In Vitro Cell.Dev.Biol.-Plant (2009) 45:162–170
DOI 10.1007/s11627-009-9201-2

# Plant regeneration through direct shoot formation from leaf cultures and from protocorm-like bodies derived from callus of *Encyclia mariae* (Orchidaceae), a threatened Mexican orchid

**María del Socorro Santos Díaz · Candy Carranza Álvarez**

Received: 16 May 2008 / Accepted: 24 February 2009 / Published online: 8 April 2009 / Editor: Edward C. Yeung


**Abstract** Two procedures for the *in vitro* propagation of *Encyclia mariae*, a threatened Mexican orchid, were developed. In the first procedure, leaves from *in vitro* germinated seedlings were cultured on Murashige and Skoog medium (MS) supplemented with the range of 2.21–4.4 μM 6-benzylaminopurine (BA) in combination with 2.69–10.74 μM naphthalene acetic (NAA), 2.07–8.29 μM indole-3-butyric (IBA), or 2.85–11.42 μM indole-3-acetic acid (IAA) to determine the best medium for the induction of shooting. Maximum direct shoot formation from leaves was observed on MS containing 22.21 μM BA and 10.74 μM NAA (25 shoots/explant). The second procedure began with the culture of protocorms on media containing NAA, IBA, or IAA, which induced callus formation with high regenerative potential in the form of protocorm-like-bodies (PLBs) that eventually differentiated into shoots. The optimal response was attained when these structures were cultured on medium with 4.14 μM IBA (30 shoots/PLB). To promote the elongation of shoots derived from PLBs, the material was subcultured onto MS medium containing 22.21 μM BA and 5.37 μM NAA. Through the exploration of the effects of auxins and matrix on the rooting of shoots, it was determined that the optimal rooting occurred on media supplemented either with 5.71 μM IAA or 4.14 μM IBA either on agar-gelled medium or in liquid media with coir as the matrix. Rooting was found to be 20% higher in liquid media than in agar-gelled medium.

M. d. S. Santos Díaz (✉) · C. Carranza Álvarez
Centro de Investigación y Estudios de Postgrado,
Facultad de Ciencias Químicas de la Universidad
Autónoma de San Luis Potosí,
Manuel Nava 6,
CP 78210 San Luis Potosí, Mexico
e-mail: ssantos@uaslp.mx

**Keywords** *Encyclia mariae* · *Euchile mariae* · Micropropagation · Orchids · Protocorm

## Introduction

The tropical and subtropical areas of Mexico are especially rich in biodiversity. Unfortunately, due to illegal collection and trade, agricultural activities, and fires, many tropical species are considered endangered or threatened with extinction (Norma Oficial de la Federación 2001). In particular, the orchids have been overcollected to meet the demands for ornamental plants in the commercial market place (Norma Oficial Mexicana 2002).

The Orchidaceae family, while one of the most diverse plant families, is also one of the most vulnerable due to its habitat destruction and high harvest rates. In Mexico, there are about 1,400 species of orchids (Bastida-Tapia et al. 2007) of which 181 species are classified as rare, threatened, or endangered (Norma Oficial Mexicana 2002). One such species, *Encyclia mariae* (Ames) Hoehne, also known as *Euchile mariae*, is a tropical epiphytic orchid that grows in Central and West Mexico (Mora-Olivo et al. 1992; Sánchez-Ramos et al. 1993). It is a species with small pseudobulbs and white flowers with central green veins. Seed maturation requires about 9 mo and *in vitro* development of embryos takes 4–5 mo. Vegetative propagation is carried out by dividing pseudobulbs, which usually contain two to three leaves. Neither of these methods provides a sufficient number of plants to support commercialization (Arditti 1967).

*E. mariae* grows associated with canopy tress like *Quercus oleoides*, *Acacia farnesiana*, and *Guazuma ulmifolia* (Rzedowski 1978). According to federal laws and Mexican

regulations, this species is considered threatened (Norma Oficial de la Federación 2001).To our knowledge, no information is available regarding seed germination, growth rates or proliferative capacity under natural conditions. In addition, recent satellite image analysis shows a dramatic deforestation of tropical areas (Ibarra-Zapata 2008), a situation that has increased the pressure of extinction for many species, including *E. mariae*. Unless an effective method of propagation can be developed to provide plants for restoration purpose and/or legal commercial trade, it is predicted that *E. mariae* will disappear from its natural habitat in a few years (Ibarra-Zapata 2008).

*In vitro* culture has been used to successfully propagate different species of terrestrial and epiphytic orchids. Such protocols include asymbiotic seed germination (Sheehan 1983; Arditti and Ernst 1993; Stewart and Kane 2006; Johnson et al. 2007) and asexual methods employing shoot tips, rhizomes, protocorms, pseudobulbs, and even cell suspension as explant starting material (Morel 1964; Sagawa 1966; Arditti 1977; Geetha and Shetty 2000; Sheelavantmath et al. 2000; Park et al. 2002; Chen et al. 2004; De Melo Ferreira et al. 2006; Teixeira da Silva et al. 2006).

The objectives of this research were: (1) to provide an efficient seed germination technique, (2) to induce direct shoot formation from leaves, (3) to promote callus formation from protocorms with high regenerative capacity, and (4) to analyze the effect of different auxins and matrices on rooting of regenerated shoots for the orchid *E. mariae.* The data collected from this study will be used for further investigations into amplified production of *E. mariae* for potential reintroduction into its natural habitat and for commercialization purposes.

## Materials and Methods

*Plant material and culture conditions*. Young *E. mariae* plants were obtained from a local greenhouse at Huichihuayán, San Luis Potosí, and mature capsules were provided by La Joya greenhouse at Atlixco, Puebla.

Murashige–Skoog (MS; Murashige and Skoog 1962) basal medium was supplemented with 116 μM myo-inositol, 1.2 μM thiamine–HCl, 30 g $L^{-1}$ sucrose, and 8 g $L^{-1}$ agar (Phyto Technology, Shawnee Mission, KS; Plant Tissue Culture grade). The pH of the media was adjusted to 5.7 after the addition of the growth regulators. Media were sterilized at 120°C for 20 min ($1.37\times10^5$ Pa). The cultures were maintained at 25°C or in a photoperiod of 16 h light at 45 μmol $m^{-2}$ $s^{-1}$ and 8 h darkness with light supplied by cool-white fluorescent lamps (Phillips, Saltillo, Mexico).

