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# EFFICIENT PRODUCTION OF PROTOCORM-LIKE BODIES AND PLANT REGENERATION FROM FLOWER STALK EXPLANTS OF THE SYMPODIAL ORCHID *EPIDENDRUM RADICANS*

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## Summary

A simple and efficient micropropagation method was established for direct protocorm-like body (PLB) formation and plant regeneration from flower stalk internodes of a sympodial orchid, *Epidendrum radicans*. Small transparent tissues formed on surfaces and cut ends of flower stalk internodes on a modified half-strength Murashige and Skoog basal medium with or without thidiazuron (TDZ) after 1–2 wk of culture. In the light, the transparent tissues enlarged and turned into organized calluses on most of the explants. However, PLBs formed only on a medium supplemented with 0.45 μ*M* TDZ within 2 mo. of culture. Sucrose, $NH_4NO_3$, and $KNO_3$ were used in media to test their effects on PLB proliferation and shooting. The best response on number of PLBs per tube was 23.6 at 40 g $l^{-1}$ sucrose, 825 mg $l^{-1}$ $NH_4NO_3$, and 950 mg $l^{-1}$ $KNO_3$, and the highest number of PLBs with shoots was found at 10 g $l^{-1}$ sucrose, 825 mg $l^{-1}$ $NH_4NO_3$, and 950 mg $l^{-1}$ $KNO_3$. Homogenized PLB tissues produced by blending were used to test the effects of four cytokinins [TDZ, $N^6$-benzyladenine (BA), zeatin-riboside, and kinetin] on PLB proliferation and shoot formation. The best responses on number of PLBs per tube, proliferation rate, and number of PLBs with shoots per tube were obtained at 4.44 μ*M* BA, 0.28 μ*M* zeatin-riboside, and 1.39 μ*M* kinetin, respectively. Normal plantlets converted from PLBs on the same TDZ-containing medium after 1 mo. of culture. The optimized procedure required about 12–13 wk from the initiation of PLBs to plantlet formation. The regenerated plants grew well with an almost 100% survival rate when acclimatized in a greenhouse.

*Key words:* cytokinin; *Epidendrum radicans*; flower stalk; medium composition; protocorm-like body.

## Introduction

*Epidendrum* is the largest genus of orchids with over 1000 species, many of which occur in great abundance in Central America. The Epidendrae are sympodial orchids that form flowers in clusters on a long inflorescence, which come in various shades of orange, yellow, white, light green, and tan. They are easy to grow and require little care, and some species including *Epidendrum radicans* were used commercially as cut flowers and potted plants (Teob, 1989). But *Epidendrum* growers face a number of problems, including the slow rate of sexual and vegetative propagation. *In vitro* propagation methods have been attempted on shoot-bud or PLB formation from callus derived from roots and leaf tips (Churchill et al., 1972; Stewart and Button, 1978) and floral tissues (Stewart and Button, 1976; Kuhn, 1981). However, the morphogenetic efficiencies were low, few normal plantlets were obtained, and establishment of *in vitro* cultures usually depended on the quality of natural additives such as coconut milk. Here we report a simple and efficient method for plant regeneration through direct formation of protocorm-like bodies (PLBs) from flower stalk internodes of *Epidendrum radicans*. Different concentrations of sucrose, $NH_4NO_3$, $KNO_3$, and cytokinins [thidiazuron (TDZ; 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea), $N^6$-benzyladenine (BA), zeatin-riboside (6-[4-hydroxy-3-methylbut-2-enylamino]purine), and kinetin (6-furfurylaminopurine)] were supplemented to media to test their effects on PLB proliferation and shoot formation. The effect of homogenization by blender on PLB proliferation and shoot formation was also investigated.

