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CALLUS GROWTH AND PLANT REGENERATION IN *LAELIA SPECIOSA* (ORCHIDACEAE)

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Resumen. *Laelia speciosa* es una orquídea epífita amenazada, endémica de México. Se considera que la reproducción asexual *in vitro* puede ser una de las acciones para contrarrestar la extracción masiva de individuos de sus poblaciones naturales, al ofrecer plantas de calidad en el mercado. El crecimiento y diferenciación de callo derivado de explantes de hojas de *L. speciosa* fueron investigados en el medio de Murashige y Skoog (MS) con 30 g l^{-1} de sacarosa y cinco concentraciones (0.0, 0.25, 0.5, 1.0, y 2.5 g l^{-1}) de ácido naftalenacético (ANA) en combinación con benziladenina (BA, 0.0, 0.25, 0.5, 1.0, y 2.5 g l^{-1}). Explantes de hojas de plántulas cultivadas *in vitro* fueron efectivos para la formación de callo en el medio MS suplementado con 2.5 mg l^{-1} BA, mientras que explantes de hojas maduras no respondieron. El diámetro del callo en promedio por explante de hoja fue de 1.25 cm después de ocho semanas de cultivo. El mejor desarrollo de PLBs se reportó en el medio MS suplementado con 2.5 mg l^{-1} ANA and 1 mg l^{-1} BA. La formación de plántulas se logró exitosamente en MS suplementado con 0.5 mg l^{-1} de ANA y 0.1 mg l^{-1} de GA_3. Dichas plántulas fueron aclimatadas exitosamente en invernadero con una tasa de supervivencia de 70%.

Abstract. *Laelia speciosa* is an endangered epiphytic orchid, endemic to México. It is thought that the asexual reproduction *in vitro* could be one of the actions to counteract the massive extraction of individuals from their natural populations. The growth and differentiation of callus tissues derived from leaf explants of *L. speciosa* were investigated in Murashige and Skoog medium (MS) with 30 g l^{-1} sucrose and five concentrations (0.0, 0.25, 0.5, 1.0, and 2.5 g l^{-1}) of naphthaleneacetic acid (NAA) with benzyladenine (BA) (0.0, 0.25, 0.5, 1.0, and 2.5 g l^{-1}). Leaf explants from *in vitro* plantlets formed callus tissue on MS medium supplemented with 2.5 mg l^{-1} BA while mature leaves did not respond. Diameter of the callus tissues averaged 1.25 cm after eight weeks of culture. PLBs development was achieved on MS medium supplemented with 2.5 mg l^{-1} NAA and 1 mg l^{-1} BA. The formation of plantlets was successfully obtained in MS supplemented with 0.5 mg l^{-1} of NAA and 0.1 mg l^{-1} of gibberellic acid (GA_3). Obtained plantlets were successfully acclimatized in a greenhouse with a survival rate of 70%.

Key Words*: Laelia speciosa*, endangered orchid, callus, plant regeneration.

Abbreviations: BA: benzyladenine; GA3: Gibberellic acid; MS: Murashige and Skoog medium; NAA, α-naphthaleneacetic acid; PLBs: Protocorm-like bodies

Introduction. It is thought that asexual reproduction is a valuable tool in the massive propagation of many orchids (Rao 1977, Arditti & Ernst 1993). Several species, varieties and hybrids have been asexually micropropagated, such as *Acampe praemorsa, Cattleya* spp., *Cymbidium* spp., *Dendrobium* spp., *Epidendrum radicans*, *Renanthera imschootiana, Laelia* spp., *Phalaenopsis* spp., *Doritaenopsis* spp.,

among others. Efficient micropropagation methods to obtain many plants for commercial purposes or for their conservation have been reported (Seeni & Latha 1992, Nayak *et al.* 1997ab, Chen *et al.* 2002, Park *et al.* 2003, Roy & Banerjee 2003, Santos-Hernández *et al.* 2005, Lavrentyeva & Ivannikov 2007).