*Disinfection and germination protocols*. Capsules of *E. mariae*, before dehiscence, were surface-sterilized with 70% ethanol for 2 min, and then in 10% sodium hypochloride containing 0.2% (*v*/*v*) Tween-20 for 10 min. The seeds were washed twice with deionized water for 1 min each and germinated on MS basal medium containing 1% (*w*/*v*) activated charcoal (Karal S.A. de C.V, Léon, Mexico). For the disinfection of shoots, roots, and bulblets, an additional treatment for 10 min with 10% $AgNO_3$ and 2% sodium hypochlorite was included.

*Shoot induction*. To initiate *in vitro* cultures, leaves and 4-wk-old protocorms generated from germinated seeds were used as explants. The leaves were cultivated on MS medium supplemented with 0, 4.4, 8.8, or 22.21 μM benzyladenine (BA) alone or in combination with the following auxins:

(a) 2.85, 5.71, or 11.42 μM indolacetic acid (IAA)
(b) 2.07, 4.14, or 8.29 μM indolbutyric acid (IBA)
(c) 2.69, 5.37, or 10.74 μM naphthalene acetic acid (NAA)

The protocorms were cultivated on MS medium supplemented with the auxins NAA (2.69, 5.37, or 10.74 μM), IBA (2.07, 4.14, or 8.29 μM), or IAA (2.85, 5.71, or 11.42 μM) to induce callus formation.

The callus formation was categorized on a scale from 0 to 4: 0 = 0% callus, type 1 = 1–25% of the explant surface with callus, 2 = 26–50% surface with callus, 3 = 51–75% surface with callus, and 4 = 75–100% surface with callus.

*Shoots elongation*. To promote elongation, the shoots were cultivated on MS medium supplemented with 22.21 μM BA and 5.37 μM NAA for 4 wk. After this time, the number of new shoots was evaluated.

*Rooting of shoots*. After 90 d, elongated, dark-green shoots were transferred to MS basal medium supplemented with 3% (*w*/*v*) sucrose, 0.3% (*w*/*v*) polyvinylpirrolidone, and either IBA (2.07 μM or 4.14 μM) or IAA (2.85 μM or 5.71 μM). Liquid media and media solidified with 0.8% (*w*/*v*) agar were employed. When liquid medium was used, coir or coconut fiber (Artifibras SA de CV, Uruapan, México) was employed as support. Prior to use, the coir was washed five times with tap water, dried in an oven, and sterilized for 1 h in the autoclave. About 30 mg of defragmented coir was placed in a Magenta box (7.5 cm×7.5 cm×10 cm) and 25 ml of sterile liquid media dispensed into each box and again autoclaved for 20 min. The number of roots and root length developing from each regenerated shoot was recorded every 30 d.

After 2 mo on rooting medium, the rooted plants were transplanted to glass vessels (55 mm×130 mm) containing a mixture of coir and bark (1:1), which had been previously autoclaved for 1 h. Basal MS medium with 3% sucrose was used as nutritive solution. The cultures were kept at 25±1°C

under a photoperiod of 16 h light and 8 h dark for 2 mo at 45 μmol $m^{-2}$ $s^{-1}$. After this time, the plants were transferred to greenhouse conditions (30–35°C, relative humidity 68.8%, light 127 μmol $m^{-2}$ $s^{-1}$). To do so, the roots were washed with tap water to eliminate the medium, treated 3 min with 1% of the commercial fungicide Benlate (Bavistin DF), and dipped onto a rooting solution (Raizal 400). The plants were placed in pots (6×7 cm) containing volcanic stone, porous stone, and a mixture (1:1) of bark and coir. All the substrates were previously sterilized and treated with fungicide. The plants were watered with a solution of commercial fertilizer "Tripe 17" (Bayer, Wuppertal, Germany) at 10% and covered with plastic domes. After 1 wk, the covers were removed progressively to allow for the acclimatization of the plants.

*Statistical analysis*. All tests were carried out using between 40 explants per experiment and two independent experiments were included per analysis. To evaluate statistically significant differences among mean values, an ANOVA and Tukey test were used at the significance level of 95%, employing the Instat II and MINITAB programs.

## Results and Discussion

*Seed germination*. Orchid seeds are minute, contain an undifferentiated embryo, and lack endosperm. Due to the non-endospermic nature of the seeds, germination in natural conditions requires fungal infection. The use of *in vitro* culture can substitute for the co-culture with a mycobiont by providing cytokinins, since many mycorrhizal fungi produce these compounds (Crafts and Miller 1974; Masuhara and Katsuya 1989; Wilkinson et al. 1989). Successful *in vitro* asymbiotic seed germination of terrestrial and epiphyte orchids has been reported for many species (Malmgren 1992; Arditti and Ernst 1993; Chou and Chang 2004; Stewart and Kane 2006; Johnson et al. 2007).

In the present work, the seeds were also selected as explants since establishment of *in vitro* cultures was not successful from sterilized leaves, pseudobulbs, or rhizomes (results not shown), due to the lack of response of the tissues on media containing auxins and cytokinins. After 10 d of culture on MS basal medium, the seeds began to swell and the embryos turned from yellow to yellowish green in color, finally becoming green as they grew into protocorms. The germination of *E. mariae* seeds reached 95% (Fig. 1), with the formation of protocorms initiating at 20 d and the first leaves appearing at 25 d. The length of germinated seedlings increased with time and reached about 25 mm after 45 d of culture on the basal medium (Fig. 1). The highest growth rate was observed at 30 d (0.66 mm/d). Maintaining seedlings on this medium for more than 60 d induced oxidation and necrotic tendency. As the continuous subculture of protocorms of *E. mariae* on basal medium did not improve their development, we developed a micropropagation protocol.

