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ORIGINAL ARTICLE

In vitro regeneration of *Lycaste aromatica* (Graham ex Hook) Lindl. (Orchidaceae) from pseudobulb sections

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Abstract A protocol for in vitro propagation from pseudobulb sections of *Lycaste armomatica* (Graham ex Hook) Lindl., an ornamental and fragrant orchid, was developed. The effect of four cytokinins: kinetin (K), metatopolin (mT), N^6-benzyladenine (BA), and thidiazuron (TDZ), in equimolar concentrations, was investigated. Shoot formation from apical and basal pseudobulb sections was obtained in all treatments. A few medial sections cultured in media supplemented with BA formed protocorm-like bodies. Shoot formation was greater from the basal section than the apical, and mainly occurred in explants cultured in media containing TDZ. The highest average numbers of shoots per explant were achieved from basal sections cultured in media supplemented with TDZ at 4.4, 8.87 and 2.2 μM, forming on average 9.9, 8.6 and 7.3 shoots per explant, respectively. Since the medial pseudobulb section was the worst explant for propagation of *L. aromatica*, we recommend that pseudobulbs be divided into two sections; the basal half should be cultured in MS medium supplemented with TDZ at 4.4 μM and the apical half with TDZ at 2.2 μM. Subculturing individual shoots in MS medium without plant growth regulators allows further development and rooting. A survival rate of more than 90% under greenhouse conditions was achieved. This research represents a direct contribution to the conservation and sustainable use of this valuable natural resource.

Keywords Micropropagation · Orchids · Organogenesis · Conservation

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Introduction

The genus *Lycaste* contains about 36 species ranging from Mexico to southern Brazil, occurring between altitudes of 500 and 2,800 m (Campacci et al. 2006). Several species possess great ornamental value, for the large size of their flowers, as in the case of *L. skinneri*, or because they are highly fragrant, like *L. aromatica*. *L. aromatica* is an epiphytic, lithophytic, or occasionally terrestrial species, reaching about 50 cm tall with yellow-orange flowers that have a strong cinnamon scent (Fig. 1a). It occurs from southern Mexico to Nicaragua. In Mexico, the native habitats of this species include tropical semi-deciduous forests, temperate oak forests, high elevation rain forests and pine-oak-liquidambar cloud forests (Hágsater and Salazar 1990).

Many species and wild populations are endangered as direct or indirect results of two human activities: habitat alteration and over-collection (Flores-Palacios and Valencia-Díaz 2007). *Lycaste* is included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (2009), as are most orchid genera. Nevertheless, *L. aromatica* is abundant in some regions of Mexico. In others, however, populations have been severely affected by habitat destruction and illegal

