Propagation and Establishment of Three Endangered Mexican Orchids from Protocorms

Martín Mata-Rosas¹ and Víctor M. Salazar-Rojas

Instituto de Ecología, A.C., Unidad de Recursos Forestales, Km 2.5 carretera antigua a Coatepec 350, Xalapa, Veracruz 91070, Mexico

Additional index words. micropropagation, protocorm-like bodies, ex vitro establishment, conservation

Abstract. Protocols for in vitro propagation from protocorms of *Mormodes tuxtlensis* Salazar, *Cuitlauzina pendula* La Llave & Lex., and *Lycaste skinneri* (Batem. Ex. Lind.) Lind., three endangered species distributed in Mexico and highly appreciated as ornamentals, were developed. The effect of two different culture media, Murashige and Skoog (MS) and modified Knudson (KCM), combined with varying concentrations of N⁶-benzyladenine (0, 2.2, 4.4, 8.9, and 13.3 μ M) and α -naphthaleneacetic acid (0, 0.5 and 2.7 μ M), were investigated. Shoot formation and development of protocorm-like bodies were observed. For all three species, cultures in MS produced more shoots per explant than those in KCM, and those shoots were longer and more robust in appearance. Maximum number of shoots for *M. tuxtlensis* (1.5) and *C. pendula* (24.3) were obtained in media supplemented with 13.3 μ M and 2.2 μ M N⁶-benzyladenine, respectively. Conversely, for *L. skinneri* the greatest shoot production (16.4) was achieved in medium supplemented with 2.7 μ M α -naphthaleneacetic acid. Subculturing explants in MS basal medium allowed further development and rooting of the shoots as well as growth of protocorm-like bodies. The effect of different potting mixes on ex vitro survival plantlets was also investigated; pine bark:oak charcoal:pumice (3:1:1) allowed the highest survival rates in all three species.

Mexico has $\approx$ 1200 of the 25,000 orchid species that have been described (Dixon et al., 2003); 40% of these are endemic species (Espejo and López, 1998). Several species are endangered as a direct or indirect result of two human activities: habitat alteration and overcollecting (Flores-Palacios and Valencia-Díaz, 2007; IUCN/SSC Orchid Specialist Group, 1996). One hundred seventy-nine orchid species are protected by the Mexican government and research aimed at finding new methods to conserve and propagate these species is a high priority. Among these are three highly sought-after species: *Mormodes tuxtlensis*, *Cuitlauzina pendula*, and *Lycaste skinneri*.

Mormodes tuxtlensis Salazar is an epiphytic species first described in 1988. It is endemic to Mexico and grows in small areas of the tropical rain forest in Veracruz, Oaxaca, and Chiapas (Fig. 1A) (Salazar, 1988). *Cuitlauzina pendula* La Llave & Lexarza is an epiphytic species endemic to the southern and western parts of Mexico (Fig. 2A). Its native range has been reduced, and the size of the type population has

plummeted (Jiménez et al., 1998). *Lycaste skinneri* (Batem. Ex. Lind.) Lind. is an epiphytic, lithophytic, or sometimes terrestrial species that occurs from Mexico to Guatemala and Honduras (Fig. 3A) (Jiménez et al., 1998). In recent years, this species has not been reported from Mexico, suggesting that it may be locally extinct.

Efficient in situ conservation of threatened or endangered species has proven difficult to achieve; however, ex situ conservation can complement global conservation strategies. Plant tissue culture represents an excellent option for the propagation and conservation of endangered species (Gangaprasad et al., 1999; Ket et al., 2004; Rubluo et al., 1993).

Although micropropagation of orchids has been a recognized technique for many decades (Arditti and Ernst, 1993), its use has been largely confined to species and hybrids of the genera *Phalaenopsis*, *Cattleya*, and *Oncidium* (Arditti and Ernst, 1993; Chen et al., 1999, 2000; Chen and Chang, 2001; Tokuhara and Mii, 2001; Wu et al., 2004). Reports of successful propagation of wild Mexican species are rare. Germination and development protocols for three Mexican species, *Cattleya aurantica*, *Encyclia chachanoensis*, and *Brassavola nodosa* were described by Damon et al. (2004). Santos-Hernández et al. (2005) reported high in vitro germination rates as well as bud and protocorm-like body (PLB) formation for *Laelia albida*, whereas Lee-Espinosa et al. (2007) reported the germination and organogenic proliferation of *L. anceps* ssp. *dawsonii*.

Ávila-Díaz and Oyama (2002) also reported in vitro germination of *Laelia speciosa*.

The present study describes the effect of modified Knudson C medium (KCM) and Murashige and Skoog medium (MS) as well as the action of different concentrations of N⁶-benzyladenine (BA) and α -naphthaleneacetic acid (NAA) on the induction of shoots and PLBs from protocorms and also the survival of ex vitro plantlets.

Materials and Methods

Seeds of the three species were donated to the Francisco Javier Clavijero Botanical Garden. Open, ripe capsules (obtained from hand-pollination) were dried for 24 to 48 h at 25 °C. The seeds were then extracted and stored in paper envelopes inside jars over silica gel for 15 to 30 d at 4 °C before experimentation.