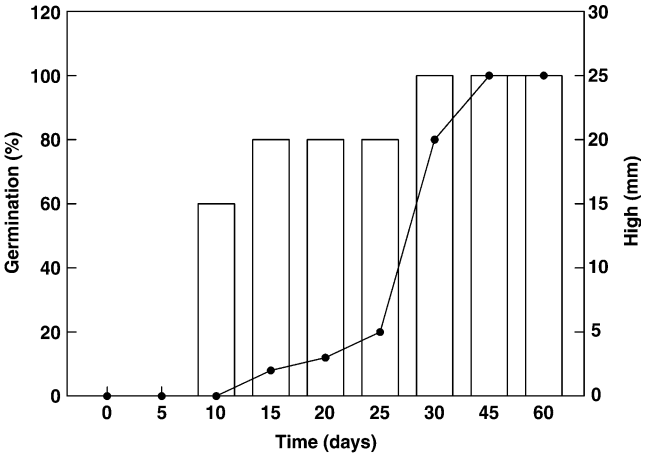


**Figure 1.** Seed germination of *E. mariae* and seedling growth. The seeds were germinated on MS basal medium containing 1% (*w*/*v*) activated charcoal.

*Direct shoot formation from leaf explants*. To induce *de novo* shoot regeneration, individual leaves of at least 1 cm in length were transferred to MS medium supplemented with BA alone or in combination with the auxins NAA, IBA, or IAA. New shoots first appeared as small, green protuberances at the leaf bases and developed into shoots without any callus or protocorm-like body (PLB) formation after 2 wk. Bud-break occurred 3–4 wk after placing the leaves on culture media. At 60 d, shoots formation was two- to threefold higher from explants cultured on media supplemented with auxins and BA, in comparison to media containing exclusively BA (Fig. 2*a*). The induction of new shoots was independent of IBA concentration ($P>0.05$), but increased proportionally to BA concentration, generating about 25 new shoots per explant on media supplemented with 22.21 μM BA. When cultured on medium containing a combination of BA and IAA, a maximum of 22 shoots per explant was observed in the presence of 8.8 μM BA and either 5.7 or 11.42 μM IAA; however, a reduction in the number of new shoots, to 16 shoots per explant, was observed when 22.21 μM BA was used (Fig. 2*a*). From 17 to 24 regenerated shoots per explant were obtained on media containing different concentrations of NAA and BA (Fig. 2*a*). Development of shoots derived from leaves after 60 d of culture on media with 5.37 μM NAA and 22.21 μM BA is shown on Fig. 3*a*. The synergistic action of auxins and cytokinins and the beneficial effect of BA have also been described for the regeneration of several orchids, including the genera *Oncidium* (Kalimuthu et al. 2007), *Geodorum* (Sheelavantmath et al. 2000), *Vanda* (Decruse et

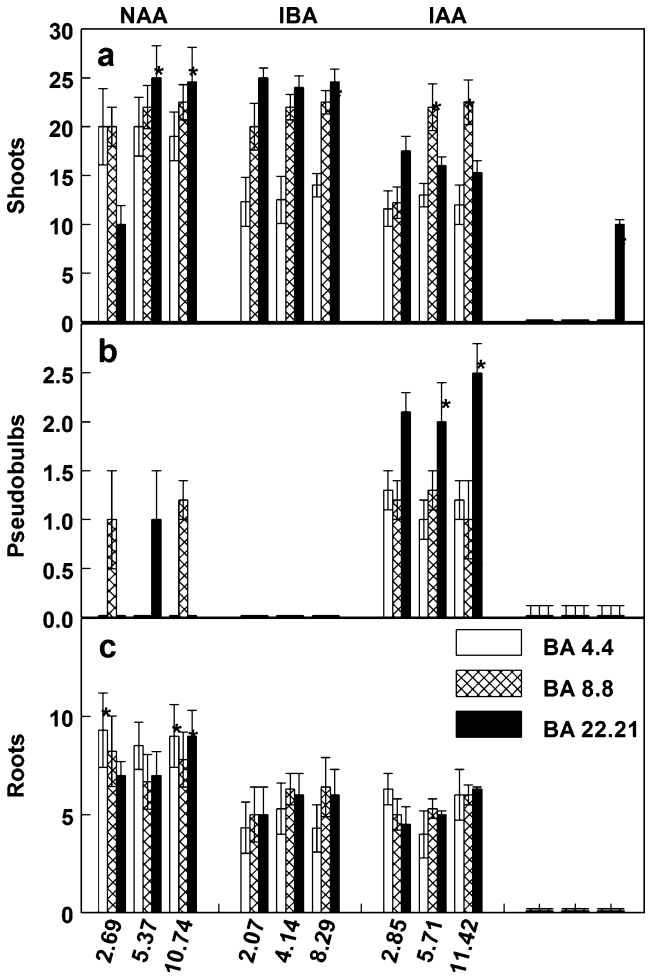


**Figure 2.** Effect of growth regulators on the morphogenetic response of *E. mariae.* Individual leaves were transferred to MS medium containing BA alone or in combination with the auxins NAA, IBA, or IAA. *a*, Induction of shoots; *b*, number of pseudobulbs formed; and *c*, presence of incipient roots. *Columns with different letters* are statistically significant ($P<0.05$).

al. 2003), *Dendrobium* (Roy et al. 2007), *Vanilla* (Geetha and Shetty 2000), and *Cymbidium* (Paek and Yeung 1991; Teixeira da Silva et al. 2006). Particularly, in *Cymbidium forrestii* species, BA was reported to induce the cytoplasmic zone of the apical meristem to enlarge and enhance leaf development (Paek and Yeung 1991).

The formation of both pseudobulbs and roots on media supplemented with BA and auxins was also evaluated. The number of pseudobulbs per shoot was approximately 1.2 on media supplemented with BA and NAA, without a clear correlation either with auxin or cytokinin concentration (Fig. 2*b*). When cultured on media containing BA and IAA, the average number of pseudobulbs varied from 0.7 to 2.7 and increased proportionally to BA concentration. No pseudobulbs were formed when IBA was used as the source of auxin. On the other hand, the presence of small roots, about 2 mm length, over the rhizome surface was observed at the end of the experiment (Fig. 2*c*). The generation of roots was influenced mainly by the type, but not the concentration, of auxin. The greatest degree of rhizogenesis was attained when tissues were cultured on medium containing a combination of 4.4 μM BA and 10.7 μM NAA, yielding seven to nine new roots per shoot. The formation of roots on media containing either BA and IBA or BA and IAA ranged between four and six roots per shoot. The roots continued to grow for 2 mo, after which the radical growth was arrested.