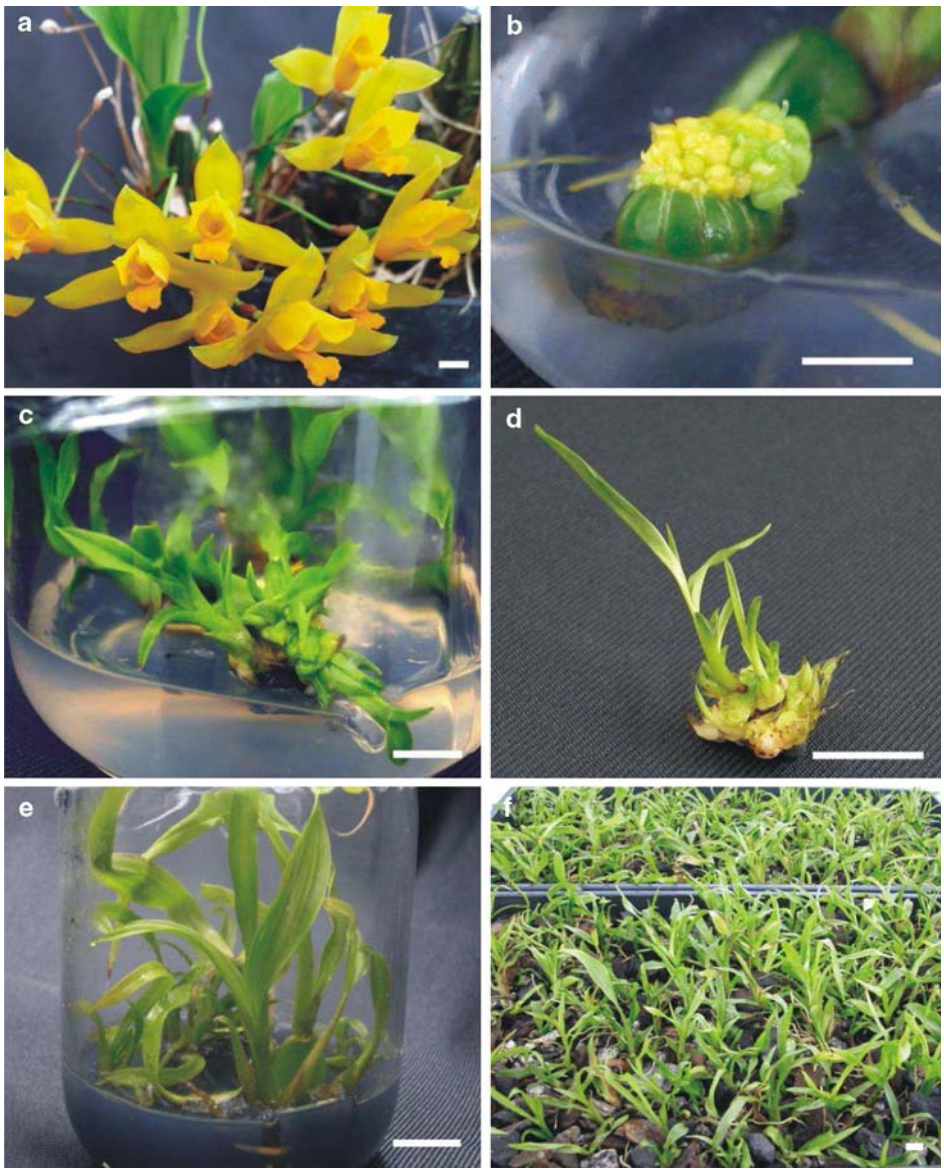

Fig. 1 **a** Blooming plant of *Lycaste aromatica*, **b** Protocorm-like bodies (PLBs) obtained from medial pseudobulb sections of *L. aromatica* cultured in MS with BA at 0.46 μM. **c** Direct organogenesis from pseudobulb section of *L. aromatica* cultured in MS with TDZ at 4.4 μM. **d** Shoot obtained from apical pseudobulb section in control. **e** In vitro rooted shoots ready for ex vitro culture. **f** Plantlets of *L. aromatica* after successfully established in soil for 3 months. *Bar* 1 cm

collecting (Hágsater and Salazar 1990). In Mexico, as in many countries, there is great interest and concern for new alternatives to conserve and propagate their native orchid species.

Especially for orchids, tissue culture represents an excellent tool for helping achieve conservation and sustainable use goals for this resource. In vitro seed germination techniques are vital, since orchid seeds have an extreme low germination rate in the wild. Augmenting in vitro germination techniques with the development of micropropagation protocols has allowed the commercial orchid production (Govil and Gupta 1997).

It is necessary to experimentally derive the specific parameters for each micropropagation protocol, since morphogenetic responses are strongly related to species, explant type, physiologic age, culture medium, and plant growth regulators (PGR) utilized (Fay 1994). Generally, the effect of cytokinins, alone or in combination with auxins, has been explored. Although the cytokinins BA and K, and the auxins NAA and indole butyric acid (IBA), are the PGRs most commonly used (Arditti and Ernst 1993; George and Ravishankar 1997; Zhao et al. 2007), the regenerative potential of other cytokinins such as 6-(α,α-dimethylallylamino)-purine (2iP), TDZ, zeatin, and mT has also been investigated (Chen et al. 1999, 2004; Nayak et al. 1997; Ogura-Tsujita and Okubo 2006).