Seed surface sterilization. The seeds were placed in a filter paper envelope (Whatman No. 1, 110 mm diameter). The envelopes were submerged in sterile distilled water for 30 min, then dipped in 70% (v/v) ethanol for 1 min, and then soaked in 30% (v/v) commercial bleach solution (1.8% NaOCl) with two drops of Tween-80 per 100 mL (Sigma, St. Louis, MO) for 30 min. This was followed by four rinses with distilled sterilized water under aseptic conditions. The seeds were then sown in 125-mL baby food jars containing 25 mL of KCM (Knudson, 1946) supplemented with 37.3 mg·L⁻¹ Na₂EDTA and 27.8 mg·L⁻¹ FeSO₄·7H₂O (Murashige and Skoog, 1962) plus 20 g·L⁻¹ sucrose.

The pH of all culture media was adjusted to 5.0 $\pm$ 0.1 with 0.5 N NaOH and 0.5 N HCl before adding 5.5 g·L⁻¹ Agargel™ (Sigma, St. Louis, MO) and autoclaving at 1.2 kg·cm⁻² and 120 °C for 15 min. All cultures were incubated in a growth chamber at 25 $\pm$ 1 °C under a 16-h photoperiod provided by cool-white fluorescent lamps (50 μ mol·m⁻²·s⁻¹).

Protocorm culture. After germination, protocorms with a height of 2 to 3 mm were selected and transferred to two different treatment media: 1) MS medium with 2 mg·L⁻¹ glycine, 100 mg·L⁻¹ myoinositol, and 30 g·L⁻¹ sucrose; and 2) KCM. Both media were supplemented with a combination of BA (0, 2.2, 4.4, 8.9, or 13.3 μ M) and NAA (0, 0.5, and 2.7 μ M); three protocorms were cultured in each jar. There were with 10 replicates (i.e., 30 protocorms per treatment). The induction period was 120 d.

After the induction period, the protocorms were subcultured every 60 d to their respective basal medium without plant growth regulators (PGRs). The number of shoots per protocorm, shoot height, and PLB production were recorded.

Number of shoots per protocorm and shoot height were analyzed using one-way analysis of variance followed by a least significant difference test ($P \leq 0.05$).

Ex vitro culture. Plantlet survival was assessed using individuals that had attained a height of 3 to 5 cm and were at least 10 months old. In the case of *M. tuxtlensis*, the

Received for publication 5 June 2008. Accepted for publication 16 Apr. 2009.

We thank Pamela Moon, Philip J. Brewster, and Guillermo Angeles for the suggestions and corrections of the English text. Víctor Salazar thanks Instituto de Ecología, A.C. for the grant offered to conduct his professional studies.

¹To whom reprint requests should be addressed; e-mail martin.mata@inecol.edu.mx.

Fig. 1. Plant regeneration of *Mormodes tuxtlensis* through direct shoot formation. (A) Flowering plant. (B) Multiple shoot formation from protocorms in Murashige and Skoog medium + 13.3 μM N^6 -benzyladenine. (C) In vitro-rooted shoots ready for ex vitro culture. (D) Plants obtained from in vitro culture after 4 months in potting mix. Bars = 2 cm.

Fig. 2. Plant regeneration of *Cuatrecasaya pendula* through direct shoot formation and protocorm-like bodies (PLBs). (A) Closeup of the flower. (B) Multiple shoot formation from protocorms in Murashige and Skoog medium + 2.2 μM N^6 -benzyladenine. (C) In vitro-rooted shoots ready for ex vitro culture. (D) Plants obtained from in vitro culture after 4 months in potting mix. Bars = 2 cm.

plantlets used were smaller, ranging from 1 to 2 cm depending on the treatment they were previously given. The plantlets were removed from the jars, washed thoroughly under tap water to remove all adhering culture medium, then transferred to propagation trays (Hummert International, Earth City, MO) and placed in a greenhouse with an average temperature of 30 °C. For the first 30 d, a high relative humidity (80% to 90%) was maintained by keeping the trays covered with plastic, translucent lids. The relative humid-

ity was then decreased to 50% to 60%. Three different substrates were used: 1) pumice; 2) pine bark, oak charcoal, and pumice (3:1:1); and 3) shredded long tree-fern fiber. Survival of plantlets and their height were recorded for 4 months.

Results

Germination

For *M. tuxtlensis* and *C. pendula*, germination was first observed 100 to 120 d after

planting; for *L. skinneri*, it began between 70 to 90 d. The germination rate approached 100% for all three species, because in the different observations under stereomicroscopy, the number of nongerminated seeds was null; but in some cases, the density of seeds was too high making it impossible to affirm that 100% of seeds had already germinated.

Mormodes tuxtlensis

Organogenesis and shoot formation.

Seventy-one percent of protocorms cultured in KCM grew and began to form shoots. In contrast, only 61% of protocorms cultured in MS showed any morphogenic response. Those that did not respond turned brown.