The growth of *in vitro* shoots was also assessed (Fig. 4). In general, the length of the shoots was not statistically different ($P<0.05$) when tissues were cultured at different concentrations of auxins, but increased proportionally to BA concentration. The longest shoots, 45 to 48 mm in length, were attained in the presence of 10.7 μM NAA and 22.2 μM BA (Fig. 4*a*), while shoots 40 to 42 mm long were observed on medium containing 4.1 μM IBA and 22.2 μM BA (Fig. 4*b*), and shoots 38 to 40 mm long were produced on medium containing 5.7 μM IAA and 22.2 μM BA (Fig. 4*c*).

*Shoots induction from protocorms*. Induction of shoots from protocorms was also attempted. These explants were transferred onto media supplemented with different concentrations of NAA, IBA, or IAA (Table 1). After 60 d of culture on media supplemented with auxins, an average of 77.5% of explants cultured on 4.14 μM IBA developed type 4 callus and 17.5% of explants developed type 3 callus. The response decreased when the protocorms were cultured on media containing higher or lower concentrations of IBA, in which case most of the explants developed callus to a level of type 2. Protocorms maintained on media with NAA displayed an inverse relationship between auxin concentration and callus formation. On media with 5.37 μM NAA and 10.74 μM NAA, callus type 4 was not developed, while on media with 2.7 μM NAA, 17.5% of the explants developed type 4 callus. When explants were cultured on media with IAA, type 4 callus formation was not observed at any concentration. Approximately 75% and 85% of explants cultured on media with 2.85 and 5.71 μM IAA developed type 1 callus, respectively, while 85% of the explants cultured on media with 11.42 μM IAA developed type 2 callus. Only a small percentage of protocorms (2–5%) did not produce callus at all (Table 1). In conclusion, the auxin which induced the highest callus formation from protocorms in this study was IBA.

After 60 d of culture on media with auxins, PLBs appeared on the callus surface in all treatments. These PLB differentiated into shoots (Fig. 3*b*), at different efficiencies (Table 1), with a high concentration of NAA stimulating shoot formation but a high concentration of IAA inhibiting shoot formation. Moderate concentrations of IBA were the most effective for inducing shoot formation. Other reports also describe the role

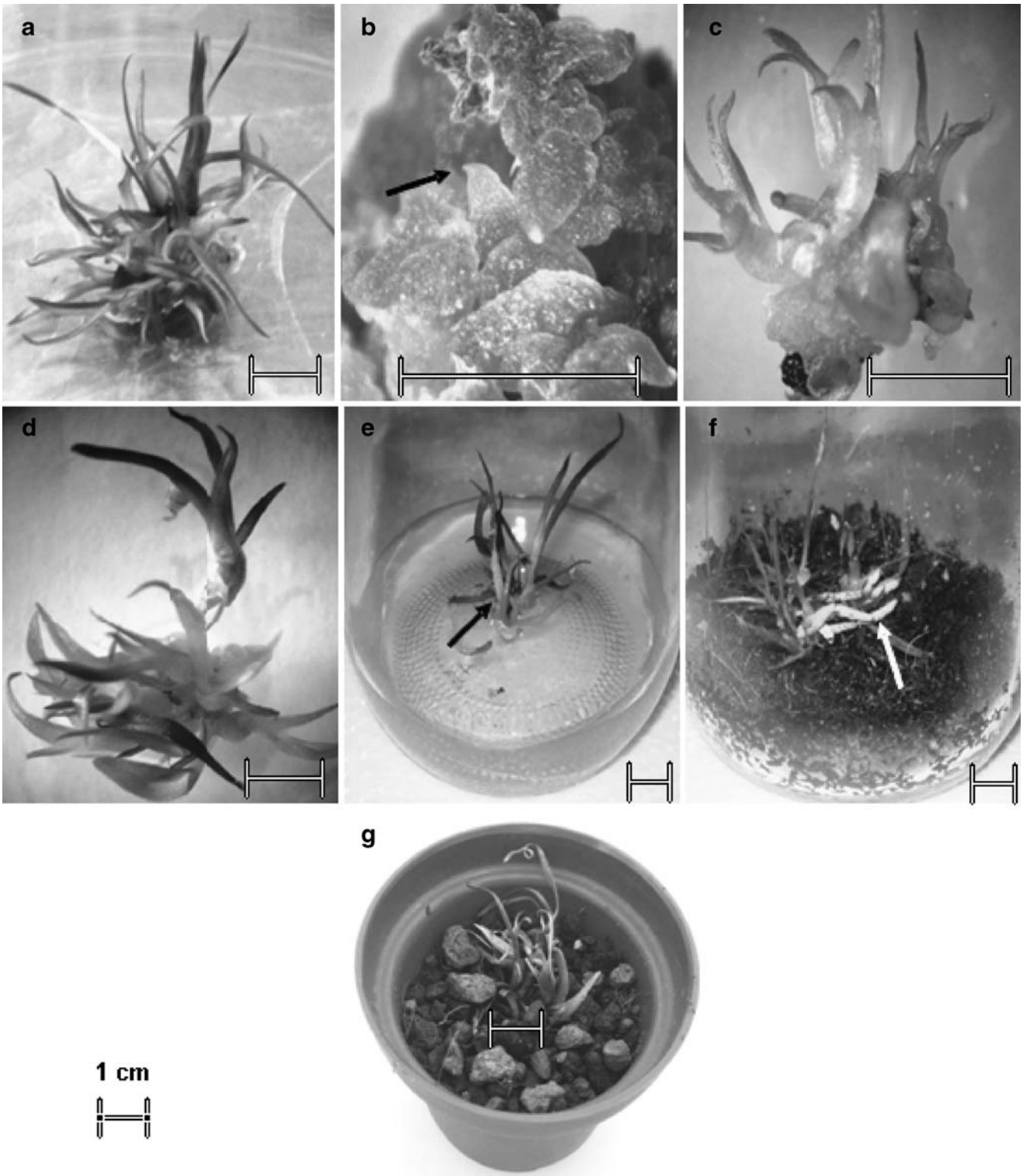


**Figure 3.** Plant regeneration of *E. mariae*. *a*, Shoots derived from leaves; *b*, callus mass showing protocorm-like bodies (PLBs) and emerging primordial; *c*, well-defined shoots derived from PLB; *d*, elongation of shoots on MS medium with 22.21 μM BA and 5.37 μM NAA; *e*, rooted plants on agar-gelled media (*arrow* shows new roots); *f*, rooted plants on liquid media with coir (*arrow* shows the new shoots); and *g*, adapted plants to soil.