Successful orchid micropropagation has been achieved using several different explants, such as apical and lateral shoot tips, flower stalks, leaf segments, rhizome segments, and protocorm sections (Arditti and Ernst 1993; Chen et al. 1999; Chugh et al. 2009; Ishii et al. 1998; Lu 2004; Tokuhara and Mii 2001). The potential of using

pseudobulb sections has not been fully explored, and in some species like *L. aromatica* in which the pseudobulb is obtained in an early stage of plant development, could be an excellent source of explants. In the present study, the effect of four different cytokinins on the in vitro culture of three different types of pseudobulb explants of *L. aromatica* was evaluated.

Materials and methods

Plants derived from in vitro seed germination with pseudobulbs of 1 cm in height were selected as explant donors. Under aseptic conditions, the plants were removed from the culture containers and the leaves and roots were removed. The pseudobulbs were then divided in three equal sections: basal, medial, and apical. These sections were cultured on MS medium (Murashige and Skoog 1962) augmented with 2 mg l^{-1} glycine, 100 mg l^{-1} myo-inositol, and 30 g l^{-1} sucrose, and further supplemented the cytokinins K, mT, BA, or TDZ at 0, 0.46, 1.1, 2.2, 4.4, or 8.9 μM. The pH of all culture media was adjusted to 5.5 ± 0.1 with 0.5 N NaOH and 0.5 N HCl before adding 3.0 g l^{-1} gellan gum (CultureGel™ Phytotechnology Laboratories) and autoclaving at 1.2 kg/cm^2 and 120°C for 15 min.

Two explants per jar were cultured and there were 6 replications per treatment. The induction period was 30 days. All cultures were incubated in a growth chamber at 25 ± 1°C, under a 16-h photoperiod provided by cool-white fluorescent lamps (50 μmol m^{-2} s^{-1}).

Growth stage

After the induction period, explants were subcultured every 60 days to basal MS medium without PGRs but with activated charcoal at 1 g l^{-1}. Every month, the number of shoots per explant, the plantlet height, and the number of protocorm-like bodies (PLBs) produced were recorded; subsequently, individual shoots were detached from shoot clumps and transferred to MS basal medium for rooting.

Ex vitro culture

Plantlets with a height of at least 3 cm were removed from the culture jars and washed thoroughly under tap water to eliminate all media attached to the roots. The plantlets were then transferred to propagation trays (Hummert International, Earth City, MO) containing a mixture consisting of pine bark, oak charcoal, and pumice (3:1:1) and placed under greenhouse conditions (average temperature 30°C, relative humidity 80–90%). The trays were kept covered with plastic translucent lids for the first 30 days. The lids were then gradually removed to allow the relative humidity to decrease to 50–60%. Survival was recorded monthly for 4 months.

Statistical analysis

Shoot formation was analyzed for significance by the Chi-square test using the FREQ procedure of SAS 9.1. (SAS Institute, Cary, NC, USA). PLB formation from BA treatments were elucidated using the general linear model (GLM) and the MEANS procedures of SAS 9.1 (SAS Institute) followed by a multiple comparison of means using Duncan's ($P \leq 0.05$).

Results

Shoot formation from pseudobulb sections of *L. aromatica* occurred via direct organogenesis and development of PLBs. Direct shoot formation was observed from the apical and basal sections. PLBs were obtained only from the medial sections. The two different morphogenetic responses were analyzed separately.

PLB formation

Regeneration from the medial sections almost zero in all cases; however, in a few explants exposed to BA, PLB formation was observed. When it did occur, however, numerous PLBs arose from the upper explant surface. After 30 days, it was possible to observe small nodules on the upper edge of the explant (Fig. 1b); these were consolidated into small shoots and then into plantlets in which root and leaf primordia were evident. The plantlets were easily separated from the PLB clumps.

A statistically significant difference for PLB formation among BA treatments could not be established ($P = 0.0814$); however, it was possible to observe that treatment with BA (0.46 μM) stimulated the higher PLB formation (6.4 per explant) (Table 1).