Laelia speciosa (HBK) Schlechter, is commonly known as "flor de mayo" (flower of May); "flor grande" (big flower); "flor de corpus" or "corpo" (flower of the Day of the Holy Corpse); "tlacuxóchitl", "deantza", "itzámahua" (Purépecha) (Halbinger & Soto 1997, Ávila-Díaz pers. obs.). It is an epiphytic orchid endemic to the central part of Mexico, including the oak forests of the Sierra Madre Occidental, of the Sierra Madre Oriental, the southern part of the Altiplanicie Mexicana (Mexican Plateau), and the Eje Neovolcánico Transversal (Trans-Mexican Volcanic Belt) (Halbinger & Soto 1997, Ávila-Díaz & Oyama 2007). It blooms from April to June, and produces an inflorescence with 1 to 2 large, pale or dark pink-lilac to purplish flowers. The plants of this species are grown in home gardens and they are also used in religious ceremonies as well as to extract mucilage from pseudobulbs to make a paste with the pith of corn, which is used for making religious figures (Miranda 1997, Hágsater *et al.* 2005). Thousands of plants of *L. speciosa* are usually harvested from their natural habitats, which has caused local extinctions. *Laelia speciosa* is listed as endangered species by official Mexican law (NOM-059-ECOL) (Salazar-Chávez 1996, Halbinger & Soto 1997, Ávila-Díaz & Oyama 2007).

Therefore, it is important to develop a system for *in vitro* asexual propagation of *L. speciosa* and to obtain high-quality plants that could be an alternative for commercialization and, in this way, to diminish the pressure that exists over their natural populations.

A successful protocol for *in vitro* propagation of *L. speciosa* by seed germination has been already developed with conservation purposes (Avila-Díaz *et al.* 2009). However, plantlet regeneration from vegetative explants has not been reported for this species. Therefore, this study aimed induction of calluses and regeneration of plantlets derived from them through PLBs proliferation.

This work is part of a multidisciplinary project in which diverse aspects of conservation biology of this species have been studied. It also contemplates the work done with local human communities. It is our hope that the results from this investigation can be applied to establish a sustainable management of this highly-appreciated orchid.

Material and methods

Callus induction. — Leaf segments of 6 month-old *in vitro Laelia speciosa* plantlets obtained by seed culture (Ávila-Díaz *et al.* 2009), were used as explants. Also, leaf explants from mature plants were tested, which were surface-disinfected with 15% Neutral Plus Hyclin (concentrated liquid detergent) (HYCEL of Mexico, Mexico D.F.) for 5 min, followed by 70% ethanol for 5 min, 3% hydrogen peroxide for 5 min, 1.2% sodium hypochlorite for 15–20 min, and then rinsed three times with sterile-distilled water in a laminar flow cabinet.

Leaf segments of 0.5 cm in length and 0.3 to 0.5 cm in width, with the under surface of the leaf placed in contact with the culture medium, were cultured on MS basal medium (Murashige & Skoog 1962) supplemented with NAA (0.25, 0.5, 1.0, and 2.5 g l^{-1}) in combination with BA (0.25, 0.5, 1.0, and 2.5 g l^{-1}) using MS without plant growth regulators (PGRs) as control treatment. Each treatment consisted of five 120 ml-glass jars, and each of them contained 25 ml of the medium. Five leaf explants were placed in each jar. They were closed with clear plastic caps of Sigma, Co. St. Louis, Missouri, USA.

Microscopic observations were carried out after 60 days of culture with a SMZ800 Nikon stereo microscope (México, D.F.). The size of the diameter of callus was registered.

Data were analyzed using one way ANOVA and HSD Tukey Post Hoc test. The SPSS 15.0 program for Windows (SPSS Inc. Chicago, IL, USA) was used for data analysis.

PLBs proliferation. — Eight week-old callus sections grown on the optimal medium for callus induction were segmented into approximately 0.5 cm diameter segments. Five callus segments were placed on 10 ml of culture medium poured into 45 mm diameter disposable Petri dishes with the same combinations of NAA/BA than in the previous experiment. Five replicates were established per treatment. After

Table 1. Callus growth (diameter, cm) in leaf explants of *Laelia speciosa* after 60 days of culture.