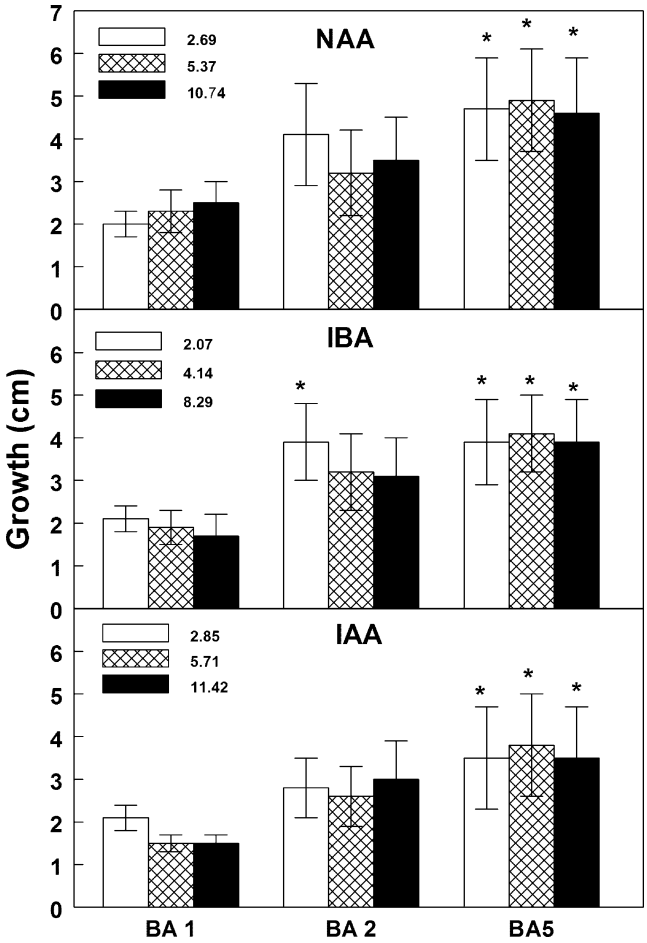


**Figure 4.** Growth of axillary shoots on media supplemented with BA and auxins at 60 d. The leaves were cultivated on MS medium supplemented with 4.4, 8.8, or 22.21 μM BA in combination with different concentrations (μM) of: *a* NAA, *b* IBA, or *c* IAA. *Bars with the same letter* are not significantly different ($P>0.05$).

of auxins (mostly NAA, IAA, and IBA) on seedling growth (Arditti 1977; Arditti 1979; Arditti and Ernst 1993). However, the exclusive use of auxins not always promotes shooting in orchid species. In *Geodorum densiflorum* (Sheelavantmath et al. 2000) and *Cymbidium faberi* (Chen et al. 2005), the NAA stimulated growth and multiplication of rhizome while in *Dendrobium chrysotoxum* NAA improved callus growth (Roy et al. 2007).

*Elongation of shoots*. While shoot regeneration was observed from PLBs derived from callus on media supplemented with auxins, the shoots did not elongate on these media. Therefore, shoots derived from PLBs were transferred to MS medium containing 22.2 μM BA and 5.4 μM NAA to promote their elongation (Fig. 3*c*). Cultivation of shoots on this medium was effective and, after 60 d, shoots from 15 mm±3 length were obtained (Fig. 3*d*). The shoots cultivated on media exclusively containing auxins had an average length of 5 mm.

A slight increase in shoot number from PLBs was obtained in comparison to shoots derived from leaves, but this process was more time-consuming because an additional elongation step was required with subculture onto a medium containing auxin and cytokinin. While plants micropropagated by means of callus or PLBs can be susceptible to somaclonal variation (Lim and Loh 2003), this effect does not seem to be general, since shoots of *Dendrobium* derived from PLBs did not exhibit any type of polymorphism (De Melo Ferreira et al. 2006). Likewise, shoots of *E. mariae* derived from PLBs in this study showed good quality and uniformity, indicating that this could be an additional strategy to increase the production of shoots in this species.

**Table 1.** Callus induction from protocorms and shoot formation from protocorm-like bodies derived from callus of *Encyclia mariae* on media with auxins[a]

| Auxin | Concentration (μM) | Callus (%)[b] | | | | | No. of shoots/PLB[c] |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | |
| NAA | 2.69 | 0 b | 65 a | 2.5 d | 15 a | 17.5 b | 12±3 b |
| | 5.37 | 0 b | 85 a | 12.5 c | 2.5 c | 0 d | 15±2 b |
| | 10.74 | 0 b | 75 a | 25.0 b | 0 d | 0 d | 28±2 a |
| IBA | 2.07 | 0 b | 10 b | 85 a | 5 c | 0 d | 8±1.5 c |
| | 4.14 | 0 b | 0 c | 5 d | 17.5 a | 77.5 a | 30±2 a |
| | 8.29 | 0 b | 0 c | 85 a | 7.5 b | 7.5 c | 6±2 c |
| IAA | 2.85 | 2.5 a | 75 a | 10 c | 12.5 b | 0 d | 16±2 b |
| | 5.71 | 0 b | 85 a | 12.5 c | 2.5 c | 0 d | 19±4 b |
| | 11.42 | 5 a | 0 c | 85 a | 10 b | 0 d | 9±1 c |

[a] Values in the same *column* with different *letters* represent statistically significant differences ($P<0.05$)

[b] The protocorms were cultivated on MS medium supplemented with the indicated auxins, and callus formation was evaluated at 60 d. The callus formation was categorized on a scale from 0 to 4: 0 = 0% callus, type 1 = 1–25% of the explant surface with callus, 2 = 26–50% surface with callus, 3 = 51–75% surface with callus, and 4 = 75–100% surface with callus