Shoots, formed through direct organogenesis from protocorms that had developed rhizoids and leaves, were 5 to 8 mm long. After 90 d, small nodules formed mainly at the base of the protocorms. These nodules started to produce leaf primordia ≈ 30 d later, especially in the KCM treatments; after the protocorms were subcultured to basal medium, the nodules consolidated into adventitious shoots (Fig. 1B).

Shoot formation per protocorm differed significantly among treatments ($P \leq 0.0001$). Although not significantly different from several other treatments, the highest level of shoot formation (1.5 shoots per protocorm) was achieved in the MS medium treatment supplemented with BA (13.3 μM); 1.4 shoots per protocorm developed from explants cultured in MS medium BA (8.9 μM) either alone or in combination with 2.7 μM NAA. In these three treatments, 85% of protocorms showed some morphogenetic response (Table 1A). In MS medium, in most cases, shoot formation tended to occur in treatments containing at least BA (4.4 μM).

Although statistically significant differences could not be established among KCM treatments, the most shoot formation per protocorm (1.4) and highest percent of responding protocorms (93% to 100%) were obtained in the three treatments: BA (4.4 μM), BA/NAA (2.2/0.5 μM), and NAA (0.5 μM) (Table 1A). In all other treatments, fewer than 76.6% of the explants responded.

Height. Table 1A shows that shoot height from the MS treatments ranged from 1.5 to 2.9 cm and significant differences ($P = 0.0001$) could be established between them. Shoots from the different KCM treatments had statistically significant different heights ($P = 0.0012$), and these were slightly greater than those developed in MS, ranging from 1.8 to 3 cm (Fig. 1C).

Ex vitro survival. Plantlets of *M. tuxtlensis* obtained from the KCM treatments did not survive after outplanting; in contrast, 78% of plantlets from MS tolerated ex vitro culture (Fig. 1D). Percentage of survival varied with substrate used; there was an 89% survival rate from the pine bark:oak charcoal:pumice treatment, whereas only 75% of plantlets placed in the other substrates survived.

Fig. 3. Plant regeneration of *Lycaste skinneri* through direct shoot formation (A) Flowering plant. (B) Multiple shoot formation from protocorms in Murashige and Skoog medium + 2.7 μM α -naphthaleneacetic acid. (C) In vitro-rooted shoots ready for ex vitro culture. (D) Plants obtained from in vitro culture after 4 months in potting mix. Bars = 2 cm.

Cuitlauzina pendula

Organogenesis. Percentage of responding explants in most treatments from both culture media (MS and KCM) was $\approx 100\%$, and PLB formation was the main response in all treatments.

In protocorms cultured in different MS medium treatments, PLB formation started after 30 d of culture. The protocorms increased their size and acquired a wrinkled green surface with nodular protuberances. After 120 d, nodules were consolidated into small shoots and then into plantlets in which the root development and leaf primordial were evident. After the PLBs were subcultured, they increased their height, eventually forming pseudobulbs that eventually consolidated into plantlets (Fig. 2B). More than 50% of the new plantlets formed secondary PLBs; these originated mainly from the base of the pseudobulb.

PLB formation from protocorms cultured in KCM medium took more time than those in MS treatments; the development of nodules were perceptible after 60 to 120 d, and the consolidation of few nodules in PLBs was obtained after few monthly subcultures to KCM basal medium, but all of them were pale green in color. The conversion of PLBs into plantlets was achieved after 6 months; however, some of them retained this stage during the entire experiment.

Shoot formation differed significantly among MS treatments ($P = 0.0001$). The highest average number (24.3) of shoots was recorded in the treatment containing BA (2.2 μM); in the rest of the treatments, a lower average was registered (Table 1B).

Plantlets obtained using KCM medium treatments showed statistically significant differences between different treatments ($P = 0.0001$). A higher average number (28.8) of shoots per explant was obtained in treatment containing BA/NAA (13.3/0.5 μM). In treatments supplemented with higher concentrations of BA (8.9 and 13.3 μM) in combination

with high NAA concentrations (2.7 μM), it was also possible to induce a large number of plantlets per explant, achieving a production average of 26.5 and 24.8 plantlets per explant, respectively.

Height. Plantlets from MS medium were higher than those obtained using KCM (Fig. 2C). In both media, plantlet height was influenced by PGR treatments, and it was possible to establish statistically significant differences ($P < 0.05$). Plantlets attained an average height of 3 cm in the PGR-free MS medium as well as the ones supplemented with BA/NAA (4.4/0.5 and 4.4/2.7 μM) (Table 1B).

In KCM medium, a higher average height was also obtained from the treatment without PGR (1.7 cm), whereas the treatments including BA/NAA (4.4/2.7 μM) and BA (2.2 μM) showed average heights of 1.4 and 1.3 cm, respectively (Table 1B).

Ex vitro survival. Plantlets from the different treatments assayed with MS showed 67% survival (Fig. 2D), whereas for KCM only 2.7% of the plantlets survived.