		NAA (mg l^{-1})				
		0	**0.25**	**0.5**	**1**	**2.5**
BA (mg l-1)	**0**	0 ± 0 ^{e}	0.1 ± 0.01 ^{e}	0 ± 0 ^{e}	0 ± 0 ^{e}	0 ± 0 ^{e}
	0.25	0.25 ± 0.02 ^{d}	0 ± 0 ^{e}	0 ± 0 ^{e}	0.49 ± 0.03 ^{c}	0 ± ^{e} 0
	0.5	0.51 ± 0.04 ^{c}	0.46 ± 0.03 ^{c}	0.55 ± 0.03 ^{c}	0 ± 0 ^{e}	0 ± 0 ^{e}
	1	0.48 ± 0.04 ^{c}	0.52 ± 0.03 c ^{c}	0 ± 0 ^{e}	0 ± 0 ^{e}	0 ± 0 ^{e}
	2.5	1.25 ± 0.06 ^{a*}	0.75 ± 0.02 ^{b}	0.50 ± 0.03 ^{c}	0 ± 0 ^{e}	0 ± 0 ^{e}

Means with different letters are significantly different at $p = 0.000$

eight weeks, the average number of PLBs per callus segment was estimated. Moreover, the mean length was recorded. In addition, the overall appearance of the cultures was registered. Analysis of data for PLBs proliferation was done by one way ANOVA and HSD Tukey Post Hoc test. The SPSS 15.0 program for Windows (SPSS Inc. Chicago, IL, USA) was used for all data analysis.

Plantlet development. — PLBs were further developed on MS medium supplemented with 0.1 mg l^{-1} GA_3 and 0.5 mg l^{-1} NAA. This nutrient medium was selected from previous experiments (Avila-Díaz *et al.* 2009).

General culture conditions. — All micropropagation and plantlet development media were based on the MS formulation with 3% sucrose and 0.7% BIOXON bacteriological agar (Becton, Dickinson of Mexico, Cuautitlan Izcalli, Mexico). Growth regulators were added in different concentrations and combinations before autoclaving. The pH of the media was adjusted to 5.7±0.1 before agar was added. Media were autoclaved for 20 minutes at 121°C. All cultures were kept in a growth chamber at 25±1°C under a 16-hour photoperiod of 36 µmol m^{-2} s^{-1} provided by fluorescent tubes (60W).

Acclimatization. – Plantlets of approximately 5 cm in length, obtained on the MS medium supplemented with 0.5 mg l^{-1} NAA and 0.1 mg l^{-1} of GA_3, were transplanted in wet tezontle (volcanic gravel) - oak bark (1:1) into plastic flats, and were covered with a clear plastic lid. Lids were gradually opened every 2 days until they were completely removed after 15 days as was recommended by Ávila-Díaz *et al.* (2009). Plantlets were watering each 8 days and survival was recorded after 30 days.

Results

Callus induction. — The leaf explants from mature plants did not show any growth when cultured *in vitro* and finally turned necrotic, while those obtained from plantlets growing *in vitro* formed callus. Significant differences among the media tested were observed at 60 days following culture ($F = 178.81$, $df = 24$, $p = 0.000$). Tissues of *L. speciosa* incubated on media with NAA alone (0.25 mg l^{-1}) and with NAA/BA (1.0/ 0.25 mg l^{-1}), developed callus (Table 1) although the treatment with 2.5 mg l^{-1} of BA without NAA carried the best growth of *L. speciosa* callus (1.25 cm-diameter and the best quality with light-green color) (Fig. 1A). This treatment was significantly higher than all other investigated. Low dosis (0.25 mg l^{-1}) of NAA with 2.5 mg l^{-1} of BA was the second best treatment for callus growth (Table 1).

PLBs proliferation. — Induction of *L. speciosa* PLBs showed significant differences among the media tested ($F = 64.11$, $df = 24$, $p = 0.000$). Treatments that generated significantly higher number of PLBs were MS medium with 2.5 mg l^{-1} NAA with 1.0 mg l^{-1} of BA, MS medium with 2.5 mg l^{-1} of BA, and 0.5 mg l^{-1} NAA with 0.5 mg l^{-1} BA 60 days following culture (Fig. 1B, Fig. 2). PLBs grown on 2.5 mg l^{-1} NAA /1.0 mg l^{-1} BA showed longer mean length (0.7 mm) than the 2.5 mg l^{-1} BA (0.4 mm) and 0.5 mg l^{-1} NAA with 0.5 mg v BA (0.5 mm) treatments, also on 2.5 mg l^{-1} NAA /1.0 mg l^{-1} BA medium the development of one or two roots of up to 2 cm long with velamen was obtained (Fig. 1C), whereas the other media did not induced any root.