Table 1 Effect of concentration of N^6-Benzyladenine on PLB induction from medial pseudobulb sections of *Lycaste aromatica*

BA (μM)	PLBs per explant ± SE	% of responding explants
0	0.04 ± 0.04	4.2
0.46	6.40 ± 3.6	33.3
1.1	1.75 ± 1.5	16.7
2.2	4.20 ± 3.17	16.7
4.4	0.42 ± 0.42	8.3
8.87	2.20 ± 1.47	16.7

BA N^6-Benzyladenine, *SE* standard error

Shoot formation

The formation of shoots was significantly affected by both the explant and the PGRs used (Table 2) ($\chi^2 < 0.0001$). Shoot formation was greater from the basal section than the apical, achieving in the former a total 1,154 shoots—almost double that of apical section (Table 2). Results of the Chi-square test showed significant differences among PGRs. The largest number of shoots occurred on medium with TDZ (565) (Fig. 1c), whereas the fewest shoots were obtained from the control and K treatments (Table 2).

In Table 2, it is possible to observe that the largest frequency of shoots, as well as the greatest positive discrepancy between observed and expected counts, occurred in basal explants cultured in medium containing TDZ; the lowest frequency was recorded in basal explants exposed to K. For apical explants, the greatest numbers of shoots were obtained from the control (148), closely followed by mT and TDZ (Fig. 1d); the lowest frequency was obtained with BA.

Shoots from basal sections were derived mainly from lateral buds. At the beginning of their growth, these shoots were compact, just 1–2 mm in height. When subcultured to basal medium, they developed rapidly, producing new leaves. In a few cases, it was possible to observe the development of pseudobulbs, even while still attached to the original explants. These were detached and transferred to basal medium for rooting.

Apical pseudobulb sections had less shoot formation than basal sections, but these shoots were taller and always developed from the apical meristem. After subculture to basal medium, shoots from these explants grew faster than those from basal explants; a few shoots began to develop roots while still attached to the original explant.

Table 2 Effect of kind of explant and plant growth regulators on shoot formation from apical and basal section of pseudobulb of *Lycaste aromatica* plantlets

Explant	Plant growth regulators					
	C	BA	K	mT	TDZ	Total
Apical						
Frequency	148	108	120	144	141	661
Expected	118	128.56	85.95	122.73	205.77	
Cell chi-square	7.63	3.29	13.49	3.69	20.38	
Basal						
Frequency	176	245	116	193	424	1154
Expected	206	224.44	150.05	214.27	359.23	
Cell chi-square	4.37	1.88	7.7275	2.12	11.67	
Total	324	353	236	337	565	1815
	17.85	19.45	13.00	18.57	31.13	100

Statistics	*df*	Value	Probability
Chi-square	4	76.2468	<0.0001
Likelihood ratio chi-square	4	76.9918	<0.0001
Mantel–Haenszel chi-square	1	9.6356	<0.0019
Phi coefficient		0.2050	
Contingency coefficient		0.2008	
Cramer's V		0.2050	

PGR Plant growth regulators, *C* control, *BA* N^6-benzyladenine, *K* kinetin, *mT* meta-topoline, *TDZ* thidiazuron

Effect of PGR

Figure 2 shows the average number of shoots obtained for each PGR and concentration, separated by explant type (pseudobulb section). It is evident that the highest average numbers of shoots were induced from the basal section, and that almost no shoots formed from the medial section.

Analyzing the effect of treatments and type of explant on shoot formation, it is possible to observe that the highest averages of shoots per explant were achieved from basal sections cultured in media supplemented with TDZ at 4.4, 8.87, and 2.2 μM, forming on average 9.9, 8.6, and 7.3 shoots per explant, respectively (Fig. 2). The next most successful treatments were basal section explants exposed to BA at 0.46 μM or TDZ at 1.1 μM, with an average of 6.6 and 6.0 shoots per explant, respectively (Fig. 2). The remainder of treatments produced fewer shoots on average, ranging from zero to 4.6. Particularly in the TDZ treatments, it was evident that, except for the highest concentration tested, the number of shoots obtained per explant increased with increasing concentration.