The survival of plantlets was affected by different substrates used to culture them ex vitro. Plantlets from MS reached 76% survival with the soil mix followed by pumice with 72%, and finally using shredded long tree-fern fiber 53% of the plantlets survived. From KCM, the highest plantlet survival of 4.1% was obtained with the soil mix, 2.4% with pumice, and 1.5% with the shredded long tree-fern fiber.

Lycaste skinneri

Organogenesis. High percentages of response (growth and development) were obtained in both culture media used, MS (91.3%) and KCM (88.1%). The main morphogenetic response obtained was the shoot formation through direct organogenesis (Fig. 3B). After subculturing the explants in their respective basal media, the shoots increased their height and developed roots. Occasion-

ally, in a few treatments from KCM, PLB formation was observed.

During the first 30 d in MS medium, the protocorms increased their volume approximately three to six times and retained a pale green coloration. During the next 30 d, small shoots or small green leaf primordia appeared around the explant. Afterward, each shoot continued its growth and formed well-consolidated leaves; in a few cases, it was possible to observe small roots with velamen. Despite the shoots having developed roots, they remained joined to the original explant.

In KCM, the shoot formation and development were slower than MS cultures. The growth of protocorms was not evident until 60 d; they increased approximately two to three times their original size. Shoot formation was not achieved until the protocorms were subcultured to KCM basal media.

Shoot formation per protocorm differed significantly among treatments ($P = 0.0001$). Plantlet formation was favored by highest NAA concentrations (2.7 μM) either alone or in combination with low concentrations of BA (Table 1C). The highest shoot formation (16.4) was obtained in MS medium treatment supplemented with NAA (2.7 μM) followed by treatment with BA/NAA (2.2/2.7 μM) and BA/NAA (8.9/0.5 μM), in which 14.8 and 13.3 shoots per explant were obtained, respectively (Table 1C).

A statistically significant difference for shoot formation among KCM treatments could not be established; however, it was possible to observe that treatments with BA/NAA (0/0.5 and 0.5/2.2 μM) stimulated higher shoot formation (Table 1C).

Height. Shoots obtained from all MS treatments attained a greater height than those produced from KCM. Media supplemented with 2.7 μM NAA tended to have the tallest plantlets with the greatest average height (3.7 cm) obtained from MS supplemented with BA/NAA (4.4/2.7 μM) (Fig. 3C). For KCM, shoots with an average height of 4 cm were obtained from media supplemented with BA/NAA (13.3/2.7 μM).

Ex vitro survival. As observed for preceding species, ex vitro survival of the plantlets of *L. skinneri* was also influenced by culture media. Those from MS showed high vigor and 50.4% survival (Fig. 3D); for KCM, only 4.7% of the plantlets survived.

Survival of plantlets from MS was greatest (60.1%) in the pine bark, oak charcoal, and pumice mix followed by shredded long tree-fern fiber (48.2%) and then pumice (42.7%).

Percentage plantlet survival from KCM was very low. The pine bark, oak charcoal, and pumice mix allowed 6.5% survival. A total of 5.7% of plantlets grown in shredded tree-fern fiber survived, whereas survival in pumice was only 1.7%.

Discussion

Orchid seeds can germinate on a wide variety of culture media in a relatively short period of time (Yam and Weatherhead, 1988)

Table 1. Effect of media and plant growth regulators (BA and NAA) on the induction of shoots and their final height from in vitro culture of protocorms of (A) *Mormodes tuxtlensis*, (B) *Cuitlauzina pendula*, and (C) *Lycaste skinneri*.

