

ACCLIMATIZATION OF THE ENDANGERED MEXICAN EPIPHYTIC ORCHID, *LAELIA SPECIOSA* (H.B.K.) SCHLTR.

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ABSTRACT

In vitro propagation could be an alternative for the conservation of the endemic and endangered Mexican epiphytic orchid *Laelia speciosa* (H.B.K.) Schltr. The goal of this study was to develop a protocol that would enhance acclimatization of *in vitro* – derived *L. speciosa* plantlets – a critical stage in propagation and subsequent conservation. Observations of stomata opening during *ex vitro* acclimatization, and the time of *in vitro* culture (0, 5, 10, 15, 20, 25, 30 days) in greenhouse conditions (pre-acclimatization), on the survival and development of seedlings during the *ex vitro* acclimatization were carried out. In addition, the effect of different levels of nutrients (100%, 75%, 50%, 25% and 0%-strength salts) and sucrose (0, 10, 20, 30, 40 g l⁻¹) in the Murashige and Skoog medium (MS) on the same parameters were measured. Plantlets incubated 20 days in greenhouse conditions before *ex vitro* acclimatization also displayed the best growth with a survival rate of 97.5%, related with high stomata opening. Plantlets on MS containing 100%-strength salts (with 20 days of pre-acclimatization, 40 g l⁻¹ sucrose) had the highest rate (97.5–100%) of survival and vigor when acclimatized. By improving micropropagation through acclimatization, the sustainable management of *L. speciosa* now more likely, benefitting the conservation of this endangered species.

Keywords: acclimatization, *in vitro*, micropropagation, orchid

Introduction

The endemic Mexican epiphytic orchid *Laelia speciosa* (H.B.K.) Schltr., is threatened with extinction due to overcollecting for ornamental, cultural, and/or commercial purposes. The destruction of its habitat has also led to drastically diminished existing populations. A multidisciplinary project of study on *L. speciosa*, includes biological research and work with local people in communities to reach a sustainable management. An alternative for sustainable management of *L. speciosa* includes *in vitro* propagation. Such protocols have been developed for the establishment and *in vitro* propagation of this species through seed (Ávila-Díaz et al. 2009) and micropropagation from callus and shoot regeneration (Sarabia et al. 2010). These studies reported a survival as high as 70% to 77.5% in plantlets and seedlings of 5 cm in length after 30 days of being transplanted using tezontle (small red basaltic-volcanic-rocks) and oak bark 1 : 1 as substrate. However, plantlets survival diminished over time during cultivation with a 30% survival rate after 90 days.

Similarly, many other species of horticultural plants are easily micropropagated *in vitro* but exhibit poor acclimatization and subsequent survival *ex vitro* (Debergh and Zimmerman 1991). *In vitro* plantlets produced under high humidity conditions and transferred *ex vitro* to the

soil are usually very susceptible to desiccation (Wetzstein and Sommer 1983). Typically, low light intensities *in vitro* result in the production of leaves that resemble shade or hydrophytic leaves *ex vitro* (Lee et al. 1988). These leaves often have little epicuticular and cuticular wax formation (Grout 1975) and malfunctioning stomata (Brainerd and Fuchigami 1981). Some medium components, such as sucrose, also affect the morphology of plantlets.

Acclimatization is also a critical stage in orchid micropropagation, because there are high percentages of damaged and lost plants whenever they are transferred to *ex vitro* conditions (Torres et al. 2006; Deb and Imchen 2010). Many different approaches toward efficient *ex vitro* establishment of the *in vitro* raised orchids have been employed, such as using different *in vitro* culture media, alternate substratum (Deb and Imchen 2010), hydroponic systems (Lopes da Silva 2006) and concentration of agar and sucrose in the medium; since it is said to have an effect on plant development on *ex situ* conditions (Deb and Imchen 2010).

The usage of different potting mixtures on the transplantation stage of orchid acclimatization has been investigated to increase the survival of some orchids like *Euchile mariae* (Suárez-Quijada et al. 2007), *Laelia speciosa* (Ávila-Díaz et al. 2009), *Cattleya maxima x nobilior* (Díaz et al. 2010), *Cattleya harrisoniana* and *Laelia pur-*

purata (Seidel-Junior and Augusto-Venturieri 2011). For the genus *Phalaenopsis* and *Cattleya* (Diaz et al. 2010), as well as for *Laelia speciosa* (Ávila-Díaz et al. 2009), size of seedlings has been researched for acclimatization purposes, in the same manner, using diverse plant growth regulators during acclimatization has been useful in increasing root and vegetative development, such is the case of the application of 200 mg l⁻¹ of NAA sprayed on seedlings of *Oncidium baueri* (Sorace et al. 2007).