Rooting

Roots developed when the shoot clumps were transferred from treatment media to basal medium. After the plant material was separated into individual shoots, root development accelerated and entire plantlets were obtained (Fig. 1e). Where the shoots were rootless, the material was subcultured to medium containing activated charcoal at 1 g l^{-1} which induced root development. There was no discernible effect of the shoot induction treatments on root formation or development.

Ex vitro survival

In general, more than 90% of the plantlets survived ex vitro under the culture conditions described here. With continued watering and fertilization, the plantlets grew and developed new leaves (Fig. 1f). Additionally, those plantlets that did not possess pseudobulbs at the time of planting produced them ex vitro. In a few cases new shoots began to

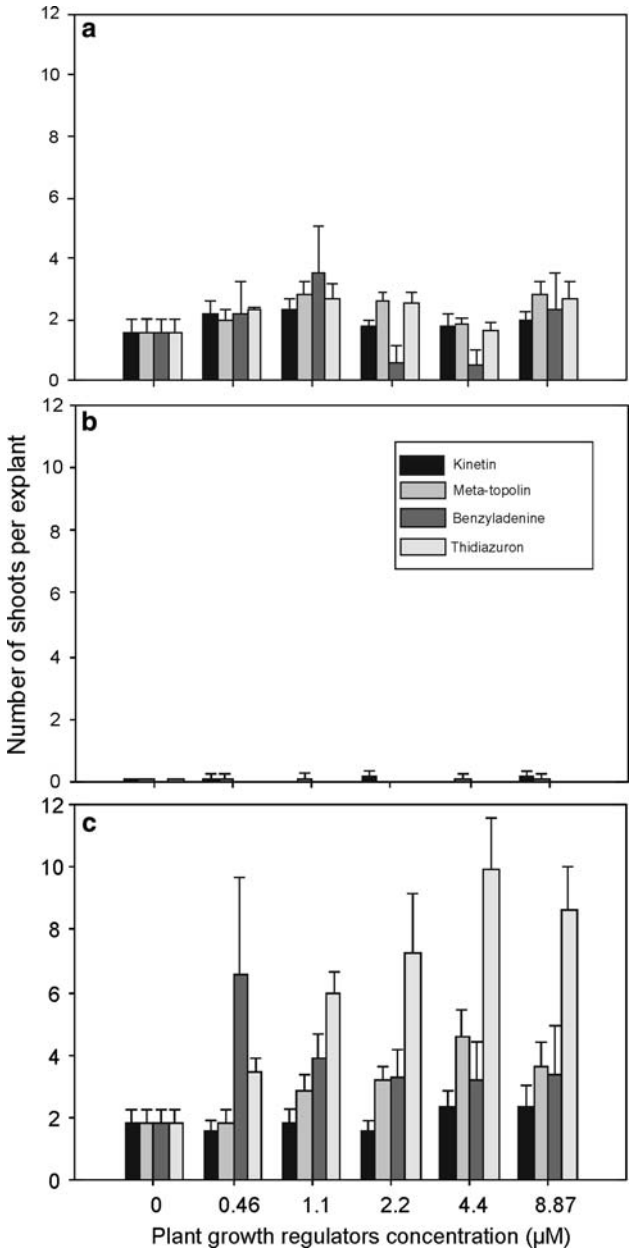

Fig. 2 Effect of pseudobulb section: **a** apical, **b** medial, **c** basal, and kind and concentration of plant growth regulator, on shoot formation of *Lycaste aromatica*, *Bar* ± SE

grow from the plantlets, demonstrating the characteristic sympodial growth habit of this species.

Discussion

Several different explants have been used for in vitro orchid propagation, the most common being seed, protocorms, plantlets, and fragments of plantlets (Arditti and Ernst 1993; Chen et al. 1999; Chugh et al. 2009: Ishii et al. 1998; Lu 2004; Tokuhara and Mii 2001). The use of pseubobulb explants is not commonly reported (Shiau et al. 2005; Zhao et al. 2007), but the present study demonstrates the high regenerative capacity of these explants. We have also established that the morphogenetic responses obtained are dependent on explant type, with the basal section always inducing the highest level of shoot formation. Cultures initiated from apical sections also gave good results, but the responses observed from medial sections were very low. The use of PGRs (TDZ, BA, and mT) was essential for shoot and PLB induction, as indicated by the lower regeneration observed in the control, but the addition of K to culture media had a negative effect, because the lowest shoot formation was recorded from these treatments.