(A) <i>Mormodes tuxtlensis</i>							
PGR (μ M)		Murashige and Skoog			Knudson C (mod.)		
BA	NAA	Responding explants (%)	Shoots per explant ^z	Shoot height ^z	Responding explants (%)	Shoots per explant ^z	Shoot height ^z
0	0	40.0	0.7 $\pm$ 0.2 a	2.6 $\pm$ 0.3 cdef	66.0	0.9 $\pm$ 0.2	1.9 $\pm$ 0.2 ab
2.2	0	50.0	0.8 $\pm$ 0.2 ab	1.5 $\pm$ 0.2 a	76.6	1.2 $\pm$ 0.2	1.8 $\pm$ 0.2 a
4.4	0	90.0	1.3 $\pm$ 0.1 bcde	2.7 $\pm$ 0.2 def	93.3	1.4 $\pm$ 0.2	2.0 $\pm$ 0.2 abcd
8.9	0	85.0	1.4 $\pm$ 0.1 cde	2.3 $\pm$ 0.2 cde	70.0	1.1 $\pm$ 0.2	2.1 $\pm$ 0.2 abcd
13.3	0	85.0	1.5 $\pm$ 0.2 e	2.9 $\pm$ 0.2 f	76.6	1.0 $\pm$ 0.1	2.2 $\pm$ 0.2 abcd
0	0.5	90.0	1.3 $\pm$ 0.1 bcde	2.2 $\pm$ 0.2 cde	93.3	1.4 $\pm$ 0.2	1.9 $\pm$ 0.2 a
2.2	0.5	45.0	0.8 $\pm$ 0.2 a	2.1 $\pm$ 0.2 abc	100.0	1.4 $\pm$ 0.1	2.2 $\pm$ 0.2 abcd
4.4	0.5	57.5	0.9 $\pm$ 0.2 abc	2.7 $\pm$ 0.2 def	70.0	1.0 $\pm$ 0.2	2.5 $\pm$ 0.3 bcdef
8.9	0.5	57.5	1.0 $\pm$ 0.2 abcd	2.1 $\pm$ 0.2 bcd	70.0	1.2 $\pm$ 0.2	3.0 $\pm$ 0.2 f
13.3	0.5	52.5	1.0 $\pm$ 0.2 abcd	2.5 $\pm$ 0.1 cdef	53.0	0.7 $\pm$ 0.2	2.9 $\pm$ 0.3 ef
0	2.7	60.0	0.9 $\pm$ 0.1 ab	1.6 $\pm$ 0.2 ab	56.6	1.0 $\pm$ 0.2	2.2 $\pm$ 0.2 abcd
2.2	2.7	50.0	0.8 $\pm$ 0.2 ab	2.2 $\pm$ 0.2 bcde	73.3	1.2 $\pm$ 0.2	2.6 $\pm$ 0.3 def
4.4	2.7	47.5	0.9 $\pm$ 0.2 abc	1.5 $\pm$ 0.1 a	63.3	0.8 $\pm$ 0.1	2.6 $\pm$ 0.4 cdef
8.9	2.7	85.0	1.4 $\pm$ 0.2 de	2.7 $\pm$ 0.2 ef	56.6	1.1 $\pm$ 0.3	2.2 $\pm$ 0.2 abcd
13.3	2.7	65.0	1.1 $\pm$ 0.2 abcde	2.5 $\pm$ 0.2 cdef	53.3	0.9 $\pm$ 0.2	1.9 $\pm$ 0.2 abc
(B) <i>Cuitlauzina pendula</i>							
PGR (μ M)		Murashige and Skoog			Knudson C (mod.)		
BA	NAA	Responding explants (%)	Shoots per explant ^z	Shoot height ^z	Responding explants (%)	Shoots per explant ^z	Shoot height ^z
0	0	100.0	14.8 $\pm$ 5.1 d	3.0 $\pm$ 0.1 i	97.1	5.7 $\pm$ 1.0 a	1.7 $\pm$ 0.1 f
2.2	0	99.0	24.3 $\pm$ 5.1 e	2.4 $\pm$ 0.1 bcde	97.8	9.1 $\pm$ 1.2 ab	1.3 $\pm$ 0.0 e
4.4	0	97.0	9.7 $\pm$ 2.0 abcd	2.6 $\pm$ 0.1 cdef	96.2	14.0 $\pm$ 2.0 bcd	1.0 $\pm$ 0.0 b
8.9	0	93.0	2.5 $\pm$ 1.0 a	2.1 $\pm$ 0.1 ab	97.8	10.4 $\pm$ 1.5 abc	1.2 $\pm$ 0.0 d
13.3	0	100.0	15.5 $\pm$ 4.9 d	2.0 $\pm$ 0.1 a	97.5	20.1 $\pm$ 2.8 defg	1.0 $\pm$ 0.0 b
0	0.5	99.0	6.9 $\pm$ 1.6 abcd	2.3 $\pm$ 0.1 bc	97.0	8.6 $\pm$ 1.7 ab	1.2 $\pm$ 0.0 cd
2.2	0.5	100.0	12.3 $\pm$ 1.8 cd	2.2 $\pm$ 0.1 b	94.8	16.6 $\pm$ 2.5 cde	1.0 $\pm$ 0.0 b
4.4	0.5	83.0	3.3 $\pm$ 1.6 abcd	3.0 $\pm$ 0.2 hi	97.2	9.2 $\pm$ 1.5 ab	1.1 $\pm$ 0.0 cd
8.9	0.5	100.0	8.6 $\pm$ 2.1 abcd	2.7 $\pm$ 0.1 efgh	100.0	16.3 $\pm$ 2.8 cd	1.1 $\pm$ 0.0 c
13.3	0.5	96.0	4.4 $\pm$ 1.5 abc	2.1 $\pm$ 0.1 ab	97.7	28.8 $\pm$ 3.4 h	0.9 $\pm$ 0.0 a
0	2.7	96.0	4.5 $\pm$ 0.9 abc	2.3 $\pm$ 0.1 bcd	99.4	23.4 $\pm$ 3.7 efgh	1.0 $\pm$ 0.0 b
2.2	2.7	78.0	7.3 $\pm$ 3.2 abcd	2.4 $\pm$ 0.1 bcde	97.3	18.5 $\pm$ 2.7 def	1.0 $\pm$ 0.0 b
4.4	2.7	95.0	8.5 $\pm$ 3.9 abcd	2.9 $\pm$ 0.1 ghi	99.0	9.5 $\pm$ 1.4 ab	1.4 $\pm$ 0.0 e
8.9	2.7	99.0	13.6 $\pm$ 3.0 d	2.7 $\pm$ 0.1 fg	98.6	26.5 $\pm$ 3.6 gh	1.0 $\pm$ 0.0 b
13.3	2.7	98.0	1.4 $\pm$ 3.8 bcd	2.6 $\pm$ 0.1 defg	99.1	24.8 $\pm$ 2.2 fgh	0.8 $\pm$ 0.0 a
(C) <i>Lycaste skinneri</i>							
PGR (μ M)		Murashige and Skoog			Knudson C (mod.)		
BA	NAA	Responding explants (%)	Shoots per explant ^z	Shoot height ^z	Responding explants (%)	Shoots per explant ^z	Shoot height ^z
0	0	80.2	2.3 $\pm$ 0.7 a	2.5 $\pm$ 0.1 abc	100.0	7.7 $\pm$ 2.0	2.1 $\pm$ 0.1 bcde
2.2	0	82.9	2.1 $\pm$ 0.7 a	3.0 $\pm$ 0.1 bcde	97.9	7.8 $\pm$ 1.5	2.0 $\pm$ 0.1 bcde
4.4	0	100.0	3.4 $\pm$ 1.8 a	2.1 $\pm$ 0.2 a	92.8	9.8 $\pm$ 2.4	1.9 $\pm$ 0.1 bcde
8.9	0	96.2	3.5 $\pm$ 0.7 a	2.9 $\pm$ 0.2 bcd	89.6	10.6 $\pm$ 2.7	1.7 $\pm$ 0.1 ab
13.3	0	63.6	2.3 $\pm$ 1.3 a	2.2 $\pm$ 0.2 ab	84.1	9.1 $\pm$ 2.2	1.7 $\pm$ 0.1 abc
0	0.5	73.2	1.5 $\pm$ 0.3 a	2.7 $\pm$ 0.1 abc	86.3	15.3 $\pm$ 3.3	1.8 $\pm$ 0.1 bcd
2.2	0.5	88.5	3.0 $\pm$ 1.0 a	2.7 $\pm$ 0.1 abc	95.1	11.3 $\pm$ 1.9	1.8 $\pm$ 0.1 bcd
4.4	0.5	85.7	3.1 $\pm$ 0.9 a	2.5 $\pm$ 0.2 abc	74.4	10.8 $\pm$ 2.8	1.8 $\pm$ 0.1 abcd
8.9	0.5	99.5	13.3 $\pm$ 3.6 bcd	2.6 $\pm$ 0.1 abc	89.5	6.4 $\pm$ 2.5	1.3 $\pm$ 0.1 a
13.3	0.5	99.4	11.0 $\pm$ 2.1 bc	3.5 $\pm$ 0.3 def	92.0	9.3 $\pm$ 2.8	1.7 $\pm$ 0.1 ab
0	2.7	100.0	16.4 $\pm$ 1.9 d	3.4 $\pm$ 0.0 def	93.0	6.4 $\pm$ 0.8	2.7 $\pm$ 0.2 f
2.2	2.7	100.0	14.8 $\pm$ 1.9 cd	3.6 $\pm$ 0.1 f	89.4	11.0 $\pm$ 1.4	2.2 $\pm$ 0.1 cde
4.4	2.7	100.0	9.9 $\pm$ 0.8 b	3.7 $\pm$ 0.1 f	87.2	9.8 $\pm$ 1.4	2.3 $\pm$ 0.1 ef
8.9	2.7	99.6	9.3 $\pm$ 1.4 b	3.6 $\pm$ 0.1 ef	78.1	6.1 $\pm$ 1.2	2.3 $\pm$ 0.1 def
13.3	2.7	100.0	9.2 $\pm$ 1.3 b	3.0 $\pm$ 0.1 cde	72.0	3.8 $\pm$ 1.0	4.0 $\pm$ 1.3 g