In the current study, we have established an efficient system of acclimatization of seedlings propagated *in vitro* of *L. speciosa*, determining the effect of difference in time of exposure in a greenhouse of *in vitro* cultures of *L. speciosa* seedlings (pre-acclimatization) on growth and stomatal dynamics during the phase of acclimatization. We also analyzed the effect of different concentrations of nutrients and sucrose on MS basal medium during *in vitro* culture, to analyze the growth and survival of *L. speciosa* seedlings in the acclimatization.

Methods

Time of *in vitro* culture in greenhouse conditions (pre-acclimatization)

Effect of pre-acclimatization on plant growth

After 10 months of *in vitro* culture, 210 seedlings of *Laelia speciosa* (H.B.K.) Schltr., were used to investigate the effect of time of *in vitro* culture in greenhouse conditions (pre-acclimatization) on the survival and development of seedlings and stomata opening during the *ex vitro* acclimatization. These seedlings were grown previously on MS medium (Murashige and Skoog 1962) without plant growth regulators, with 30 g/l of sucrose, pH 5.7 and under light conditions (suggested by Ávila-Díaz et al. 2009), proceeding from a mature capsule.

Seven study groups were carried out with different treatments of pre-acclimatization as follows: 0, 5, 10, 15, 20, 25, and 30 days. Each study group consisted of 10 (120 ml) glass jars (with 3 seedlings of ca. 5 cm in length). Each glass jar contained 25 ml of MS which was covered with clear plastic caps.

After the permanence of the *in vitro* cultures in greenhouse conditions, each plant was washed carefully with distilled water to eliminate agar residue, and was then

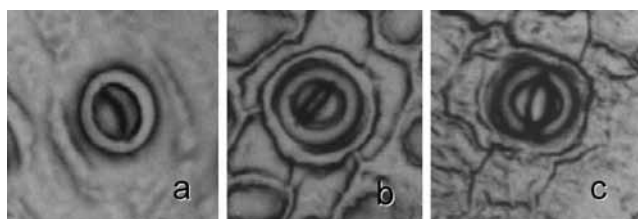


Fig. 1 Categories of stomata opening: 1, closed stomata (a); 2, partially open stomata (b) and 3, open stomata (c).

placed on absorbent paper to determine the following measurements: length and width of the new leaf, length of the new pseudobulb, length and width of the roots of this pseudobulb and vigor was recorded. The measured parts were marked with a small indelible point to identify and measure the same structures subsequently. Seedlings were transplanted into individual plastic pots with tezontle-oak bark; 1 : 1 as was suggested by Ávila-Díaz et al. in 2009. The fungicide thiabendazole (Tecto 60) was applied in a concentration of 1 g l⁻¹, to prevent any possible infection by pathogenic fungi.

Seedlings were maintained in acclimatization chambers with clear plastic bags. Bags were gradually opened every 2 days until they were completely removed after 30 days. After 7 months, the previously mentioned parameters and survival were also recorded.

Data were analyzed using one-way analysis of variance and Tukey Post Hoc test for the difference between final and initial observations for each parameter, assisted by SPSS 18.0 program for Windows (SPSS Inc. Chicago, IL, USA).

Effect of pre-acclimatization on stomata opening

Stomata opening during *ex vitro* acclimatization was evaluated on 10 randomly-selected seedlings for each pre-acclimatization treatment. Impressions on the abaxial side of new leaves were made with a dental mold pulp exactodent, at 5:00 a.m., 9:00 a.m., and 2:00 p.m. once a month (June to September), once the 30-day of pre-acclimatization seedlings were transplanted, during 4 months. The exactodent mold was covered with transparent nail polish, and once it dried, it was removed and observed under light microscopy to assess stomata opening on 10 stomata on each seedling. Categories were established based on stomata opening, assigning 1 to closed stomata, 2 to stomata partially open and 3 to open stomata (Fig. 1).

Data were analyzed in the same manner described previously.