Of the different cytokinins assayed, and across all equimolar concentrations, TDZ showed the highest capacity to induce morphogenetic responses. TDZ has been commonly used in the propagation of several orchid species due to its tremendous organogenic capacity (Ket et al. 2004). In some species, TDZ has been shown to induce more adventitious shoot regeneration and axillary shoot formation than purine-type cytokinins (BA, K, mT) even at extremely low concentrations (George et al. 2008; Nayak et al. 1997). In addition, other authors working with in vitro orchid propagation of orchids have reported that the highest PLB and shoot formation was observed with concentrations of TDZ similar to that found to be optimal in the present study—4.4 μM (Chen et al. 1999; Chung et al. 2005; Ket et al. 2004; Kuo et al. 2005; Nayak et al. 1997).

Shoot formation via direct organogenesis was associated with the presence of meristematic areas in the explant: the apical meristem for the apical explants, and lateral buds for the basal explants (Arditti 1992). The endogenous auxin/cytokinin ratio is usually modified by exogenous PGR application (George 1993). It is thus possible to obtain new shoots by both the activation of pre-existing lateral and apical buds and by the induction of multiple shoots from these areas. Presumably, these patterns of regeneration were followed by most of the shoots obtained from the explants. This kind of response has been reported in several studies with orchids (Arditti and Ernst 1993; Mata-Rosas and Salazar-Rojas 2009; Nayak et al. 1997; Shyamal and Pinaki 2004; Tinoco and Mata 2007).

As mentioned above, the portion of pseudobulb used as an explant was critical to shoot formation. The optimal explant for propagation of *L. aromatica* was the basal section and the worst was the medial section. Therefore, our proposal for micropropagation of this species is that, when pseudobulbs are used as the explant for micropropagation, they only be divided in two sections—apical and basal. We believe this would enhance shoot formation;

additionally, explanting would be more easily performed. We further recommend that basal sections be cultured in MS medium supplemented with TDZ at 4.4 μM and that apical sections are cultured with half as much TDZ (2.2 μM). Our results indicate that this would allow the formation of 12 shoots per explant.

The results of this study provide precise information delimiting the optimal conditions for *L. aromatica* shoot induction and development, with recommendations about both the type and concentration of cytokinin to use. Previous studies have reported the effect of only one or two cytokinins (Chen et al. 1999, 2004; Lu 2004; Mata-Rosas and Salazar-Rojas 2009; Santos-Hernández et al. 2005; Shiau et al. 2005), but there has always been a degree of uncertainty that the previously published protocols described were, in fact, optimal for the propagation of those species. There are only a few reports similar to this present study in which the effects of multiple cytokinins are examined simultaneously (Chung et al. 2005; Ket et al. 2004; Nayak et al. 1997). These results prove that both the type and concentration of cytokinin have a strong influence on the results obtained.

In spite of the clear results obtained, it is possible that the response could be improved by relatively slight modifications of the cytokinin concentration using the concentration identified as optimal in this study as a starting point. Another avenue of research would be to assay different cytokinins in combination with auxins. However, we found in a previous experiment that the addition of NAA or IBA at 0.1–0.5 mg l^{-1} inhibited shoot formation of *L. aromatica* (data not shown). Other authors have reported similar negative auxin effects on in vitro orchid propagation (Chung et al. 2005; Kuo et al. 2005; Peres et al. 1999).

For both propagation and conservation efforts, experimental approaches resulting in the formation of new plants through direct organogenesis are preferable, since plants obtained in this way tend to maintain their genetic stability (George 1993). The plantlets obtained in the present study could be used either as material for further research or to satisfy horticultural demand, thus reducing pressures on wild populations. Therefore, this research represents a direct contribution to the conservation and sustainable use of this valuable natural resource.

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