^zMean $\pm$ SD. Results after 8 months. Different letters within columns indicate significant difference at $P \leq 0.05$.

BA = N⁶-benzyladenine; NAA = α -naphthaleneacetic acid; PGR = plant growth regulator.

and KCM and MS are frequently used. Different responses have been reported for culture of one species on different culture media (Bhadra and Hossain, 2003). Ichihashi (1991) states that germination of orchids is best in culture media with low mineral concentration such as KCM. In this study, KCM proved to be an adequate germination medium for all three species. Growth was similar, but germination time varied. KCM has been used for germination of several diverse orchid species

(Bhadra and Hossain, 2003; Pierik et al., 1988; Yam and Weatherhead, 1988).

Morphogenic responses were strongly affected by medium formulation and PGR. For *M. tuxtlensis* and *L. skinneri*, the main response was shoot formation; and for *C. pendula*, it was PLB formation. Fay (1994) states the requirements for in vitro culture of every single species must be determined experimentally; previously established regeneration protocols can only be applied

in a general way. *M. tuxtlensis* and *L. skinneri* had different PGR concentration optima but were otherwise similar in their responses.

There are few reports with similar results as our observations with *M. tuxtlensis*. Mauro et al. (1994) found, for *Cattleya aurantiaca*, the highest shoot formation occurred in MS medium with BA/NAA (44.4/0.5 μ M). However, in several other orchid species, optimal results have been obtained using MS medium containing relatively high cytokinin

concentrations and no auxins (Gangaprasad et al., 1999; Kerbaudy and Collin, 1997; Ket et al., 2004; Nayak et al., 1997).