Effect of different levels of MS nutrients previous to acclimatization

Seedlings proceeding from seeds of a mature capsule, grown *in vitro* as previously described, were subcultured in MS medium with different levels of nutrients (100%, 75%, 50%, 25%, and 0%-strength salts). Each study group consisted of 10 (120 ml) glass jars, each one containing 4 seedlings. After 60 days, seedlings were placed in greenhouse conditions for 20 more days and transplanted into individual plastic pots which were maintained in acclimatization chambers, uncovering gradually as described above. Seedling survival, length and width of the new leaf, length of the new pseudobulb, length and width of the largest root of this pseudobulb, and vigor were recorded during transplant, and after 5 months.

Data were analyzed in the same manner described previously.

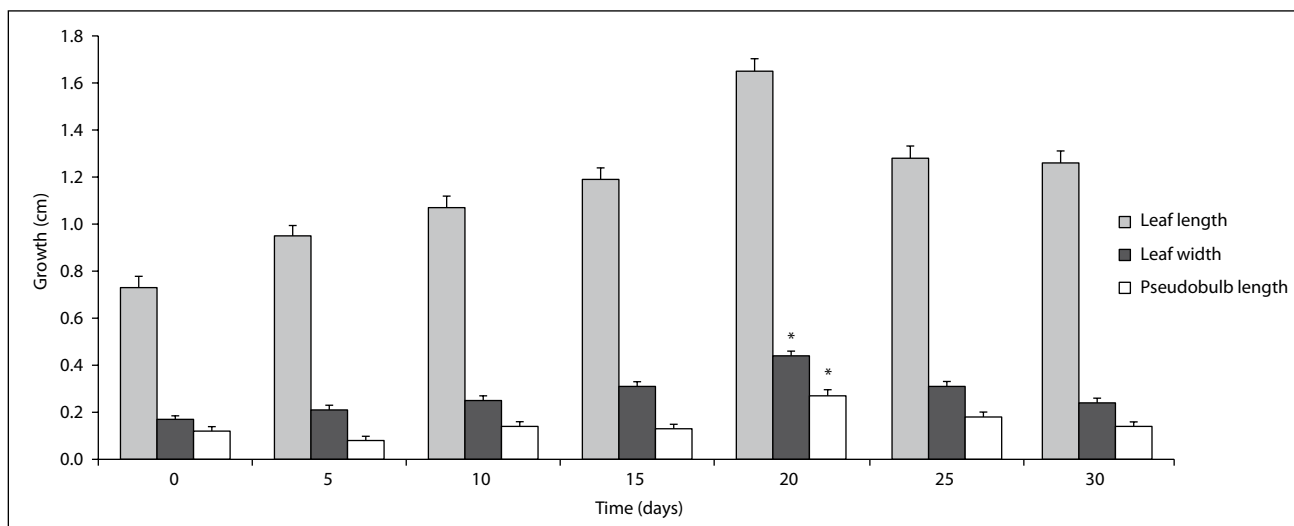


Fig. 2 Effect of the pre-acclimatization time on *Laelia speciosa* growth, * means significant differences at $P > 0.05$.

Effect of sucrose concentration in the MS medium previous to acclimatization

We studied the effect of different sucrose concentration (0, 10, 20, 30, 40 g l⁻¹) in the MS during 60 days of *in vitro* culture on the same parameters described before during acclimatization.

Seedlings used in this experiment were of the same source than those of the previous experiment. In addition, 40 similar seedlings for each study group were used, 10 (120 ml) glass jars, each one containing 4 seedlings. Before transplant, seedlings *in vitro*, were placed in greenhouse conditions for 20 more days and were transplanted and acclimatized, as in previous experiments.

Data were analyzed in the same manner described previously.

The acclimatization took place in a greenhouse with the following average temperatures 26 °C, during the day and 18 °C during the night and with an average of relative humidity of 70–80% and constant ventilation. Seedlings were maintained under this humidity and sprayed with water every four days.

Results

Time of *in vitro* culture in greenhouse conditions (pre-acclimatization)

Effect of pre-acclimatization on plant growth

After seven months of the transplant, the highest growth of the aerial region of *L. speciosa* seedlings was observed for those pre-acclimatized in greenhouse conditions. The average increase of the new leaf length was 1.66 cm ± 0.24, followed by 1.29 cm ± 0.23 in seedlings that remained 25 days in pre-acclimatization; however no significant differences in this parameter between treatments were found. Significant differences were registered

in width of the leaves ($F = 5.57$; gl 6; $P = 0.000$), in the length of the new pseudobulb ($F = 2.4$; gl 6; $P = 0.032$) and in the vigor ($F = 3.48$; gl 6; $P = 0.003$). The treatment of 20 days of pre-acclimatization was significantly higher than others (Fig. 2), also in this treatment the plants were more vigorous with healthy green leaves.