[c] Shoots derived from protocorm-like bodies (PLB) at 90 d

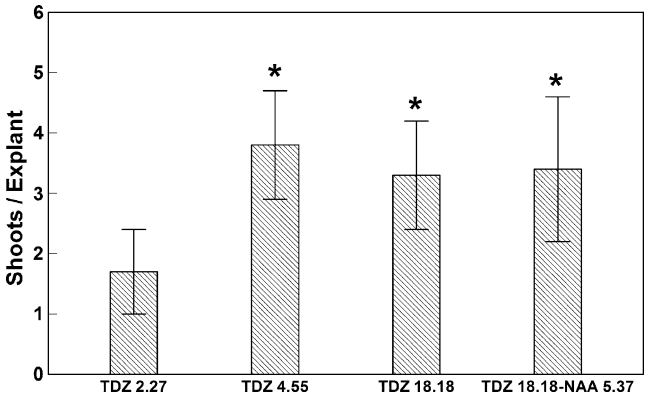


**Figure 5.** Effect of thidiazuron on shoot induction.

*Effect of thidiazuron on shoot induction*. The effect of thidiazuron (TDZ) on the morphogenic response of orchids is well documented. The beneficial effect of TDZ on shoot regeneration and proliferation (Chen and Piluek 1995; Nayak et al. 1997; Chen et al. 2004; De Melo Ferreira et al. 2006), direct somatic embryogenesis (Chen et al. 1998; Chen and Chang 2000), and induction of PLBs (Park et al. 2002; Roy et al. 2007) has been reported. Therefore, we studied the morphogenetic effect of TDZ on callus derived from protocorms. Callus was cultivated on MS media containing either TDZ alone (2.27, 4.55, or 18.18 μM TDZ) or 18.18 μM TDZ plus 5.37 μM NAA. Approximately 3.8 shoots per callus were obtained on medium supplemented with either 4.55 μM or 18.18 μM TDZ, but lower concentrations of TDZ reduced the number of new shoots significantly ($P<0.05$). The presence of NAA did not improve shoot formation (Fig. 5). Although TDZ promoted *E. mariae* shoot formation, the efficiency of the process was lower compared to media with auxins or auxins plus cytokinins.

*Rooting of in vitro derived shoots*. The effect of two types of matrix—coir and agar—and the two auxins IAA and IBA were evaluated for their impact on root formation from elongated shoots derived from leaves and PLBs (Table 2). Shoots cultivated in liquid media supplemented with IAA developed three times more roots per shoot (13.0±1.3) in comparison to those cultured on media with IBA (4.1±1.7). The difference was statistically significant ($P<0.05$) at both 90 and 120 d. The formation of new roots increased in parallel to IAA concentration but was independent of IBA concentration. In addition, longer roots were formed on media with IAA than in media with IBA (Fig. 3*e*). At 90 d, the percentage of rooted shoots reached 100% using 5.71 μM IAA and 80% using 4.14 μM IBA.

Shoots maintained on media solidified with agar developed more roots per explant on media containing IAA than on those with IBA. However, the response was independent

**Table 2.** Rooting of shoots from *E. mariae* on media with different auxins and physical supports[a,b]

| Auxin | Concentration (μM) | Time (d) | Liquid medium and coir | | | Agar | | |
|---|---|---|---|---|---|---|---|---|
| | | | Roots/shoot ±SD | Root length (cm) ±SD | Rooting (%) | Roots/shoot ±SE | Root length (cm) ±SD | Rooting (%) |
| IAA | 2.85 | 30 | 0 d | 0 d | 0 | 0 d | 0 | 0 |
| | | 60 | 4.3±1.2 c | 0.5±0.09 c | 60 | 3.3±1 b | 0.5±0.05 c | 40 |
| | | 90 | 6.3±1.33 b | 2.0±0.9 c | 80 | 4.3±1.2 b | 0.5±0.09 c | 60 |
| | | 120 | 6.3±1.33 b | 4.3±1.2 b | 80 | 4.3±1.3 b | 1.2±0.2 b | 60 |
| | 5.71 | 30 | 4.0±0.9 c | 2.5±0.07 c | 80 | 4±0.8 b | 0.5±0.07 c | 60 |
| | | 60 | 10.0±1.4 a | 2.6±0.9 c | 80 | 5±0.9 b | 1.3±0.09 b | 80 |
| | | 90 | 13.0±1.3 a | 6.8±1.4 a | 100 | 5±1.1 b | 3.2±0.5 a | 80 |
| | | 120 | 13.0±1.3 a | 6.8±1.4 a | 100 | 8±2 a | 3.5±0.7 a | 80 |
| IBA | 2.07 | 30 | 0 d | 0 | 0 | 0 d | 0 d | 0 |
| | | 60 | 3.5±1.5 c | 0.5±0.05 d | 60 | 2.8±1 b | 0.5±0.02 c | 40 |
| | | 90 | 4.1±1.7 c | 2.5±1.1 c | 80 | 3.1±0.3 b | 1±0.1 b | 40 |
| | | 120 | 4.0±1.4 c | 5.5±1.5 b | 80 | 3±0.5 b | 1±0.05 b | 60 |
| | 4.14 | 30 | 0 d | 0 d | 60 | 0 d | 0 d | 70 |
| | | 60 | 2.2±1c | 4.5±2.2 b | 80 | 1.2±0.5 c | 1.4±0.2 b | 60 |
| | | 90 | 3.2±0.5 c | 5.8±3.1 b | 80 | 1.2±0.2 c | 2.8±0.6 a | 70 |
| | | 120 | 3.2±0.5 c | 5.8±3.1 b | 80 | 2.2±0.8 c | 3.1±0.3 a | 70 |

[a] Values in the same *column* with different *letters* are different in a statistically significant manner ($P<0.05$)

[b] Elongated shoots were transferred to MS basal medium supplemented with IAA or IBA. The shoots were maintained on media solidified with agar or in liquid media with coir to induce rooting

of the IAA concentration and inverse to the IBA concentration. Longer roots were obtained on media with IAA (Fig. 3*f*) at early time points, but these results were not statistically different at the end of the experiment in comparison to those cultured on media with IBA. At 90–120 d, the maximum percentage of rooted shoots (80%) occurred on media supplemented with IAA, while 70% rooting occurred on media containing IBA. In summary, IAA was more efficient in promoting root formation than IBA. It has been described that IAA improves the number and quality of roots in several species because it stimulates the cellular division of radical primordium, promotes the synthesis of specific proteins, and increases the sugar release into the phloem (Wilson 1994).