Results similar to those obtained for *L. skinneri* have been reported for other orchid species (Hernández, et al., 2001; Kusumoto, 1978, 1979) in which the highest shoot formation was achieved in MS with a higher level of auxin and lower of cytokinin. However, it is more commonly reported that auxins have an inhibitory effect on shoot formation (Kerbaudy and Collin, 1997; Pierik and Steegmans, 1972; Rubluo et al., 1993).

In contrast, PLB formation in *C. pendula* was the primary observed response, and this kind of response is not uncommon in diverse orchid genera such as *Oncidium*, *Dendrobium*, and *Cymbidium* (Chen et al., 1999; Saiprasad and Polisetty, 2003; Teixeira da Silva et al., 2006).

For *C. pendula*, the highest average number of PLBs and shoots per explant were obtained from KCM, but the subsequent development in KCM basal medium was lower than in MS; an alternative methodology for this species could be to induce shoots and PLBs in KCM and then subculture them into MS basal medium. It is possible that more vigorous plantlets could be obtained.

In México and other tropical countries, tree-fern fiber ("maquique") is commonly used as a substrate for orchid culture and overcollection for this purpose has contributed to many tree-ferns becoming endangered (Palacios-Rios and Flores, 1992). The present study demonstrated that "maquique" is not the best substrate for ex vitro plantlet establishment, and superior alternatives such as the pine bark:oak charcoal:pumice mix used here exist. Reduction in the use of maquique could help conserve several tree-fern species.

Documented studies with Mexican orchids are scarce, and most of them focus on determination of the best culture media for the germination and development of plantlets (Damon et al., 2004; Lee-Espinosa, et al., 2007; Santos-Hernández et al., 2005). The use of protocorms as an explant is ideal for propagation, especially if the goal is to maximize genetic variability. Therefore, success in defining micropropagation protocols for these three species with high ornamental potential is one of the main contributions of the present study. Not only could this technique be used by other researchers, but also by individuals in rural communities interested in establishing nurseries to satisfy horticultural demand while reducing pressures on wild populations.