Significant differences among the treatments were not observed for length and width of the roots of the new pseudobulb. No significant differences in seedling survival among the treatments were detected.

Effect of pre-acclimatization on stomata opening

The initial observations (in June) revealed stomatal closure in the 0 treatment days of pre-acclimatization. In all treatments the majority of stomata are closed and few partially open. There were significant differences in the stomata opening during different times of day in all three pre-acclimatization treatments shown here: 0 days ($F = 6.505$, gl 2, $P = 0.002$) (Fig. 3a,b,c), 20 days ($F = 12.871$, gl 2, $P = 0.000$) (Fig. 3d,e,f), and 30 days ($F = 19.37$, gl 2, $P = 0.000$) (Fig. 3g,h,i), revealing a tendency for stomata to open during the day. Moreover, significant differences in stomata opening are registered during different time of exposure at different hours of the day: 5:00 a.m. ($F = 25.441$, gl 2, $P = 0.000$) (Fig. 3a,d,g), 9:00 a.m. ($F = 27.149$, gl 2, $P = 0.000$) (Fig. 3b,e,h), and 2:00 p.m. ($F = 41.678$, gl 2, $P = 0.000$) (Fig. 3c,f,i). At 0 day pre-acclimatization treatment, stomata opening was significantly less frequent compared to the other treatments.

At the end of the experiment (September), three months after of culture in greenhouse conditions, any significant differences were detected at different hours of the day for the 0 and 20 day pre-acclimatization treatments. Nevertheless, there was a trend of increase in the percentage of the stomata opening or partially opened (Fig. 4a,b,c and Fig. 4d,e,f), during the day. In the 30 day treatment significant differences were registered at different times of the day ($F = 4.543$, gl 2, $P = 0.011$) (Fig. 4g,h,i), with

the highest (62%) opening at 9:00 a.m. When comparing the treatments in each of the observation times, significant differences were found at 5:00 a.m. ($F = 6.010$, gl 2, $P = 0.003$), 9:00 a.m. ($F = 20.232$, gl 2, $P = 0.000$), and 2:00 p.m. ($F = 8.369$, gl 2, $P = 0.000$). For the 0 day treatment, stomata opening was significantly less frequent than in the other treatments at 9:00 a.m. and 2:00 p.m.

Effect of different levels of MS nutrients previous to acclimatization

The seedlings of *L. speciosa* cultivated in the treatment with 100% of MS nutrients recorded an increase in the length of the leaf of 1.3 cm (± 0.171), whereas the treatment lacking nutrients (0% of MS) the increase was of