Plantlet development. — PLBs developed successfully

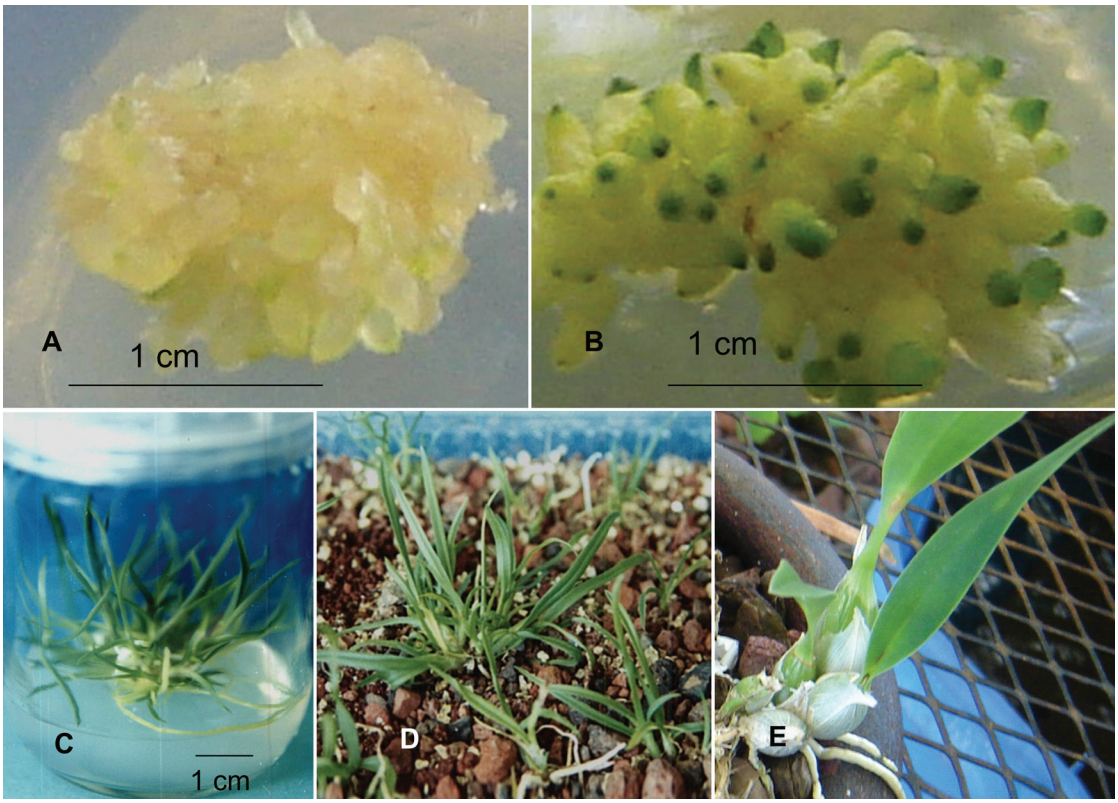

FIGURE 1. A — Callus formation in *Laelia speciosa* on MS media with 2.5 mg l^{-1} of BA. B — Proliferation of PLBs protocorm-like bodies on MS medium supplemented with 2.5 mg l^{-1} NAA and 1 mg l^{-1} BA. C — Plantlets with roots subcultured on MS supplemented with 0.5 mg l^{-1} of NAA and 0.1 mg l^{-1} of GA3. D — Plantlets acclimatized in greenhouse for 30 days. E — Three years old plantlets to be used for hand crafts.

to plantlet on the selected nutrient medium (Avila-Díaz *et al.* 2009). When they reached 3 cm in height they were transplanted to the greenhouse for their acclimatization.

Acclimatization. — Survival of plantlets of *L. speciosa* transferred in tezontle-oak bark substrate was 70% in the greenhouse (Fig. 1D, 1E).