In relation to matrix types, it has been reported that most species require a solid support for proper multiplication and rooting. However, maintenance of cultures in liquid media is a common practice for many plant systems and has often been found to be more advantageous than conventional agar-gelled media. It has been suggested than liquid culture changes the physiological nature of the explants because direct contact of the tissue with nutrients favors the development of organogenesis (Mohamed et al. 2006). The use of coir as a physical support has been described previously as a matrix that is eco-friendly, inexpensive, and with a high moisture retention capacity. A 100% root induction rate was attained in liquid media with coir in tobacco, *Chenopodium*, *Beta vulgaris*, teak, and banana species (Gangopadhyay et al. 2002). In our study, the rooting matrix also had an important effect on the rooting response. The use of coir in combination with liquid media was 20% more efficient in root promotion, yielding a higher percentage of rooting, an increased average number of roots produced per explant, and longer roots than shoots cultured on media solidified with agar (Table 2).

The combination of bark and coir forms a porous mixture that allowed good root ventilation and an additional development of roots. As *E. mariae* is an epiphyte orchid, the aeration of roots is an important requirement to survive. The transfer of rooted plants to a mixture of coir and bark under *in vitro* conditions improved the vigor of the roots, a factor which was crucial to adaptation of plants to soil. The survival of plants in greenhouse conditions was 90% at 30 d (Fig. 3*g*). Plants without a well-developed root do not survive when transferred to soil.

In summary, the present study describes two efficient pathways of shoot induction of *E. mariae*. The first protocol includes direct shoot formation from leaf (25 shoots/explant) without callus or PLB formation. The second method involves the generation of callus derived from protocorms, with a high regenerative capacity in the form of PLBs that, later on, differentiated into shoots (30 shoot/explants). Optimal rooting was attained on the media with 5.71 μM IAA. The use of coir and liquid media was an important factor in promoting root induction. The regeneration process of *E. mariae* plants required approximately 9–12 mo.

Considering that *in vitro* germination of one mature capsule generated 200 plantlets with two or three leaves, and taking into account that from each leaf it is possible to obtain 25 shoots, approximately 10,000–15,000 plants per capsule could theoretically be regenerated using the *in vitro* techniques reported here. Unfortunately, there are no official reports about the production of plants of *E. mariae* in natural conditions to make a comparison.

Future goals include the reintroduction of *E. mariae* to its natural habitat and the commercialization of *E. mariae* production to reduce the pressure of collection from the wild population. In order to reduce costs, the rooting and acclimatization of regenerated shoots could be realized in one single step, using biodegradable pots such as Jiffy 7® peat pellets or coir pots. For high-scale mass propagation, the temporary immersion systems are another potential alternative (Etienne and Berthouly 2002). Used in combination with the culture techniques described here, it should be possible to generate very large numbers of *E. mariae* at a low cost per unit.

**Acknowledgments** This work was carried out with financial support from FAI-UASLP (C06-FAI-03-25.28). The authors thank CONACYT for the scholarship to CCA (no. 173383).

## References

Arditti, J. Factors affecting the germination of orchid seeds. *Bot. Rev* 33: 1–97; 1967. doi:10.1007/BF02858656.

Arditti, J. Clonal propagation of orchids by means of tissue culture—a manual. In: Arditti J. (ed) Orchid biology, reviews and perspectives I. Comstock, Ithaca, pp 203–295; 1977.

Arditti, J. Aspects of the physiology of orchids. *Adv. Bot. Res* 7: 422–638; 1979.

Arditti, J.; Ernst R Micropropagation of orchids. Wiley, New York, p 682; 1993.

Bastida-Tapia, A.; Flores Escobar, G.; Gil Vázquez, I.; Navarro López, E. Introducción a la reproducción y manejo de orquídeas mexicanas. Serie de Publicaciones AGRIBOT, Chapingo, México; 2007.

Chen, J. T.; Chang, C.; Chang, W. C. Direct somatic embryogenesis from leaf explants of *Oncidium* "Grower Ramsey" and subsequent plant regeneration. *Plant Cell Rep* 17: 251–255; 1998. doi:10.1007/s002990050433.

Chen, J. T.; Chang, W. C. Efficient plant regeneration through somatic embryogenesis from callus cultures of *Oncidium* (Orchidaceae). *Plant Sci*. 160: 87–93; 2000. doi:10.1016/S0168-9452(00)00367-8.

Chen, J. T.; Su, Y. J.; Wu, I. F.; Chueh, C. M.; Kuo, H. J.; Chang, W. C. Somatic embryogenesis in *Oncidium*. In: Chang W. C.; Drew R. (eds) Proc. of the 2nd International Symposium on biotechnology of tropical and subtropical species. *Acta Hortic*. 692: 75–80; 2005.

Chen, T. Y.; Chen, J. T.; Chang, W. C. Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum*

orchids.. *Plant Cell Tissue Organ Cult* 76: 11–15; 2004. doi:10.1023/A:1025858211320.

Chen, Y.; Piluek, C. Effects of thidiazuron and $N^6$-benzylaminopurine on shoot regeneration of *Phalaenopsis*. *Plant Growth Regul*. 16: 99–101; 1995. doi:10.1007/BF00040513.

Chou, L. C.; Chang, D. C. N. Asymbiotic and symbiotic seed germination of *Anoectochilus formosanus* and *Haemaria* discolor and their F1 hybirds. *Bot. Bull. Acad. Sin* 45: 143–147; 2004 (SCI).

Crafts, C. B.; Miller, C. O. Detection and identification of cytokinins produced by mycorrhizal fungi. *Plant Physiol* 54: 586–588; 1974. doi:10.1104/pp.54.4.586.

De Melo Ferreira, W.; Barbante Kerbauy, G.; Pimentel Costa, A. P. Micropropagation and genetic stability of *Dendrobium* hybrid (Orchidaceae). *In Vitro Cell. Dev. Plant* 42: 568–571; 2006.

Decruse, S. W.; Gangaprasad, A.; Seeni, S.; Menon, V. S. Micropropagation and ecorestoration of *Vanda spathulata*, an exquisite orchid. *Plant Cell Tissue Organ Cult* 72: 199–202; 2003. doi:10.1023/A:1022267009531.

Etienne, H.; Berthouly, M. Temporary inmersion system in plant micropropagation. *Plant Cell Tissue Organ Cult* 69: 15–231; 2002. doi:10.1023/A:1015668610465.