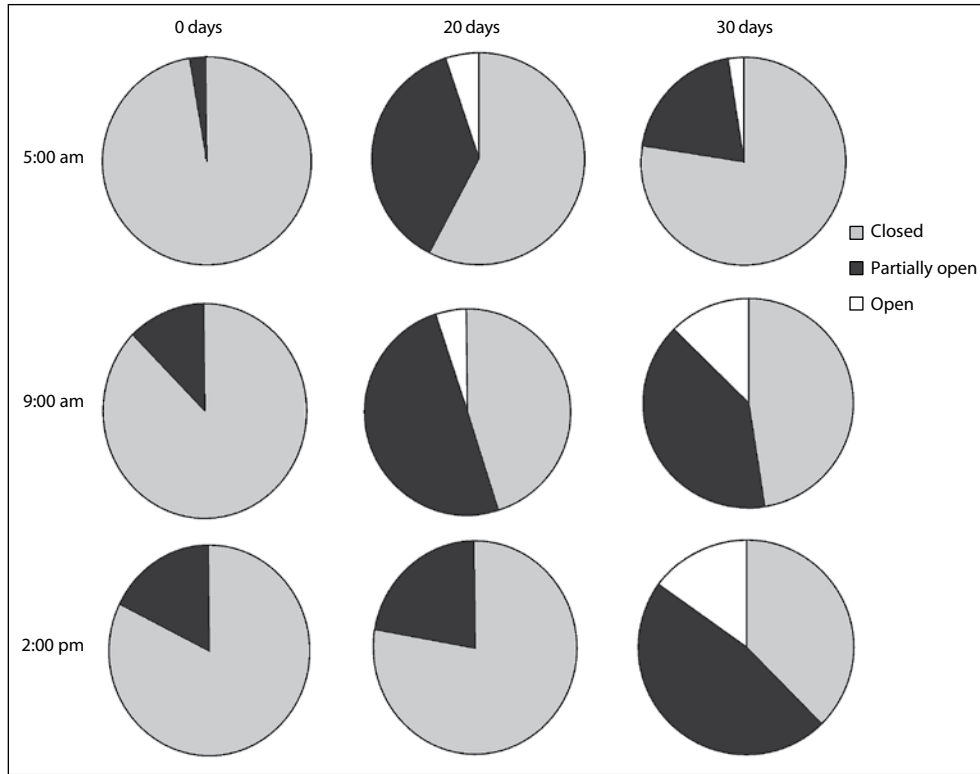


Fig. 3 Stomata opening of *Laelia speciosa* seedlings with different pre-acclimatization times: 0 days (a, b, c at 5:00 a.m., 9:00 a.m., and 2:00 p.m. respectively), 20 days (d, e, f at 5:00 a.m., 9:00 a.m., and 2:00 p.m.), and 30 days (g, h, i at 5:00 a.m., 9:00 a.m., and 2:00 p.m. respectively), at initial observations (June).

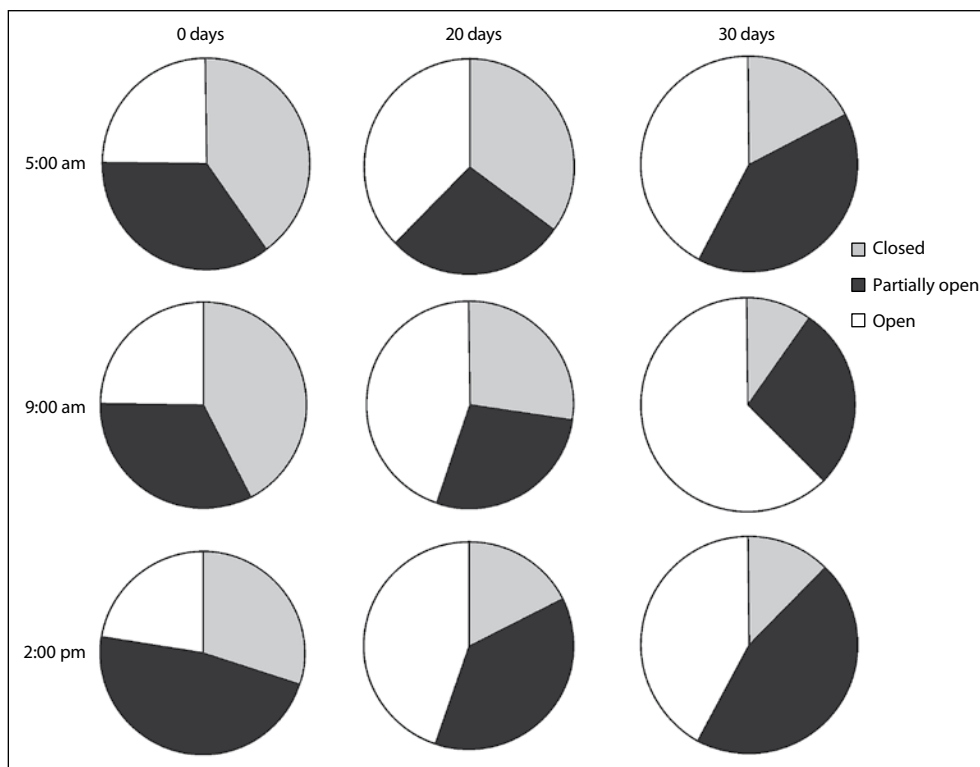


Fig. 4 Stomata opening of *Laelia speciosa* seedlings with different pre-acclimatization times: 0 days (a, b, c at 5:00 a.m., 9:00 a.m., and 2:00 p.m. respectively), 20 days (d, e, f at 5:00 a.m., 9:00 a.m., and 2:00 p.m.), and 30 days (g, h, i at 5:00 a.m., 9:00 a.m., and 2:00 p.m. respectively), after 3 months of transplanted (final observations).