Discussion

According to the results of this work, the use of *L. speciosa* leaf segments from *in vitro* grown plantlets as explants can be considered effective for the asexual propagation of this species. Other micropropagation studies in epiphytic orchids have shown that different sections of the plants can be used as explants, such as: flower stalk sections, buds, leaf primordium, tip and basal part of the leaves, shoot-tips, root tips (Arditti *et al.* 1972, Seeni & Latha 1992, Nayak *et al.* 1997a, 1997b, Chen *et al.* 2002, Park *et al.* 2003, Roy & Banerjee 2003, Santos-Hernández *et al.* 2005, Lavrentyeva & Ivannikov 2007). In general, it has been reported that the young tissues are more adequate than mature ones for the induction of PLBs or shoots (Seeni & Latha 1992, Murthy & Pyati, 2001). In many cases, such as in our experiments, explants from *in vitro* cultures have given successful results (Nayak *et al*. 1997a, Murthy & Pyati, 2001, Chen *et al.* 2002, Park *et al.* 2003, Salazar & Mata 2003, Condemarín-Montealegre *et al.* 2007, Lavrentyeva & Ivannikov 2007).

The induction of callus in *L. speciosa* on MS medium supplemented with NAA resembles the results of Avila-Díaz *et al.* (2009), using complete seedlings grown *in vitro* as explants; nevertheless, in this particular investigation, we obtained higher induction

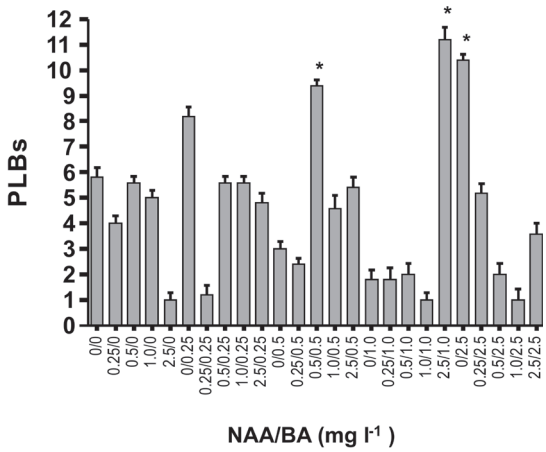

Figure 2. Mean number of PLBs after 60 days of subculture of leaf explants on media with NAA and BA. Treatments that generated significantly higher number of PLBs are marked with *.

rates when BA was added to the MS media using plantlet's leaves as explants. The induction of callus in *L. speciosa* with high concentrations of BA (2.5 mg l^{-1}), whether alone or in combination with NAA (0.25 mg l^{-1}), is similar to what has been reported for other orchid species; the callus induction is favored by the addition of cytokinins along or in combination with auxins as in the case of *Dendrobium fimbriatum*, in which optimum callusing was recorded in the presence of 1.0 mg l^{-1} BAP and 0.5 mg l^{-1} NAA (Roy & Banerjee 2003). In *Epidendrum radicans*, small transparent tissues enlarged and developed calluses when cultured with thidiazuron (TDZ) under light (Chen *et al.* 2002).

The cytokinin and auxin balance is also important in PLBs formation. A concentration of 2.5 mg l^{-1} NAA and 1 mg l^{-1} BA induced the highest formation of the largest PLBs in *L. speciosa*. In many orchids, PLB or shoot induction has been accomplished with cytokinins alone or in combination with auxins. The response to different concentrations is variable, depending on the species. For example, in *Epidendrum radicans* homogenized PLB tissues produced by blending were used as explant to test the effects of four cytokinins. The best response on number of PLBs per tube was found on a basal medium supplemented with 1 mg l^{-1} BA (Chen *et al.* 2002). However, in *Acampe praemorsa* shoot buds were induced on MS medium supplemented with 1 mg l^{-1} TDZ, while shoot elongation and leaf expansion were promoted with 0.5 mg l^{-1} BA and 2.0 mg l^{-1} NAA (Nayak *et al*. 1997a). On the other hand, in *Aerides maculosum*, *Mormodes tuxtlensis* and *Cuitlauzina pendula*, PLBs or shoots were inducted with BA alone (Murthy & Pyati 2001, Salazar & Mata 2003) and in *Lycaste skinneri* with NAA alone (Salazar & Mata 2003).

The plantlet survival rate during acclimatization (70%) is close to that reported by Ávila *et al*. (2009) for *L. speciosa* seedlings cultivated *in vitro* (77.5%). More investigation is recommended about acclimatization of this species to increase its survival.

The method of asexual propagation developed in this study for *L. speciosa is* efficient. 28 plants can be obtained from each single explant in 10 to 12 months and if the callus is subcultured, it is possible to obtain much more individuals. This is considered useful for an abundant production of orchids, which can be used for commercialization of ornamental plants or for the elaboration of arts and crafts.

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