Gangopadhyay, G.; Das, S.; Mitra, S. K.; Poddar, R.; Modak, B. K.; Mukherjee, K. K. Enhanced rate of multiplication and rooting through the use of coir in aseptic liquid culture media. *Plant Cell Tissue Organ Cult* 68: 301–310; 2002. doi:10.1023/A:1013921330155.

Geetha, S.; Shetty, S. A. *In vitro* propagation of *Vanilla planifolia*, a tropical orchid. *Curr. Sci* 79: 887–889; 2000.

Ibarra-Zapata, E. Análisis Geográfico para la Conservación de la Naturaleza. Estudio de caso Área Natural Protegida Reserva de la Biosfera Sierra Abra de Tanchipa. Tesis. Coordinación de Ciencias Sociales y Humanidades, Universidad Autónoma de San Luis Potosí; 2008: 120.

Johnson, T. R.; Stewart, S. L.; Dutra, D.; Kane, M. E.; Richardson, L. Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)—preliminary evidence for the symbiotic culture advantage. *Plant Cell Tissue Organ Cult* 90: 313–323; 2007. doi:10.1007/s11240-007-9270-z.

Kalimuthu, K.; Senthilkumar, R.; Vijayakumar, S. *In vitro* micropropagation of orchid, *Oncidium* sp. (Dancing dolls). *African J. Biotechnol* 6: 1171–1174; 2007.

Lim, W. L.; Loh, C. S. Endopolyploidy in *Vanda* Miss Joaquim (Orchidadaceae). *New Phytol* 159: 279–287; 2003.

Malmgren, S. Large scale asymbiotic propagation of *Cypripedium calceolus*—plant physiology from a surgeon's point of view. Royal Botanic Gardens Kew. *Micropropagation News* 15: 59–63; 1992.

Masuhara, G.; Katsuya, K. Effects of mycorrhizal fungi on seed germination and early growth of three Japanese terrestrial orchids. *Sci. Hortic* 37: 331–337; 1989.

Mohamed, S. V.; Sung, J. M.; Jeng, T. L.; Wang, C. S. Organogenesis of *Phaseolus angularis* L: high efficiency of adventitious shoot regeneration from etiolated seedlings in the presence of $N^6$-benzylaminopurine and thidiazuron. *Plant Cell Tissue Organ Cult* 86: 187–199; 2006.

Mora-Olivo, A.; Sifuentes Silva, R.; Margain Hernández, M.; Yáñez Pacheco, M. Recursos florísticos. En: Estudio de Declaratoria como Reserva Ecológica de la Sierra del Abra-Tanchipa, San Luis Potosí, México. Instituto de Ecología y Alimentos, Universidad Autónoma de Tamaulipas, SEDUE, pp 97–123; 1992.

Morel, G. M. Tissue culture—a new means of clonal propagation of orchids. *Am. Orchid Soc. Bull* 33: 473–478; 1964.

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

Nayak, N. R.; Rath, S. P.; Patnaik, S. N. *In vitro* propagation of three epiphytic orchids. *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. and *Dendrobium moschatum* (Buch-Ham) Sw. through thidiazuron-induced high frequency shoot proliferation. *Sci. Hort* 71: 243–250; 1997.

Norma Oficial de la Federación. NOM-059-ECOL-2001. Listado de especies raras, amenazadas, en peligro de extinción o sujetas a protección especial y sus endemismos en la República Mexicana. Tomo CDLII, No. 12; 2001.

Norma Oficial Mexicana. NOM-059-ECOL-2001. Protección ambiental. Especies de orquídeas nativas de México. Publicada 6 de marzo de 2002.

Paek, K. Y.; Yeung, E. C. The effect of 1-napthalenacetic acid and $N^6$-bencyladenine on the growth of *Cymbidium forrestii* rhizome *in vitro*. *Plant Cell Tissue Organ Cult* 24: 65–71; 1991.

Park, S. Y.; Yeung, E. C.; Chakrabarty, D.; Paek, K. Y. An efficient direct induction of protocorm-like bodies from leaf subepidermal cells of *Doritaenopsis* hybrid using thin-section culture. *Plant Cell Rep* 21: 46–51; 2002.

Roy, J.; Naha, S.; Majumdar, M.; Banerjee, N. Direct callus-mediated protocorm-like body induction from shoot-tips of *Dendrobium chrysotoxum* Lind. (Orchidaceae). *Plant Cell Tissue Organ Cult* 90: 31–39; 2007.

Rzedowski, J. La Vegetación de México. Ed. Limusa S. A., México, D. F., pp 432; 1978.

Sagawa, Y. Clonal propagation of cymbidiums through shoot meristem culture. *Am. Orchid Soc. Bull* 35: 188–192; 1966.

Sánchez-Ramos, G. L.; Hernández Sánchez, A.; Mora Olivo, J.; Vargas Contreras, M.; Lara Vázquez, C.; Zamora Tapia, A.; Cardona, E. Sierra del Abra-Tanchipa. Estudio de caso. En: Gómez-Pompa, R. Proyecto de Evaluación de Áreas Naturales Protegidas de México. SEDESOL, México1993.

Sheehan, T. J Recent advances in botany propagation and physiology of orchids. *Hortic. Rev* 5: 279–315; 1983.

Sheelavantmath, S. S.; Murthy, H. N.; Pyati, A. N.; Ashok, K. H.; Ravishankar, B. V. *In vitro* propagation of the endangered orchid *Geodorum densiflorum* (Lam.) Schltr. trough rhizome section culture. *Plant Cell Tissue Organ Cult* 60: 151–154; 2000.

Stewart, S. L.; Kane, M. E. Asymbiotic seed germination and *in vitro* seedling development of *Hibernaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell Tissue Organ Cult* 86: 147–158; 2006.

Teixeira Da Silva, J. A.; Singh, N.; Tanaka, M. Priming biotic factors for optimal protocorm-like body and callus induction in hybrid *Cymbidium* (Orchidaceae), and assessment of cytogenetic stability in regenerated plantlets. *Plant Cell Tissue Org Cult* 84: 100119–100128; 2006..

Wilkinson, K. G.; Dixon, K. W.; Sivasithamparam, K. Interaction of soil bacteria, mycorrhizal fungi and orchid seed in relation to germination of Australian orchids. *New Phytol* 112: 429–435; 1989.

Wilson, P. J. The concept of a limiting rooting morphogen in woody stem cuttings. *J. Hortic. Sci* 69: 591–600; 1994.