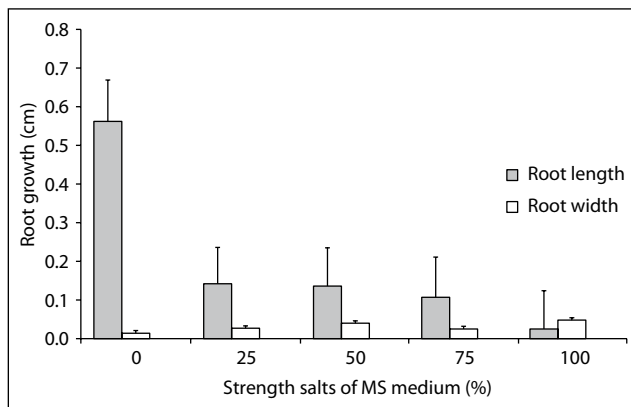


Fig. 5 Effect on root growth (cm) of *Laelia speciosa* seedlings during acclimatization with different percentages of strength salts in MS medium.

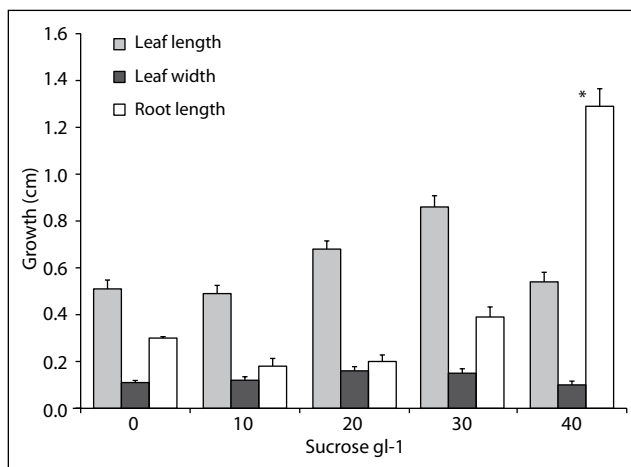


Fig. 6 Growth of seedlings of *Laelia speciosa* during acclimatization after cultured *in vitro* with different sucrose concentration in the MS medium.

0.78 cm (± 0.187), however, no significant differences were observed. No significant differences were also not detected in the width or length of the leaf of the new pseudobulb either.

Seedlings cultivated in the 0% MS treatment developed significantly longer roots ($F = 3.96$, $gl\ 4$, $P = 0.004$) with an increase of 0.56 cm (± 0.23) (Fig. 5). The treatments applied did not show a significant increment in the width of the root. In this treatment, the plantlets showed a low vigor, because the plants were chlorotic or reddish, thin and flexible leaves. In contrast, the plantlets cultivated in treatment 100% MS, were vigorous with optimal growth. No significant differences were revealed in the survival of seedlings cultivated to different levels of MS nutrients, with high percentages of 97.5% to 100% recorded.

Effect of sucrose concentration in MS medium previous to acclimatization

The effect of sucrose concentration was determined in the acclimatization of *L. speciosa* plantlets, measuring growth of the aerial part and roots during greenhouse

cultivation. The results of the growth of aerial part of seedlings did not present significant differences between treatments (different concentrations of sucrose; Fig. 6). However, there were significant differences in the length of the roots of these seedlings ($F = 3.96$, $gl\ 4$, $P = 0.005$). Seedlings cultivated on MS with 40 g/l sucrose prior to transplantation displayed a significant increase in root length (Fig. 5). Plantlets also displayed more vigor using this treatment evident by high (97.5%) survival after 60 days of being transplanted in the greenhouse.

Discussion

Time of *in vitro* culture in greenhouse conditions (pre-acclimatization)

Seedlings of *L. speciosa* with 20 days of pre-acclimatization registered the highest growth and vigor compared to those without it. Acclimatization of orchids and *in vitro* hardening has also been successful for other epiphytic orchids such as *Arachnis labrosa* and *Cleisostoma racemiferum*, in which the 1/10 liquid MS basal medium was employed and subsequently replaced by an alternate substratum. Such plants were maintained under normal laboratory photoperiod and temperature conditions for three months and showed a significant increase on survival rate at *ex vitro* conditions (Deb and Imchen 2010). In the same way an *in vitro* hydroponic system for *Cattleya tigrina* increased survival and growth of *ex vitro* plants (Lopes da Silva et al. 2006).

