2

Prasad, T. K., Anderso, M. D., Martin, B. A., & Steward, C. R. (1994). Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. *Plant Cell*, *6*, 65–74. https://doi.org/10.2307/3869675

Selye, H. (1950). *Stress: The physiology and pathology of exposure to stress*. ACTA Publications.

Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany*, *2012*, Article 217037. https://doi.org/10.1155/2012/217037

Stefanova, M. A., & Koleva, D. P. (2014). Leaf's structural response of in vitro cultured *Leonurus cardiaca* plants to N^6-benzyladenine and indole-3-butyric acid. *Journal of Pharmacy Research*, *8*, 1014–1021.

Stern, W. L., & Carlsward, B. S. (2009). Comparative vegetative anatomy and systematics of Laeliinae (Orchidaceae). *Botanical Journal of the Linnean Society*, *160*, 21–41. https://doi.org/10.1111/j.1095-8339.2009.00818.x

Teixeira da Silva, J. A., Hossain, M. M., Sharma, M., Dobranszki, J., Cardoso, J. C., & Zeng, S. (2017). Acclimatization of in vitro-derived *Dendrobium*. *Horticultural Plant Journal*, *3*(3), 110–124. https://doi.org/10.1016/j.hpj.2017.07.009

Ticktin, T., Mondragon, D., Lopez-Toledo, L., Dutra-Elliot, D., Aguirre-Leon, E., & Hernandez-Apolinar, M. (2020). Synthesis of wild orchid trade and demography provides new insight on conservation strategies. *Conservation Letters*, *13*, Article e12697. https://doi.org/10.1111/conl.12697

Torres, J., Laskowski, L., & Sanabria, M. (2006). Efecto del ambiente de desarrollo sobre la anatomía de la epidermis foliar de *Cattleya jenmanii* Rolfe [Environmental effect during growth on anatomical characteristics of leaf epiderm in *Cattleya jenmanii* Rolfe]. *Bioagro*, *18*(2), 93–99.

Torres, J. A., & Sanabria, M. (2011). Effect of the development environment on the leaf anatomy of *Cattleya jenmanii* Rolfe and *C. lueddemanniana* Rchb. f. (Orchidaceae). *Acta Botanica Venezuelica*, *34*(1), 199–214.

van den Berg, C., Higgins, W. E., Dressler, R. L., Whitten, W. M., Soto Arenas, M. A., Culham, A., & Chase, M. W. (2000). A phylogenetic analysis of Laeliinae (Orchidaceae) based on sequence data from internal transcribed spacers (ITS) of nuclear ribosomal DNA. *Lindleyana*, *15*, 96–114.

Wettstein, D. (1957). Chlorophyll-letale und der submikroskopische Formwechsel der Plastiden [Chlorophyll lethals and submicroscopic morphological changes in plastids]. *Experimental Cell Research*, *12*(3), 427–433. https://doi.org/10.1016/0014-4827(57)90165-9

Woodward, F. I., & Williams, B. G. (1987). Climate and plant distribution at global and local scales. *Vegetatio*, *69*, 189–197. https://doi.org/10.1007/BF00038700

Zhang, W., Hu, H., & Zhang, S.-B. (2016). Divergent Adaptive strategies by two co-occurring epiphytic orchids to water stress: Escape or avoidance? *Frontiers in Plant Science*, *7*, Article 588. https://doi.org/10.3389/fpls.2016.00588