Literature Cited

- Arditti, J. and R. Ernst. 1993. Micropropagation of orchids. Wiley, New York, NY.
- Ávila-Díaz, I. and K. Oyama. 2002. Manejo sustentable de *Laelia speciosa* (Orchidaceae). Biodiversitas. 7:9–12.
- Bhadra, S.K. and M.M. Hossain. 2003. *In vitro* germination and micropropagation of *Geodorum densiflorum* (Lam.) Schltr., an endangered orchid species. Plant Tiss. Cult. 13:165–171.
- Chen, J.T., C. Chang, and W.C. Chang. 1999. Direct somatic embryogenesis on leaf explants of *Oncidium* Gower Ramsey and subsequent plant regeneration. Plant Cell Rep. 19:143–149.
- Chen, J.T. and W.C. Chang. 2001. Effects of auxins and cytokinins on direct somatic embryogenesis on leaf explants of *Oncidium* 'Gower Ramsey'. Plant Growth Regulat. 34:229–232.
- Chen, Y., Ch. Chang, and W. Chang. 2000. A reliable protocol for plant regeneration from callus culture of *Phalaenopsis*. In Vitro Cell. Dev. Biol. Plant 36:420–423.
- Damon, A., E. Aguilar-Guerrero, L. Rivera, and V. Nikolaeva. 2004. Germinación *in vitro* de semillas inmaduras de tres especies de orquídeas de la región del Soconusco, Chiapas, México. Revista Chapingo Serie Horticultura 10:195–204.
- Dixon, K., S. Kell, R. Borret, and P. Cribb. 2003. Orchid conservation. Natural History Publications (Borneo), Kota Kinabalu, Sabal.
- Espejo, S.A. and F.A.R. López. 1998. Las Monocotiledóneas Mexicanas una Sinopsis Florística 1. Lista de Referencia Parte VII. Orchidaceae I. Consejo Nacional de la Flora de México, A.C. Universidad Autónoma Metropolitana-Iztapalapa, Comisión Nacional para el Conocimiento y Uso de la Biodiversidad México.
- Fay, M.F. 1994. In what situations is *in vitro* culture appropriated to plant conservation? Biodivers. Conserv. 3:176–183.
- Flores-Palacios, A. and S. Valencia-Díaz. 2007. Local illegal trade reveals unknown diversity and involves a high species richness of wild vascular epiphytes. Biol. Conserv. 136:372–387.
- Gangaprasad, A.N., W.S. Decruse, S. Seen, and S. Menon. 1999. Micropropagation and restoration of the endangered Malabar daffodil orchid *Ipsea malabarica*. Lindleyana 14:38–46.
- Hernández, J., S. Hernández, and M. Mata. 2001. Regeneración de plántulas a partir del cultivo *in vitro* de mitades de protocormos de *Laelia anceps* Lindl. y *Catasetum integerrimum* Hook. Amaranto. 14:3–12.
- Ichihashi, S. 1991. Development of media for aseptic seed germination of *Dendrobium*. The Bull. Aichi of Education 40:95–100.
- IUCN/SSC Orchid Specialist Group. 1996. *Orchids*—Status survey and action plan. IUCN, Gland, Switzerland, and Cambridge, UK.
- Jiménez, M.R., S.L. Sánchez, and J. García-Cruz. 1998. Familia Orchidaceae. Tribu Maxillarieae. Flora del Bajío y de regiones adyacentes. Fascículo 67.
- Kerbaudy, B.G. and S. Collin. 1997. Increased conversion of root tip meristems of *Catasetum fimbriatum* into protocorm-like bodies mediated by ethylene. Lindleyana 12:59–63.
- Ket, N.V., E.J. Hahn, S.Y. Park, D. Chakrabarty, and K.Y. Paek. 2004. Micropropagation of an endangered orchid *Anoectochilus formosanus*. Biol. Plant. 48:339–344.
- Knudson, L. 1946. A new nutrient solution for the germination of orchid seeds. Am. Orchid Soc. Bull. 15:214–217.
- Kusumoto, M. 1978. Effects of combinations of growth regulating substances, and of organic matter on the propagation and organogenesis of *Cymbidium* protocorms cultured *in vitro*. J. Jpn. Soc. Hort. Sci. 47:391–400.
- Kusumoto, M. 1979. Effect of combination of growth regulators, and of organic supplements on growth of *Cattleya* plantlets cultured *in vitro*. J. Jpn. Soc. Hort. Sci. 47:492–501.
- Lee-Espinosa, H.E., A. Laguna-Cerda, J. Murguía-González, P. Elorza-Martínez, L. Iglesias-Andreu, B. García-Rosas, F.A. Barredo-Pool, and N. Santana-Buzzy. 2007. Regeneración *in vitro* de *Laelia anceps* ssp. *dawsonii*. Revista Científica UDO Agrícola. 7:58–67.
- Mauro, M., D. Sabapathi, and R. Smith. 1994. Influence of benzylaminopurine and alpha-naphthalenetic acid on multiplication and biomass production of *Cattleya aurantiaca* shoot explants. Lindleyana 9:169–173.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473–494.
- Nayak, N.R., S. Patnaik, and S.P. Rath. 1997. Direct shoot regeneration from foliar explant of an epiphytic orchid, *Acampe praemorsa* (Roxb.) Blatter and McCann. Plant Cell Rep. 16:583–586.
- Palacios-Rios, M. and A.P. Flores. 1992. Notas sobre el maquique y cómo afecta su uso a los helechos arborescentes. Boletín de la Asociación Mexicana de Orquideología, A.C. México, D.F. 92:2–5.
- Pierik, R.L. and M.H.H. Steegmans. 1972. The effect of 6-benzilaminopurine on growth and development of *Cattleya* seedlings grown from unripe seeds. Z. Pflanzenphysiol. 68:228–234.
- Pierik, R.L.M., P.A. Sprenkles, B. Van Der Harst, and Q.G. Van Der Meys. 1988. Seed germination and further development of plantlets of *Paphiopedilum ciliolare* Pfitz. *in vitro*. Scientia Hort. 34:139–153.
- Rubluo, A., V.M. Chávez, A. Martínez, and O. Martínez-Vázquez. 1993. Strategies for the recovery of endangered orchids and cacti through *in vitro* culture. Biol. Conserv. 63: 163–169.
- Saiprasad, G.V.S. and R. Polisetty. 2003. Effect of growth regulators on production of PLBs and multiple shoots in orchid: *Dendrobium* 'sonia': Assessment of role of methane and ethylene. Phytomorphology. 53:67–71.
- Salazar, G. 1988. *Mormodes tuxtensis*, a new species from Veracruz, México. Orq. (Mex.) 11:59–62.
- Santos-Hernández, L., M. Martínez-García, J.E. Campos, and E. Aguirre-León. 2005. *In vitro* propagation of *Laelia albida* (Orchidaceae) for conservation and ornamental purposes in Mexico. HortScience 40:439–442.
- Teixeira da Silva, J.A., N. Singh, and M. Tanaka. 2006. Priming biotic factors for optimal protocorm-like body callus induction in hybrid *Cymbidium* (Orchidaceae), and assessment of cytogenetic stability in regenerated plantlets. Plant Cell. Tiss. Org. Cult. 84:135–144.
- Tokuhara, K. and M. Mii. 2001. Induction of embryogenic callus and cell suspension culture from shoot tips excised from flower stalk buds of *Phalaenopsis* (Orchidaceae). In Vitro Cell. Dev. Biol. Plant 37:457–461.
- Wu, I.F., J.T. Chen, and W.C. Chang. 2004. Effects of auxins and cytokinins on embryo formation from root-derived callus of *Oncidium* 'Gower Ramsey'. Plant Cell. Tiss. Org. Cult. 77:107–109.
- Yam, T.W. and M.A. Weatherhead. 1988. Germination and seedling development of some Hong Kong orchids. Lindleyana 3:156–160.