In this study, we found that seedlings of *L. speciosa* without pre-acclimatization, after 30 days of being transplanted, did not present fully open stomata at any part of the day and just a small percentage of stomata were partially opened. These results agree with data reported by Roberts and Smith (1990), who reported that *in vitro* *Chrysanthemum* plantlets showed partially open stomata, and that rapid *ex vitro* transfer led to high mortality. Similarly, *in vitro* *Malus pumila* plantlets had low stomatal conductance (indicating stomata closed or slightly open) after transfer *ex vitro* culture (Díaz-Pérez et al. 1995). General plant literature has reported changes during acclimatization that include: development of cuticle, epicuticular waxes, and effective stomatal regulation of transpiration (Pospíšilová et al. 2007). Particularly, for orchids morphological and functional changes during acclimatization had been reported. For example in *Cattleya jenmanii*; leaves that developed during this process showed reversion of some abnormalities observed *in vitro*, in addition to an increase of stomata size (Torres et al. 2006). For *Dendrobium* hybrids, an increase of stomata size in acclimatized plants in relation to *in vitro* micro-propagated plants was also observed (Anita et al. 2000).

In this study, we provide evidence of functional changes in *L. speciosa* seedlings during acclimatization, by recording an increase in the percentage of open stomata

over time for all pre-acclimatization treatments. Nevertheless, in the treatment without pre-acclimatization and on the 5th day of treatment, a significantly lower percentage of stomata were open compared to seedlings with a larger pre-acclimatization period. In those seedlings subjected to a 20 day period of pre-acclimatization, a higher percentage of open stomata were observed coupled with significantly improved growth and vigor compared to other treatments. We suggest that pre-acclimatization is an important factor in promoting stomatal functioning prior to transfer, resulting in seedlings that are more well-suited to survival under greenhouse conditions.

Effect of different levels of MS nutrients previous to acclimatization

Prior to transplantation of plantlets, it has been suggested that a decrease in nutrient concentration within *in vitro* culture media can be used to obtain more vigorous roots and higher survival rates during acclimatization (Preece and Sutter 1991; Cusi, pers. comm.). Nevertheless, although *L. speciosa* had significantly larger roots in the treatment lacking nutrients, vigor of plantlets was low and leaves were reddish in color, thin, and less turgid compared to the treatment containing more nutrients. Thus, we advocate the use of a higher nutrient content prior to acclimatization.

No significant differences were detected in the survival of seedlings during acclimatization, possibly because the acclimatization used in the previously-developed method (Ávila-Díaz et al. 2009), and the use of a fungicide, favored survival of *L. speciosa*.

Effect of sucrose concentration in MS medium previous to acclimatization

Seedlings in the 40 g l⁻¹ of sucrose treatment yielded a significantly greater increment in root length (Fig. 6).

Serret et al. (2001) reported optimal growth in the acclimatization stage using high sucrose concentrations during the previous *in vitro* culture phase. The hardening of *in vitro* plantlets by decreasing the osmotic potential in the medium by the addition of sugars can ameliorate wilting of plants after transplantation, but very high concentrations of sucrose in the medium can slow development (Pospíšilová et al. 2007).

Implications for conservation

The *in vitro* propagation and acclimatization of *L. speciosa* and other orchids to produce plants of exceptional quality, is an alternative that might help by decreasing pressure on natural populations. *In vitro* propagated plants could also be used for re-introduction, but it is important to use a large quantity of seeds from as many capsules as possible from the local plants or from the closest population in order to maintain the natural

genetic variation and structure (Ávila-Díaz and Oyama 2007).

Moreover, *L. speciosa* micropropagated plants that are kept under greenhouse conditions may be used as a source in Mexican religious art; thereby diminishing the impact on wild populations, promoting its sustainable management with a central issue for an orchid that has been used for centuries in many different ways.

Conclusions

This study provides an efficient protocol for *in vitro* propagation and acclimatization for the conservation of *Laelia speciosa*, via 20 days pre-acclimatization in greenhouse conditions coupled with high concentrations of nutrients and sucrose within the *in vitro* culture prior to its transplant.

This acclimatization *L. speciosa* work has great application possibilities for obtaining seedlings with high survival percentages and growth with the purpose of accomplishing a sustainable management of this threatened species.

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