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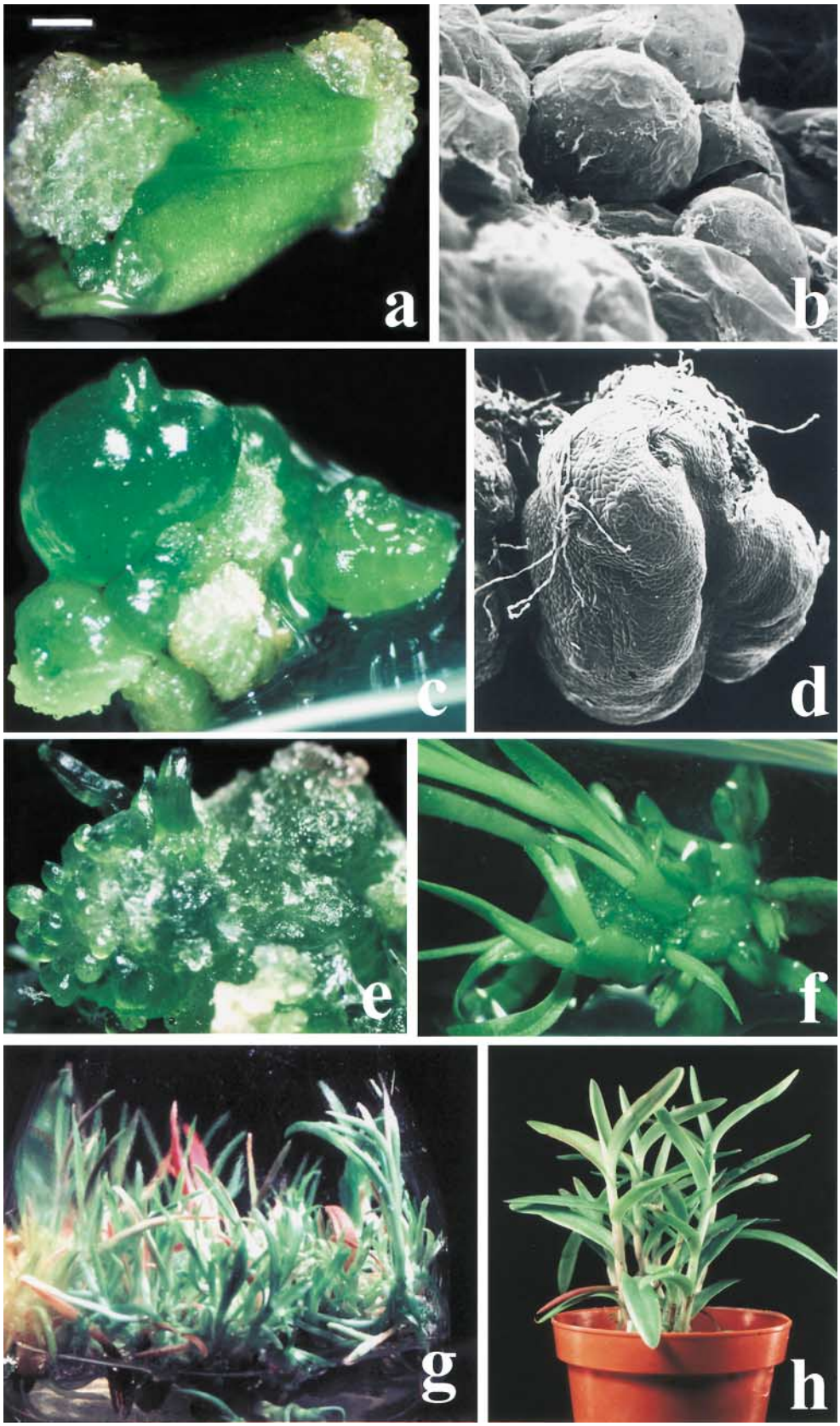
## Materials and Methods

*Plant Materials and Culture Conditions.* Two to 3-yr-old potted plants of a local accession of *Epidendrum radicans* that were grown in a greenhouse were donor plants. Explants, 5–7 mm in length, were excised from flower stalk internodes (*ca.* 10–15 cm in length) at the moment the flower buds were fully formed with no flower color showing. The explants were surface-sterilized with 1% (w/v) $NaOCl_3$ for 12 min followed by three rinses in sterile distilled water. Explants were placed on the surface of a 1/2MS basal medium containing half-strength macro- and micro-elements of Murashige and Skoog (Murashige and Skoog, 1962) supplemented with (mg $l^{-1}$): *myo*-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), peptone (1000), $NaH_2PO_4$ (170), sucrose (20 000), and Gelrite (2200). Plant growth regulators were added prior to autoclaving as optional additives according to the experimental objectives. The MS nutrients and additive compounds were modified according to each treatment as described below. The pH of the media was adjusted to 5.7 with 1 *N* KOH or HCl prior to autoclaving for 15 min at 121°C. Explants were incubated in 20 × 150 mm culture tubes under a 16:8-h photoperiod at 28–36 μmol $m^{-2}$ $s^{-1}$ (daylight fluorescent tubes FL-30D/29, 40 W, China Electric Co., Taipei) and 26 ± 1°C.

*Experimental Treatments. (1) Induction of PLBs from flower stalk*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*explants.* About 5–7-mm flower stalk explants were placed on a 1/2MS basal medium supplemented with 0, 0.45, 1.36, 4.54, and 13.62 μ*M* TDZ. Five replicates were taken for each treatment, and one explant was planted in each culture tube. Differences between means were scored with Duncan's multiple range test (Duncan, 1955).

*(2) Effects of sucrose and nitrogen source on PLB proliferation and shooting.* Sucrose (0, 10, 20, 30, and 40 g $l^{-1}$), $NH_4NO_3$ (0, 165, 330, 495, 660, and 825 mg $l^{-1}$), and $KNO_3$ (0, 190, 380, 570, 760, and 950 mg $l^{-1}$) were added to a hormone-free basal medium and tested for their effects on PLB proliferation and shooting. About 3 × 3 $mm^2$ green organized calluses with young PLBs were placed on the medium surface of each tube. Numbers of PLBs and PLBs with shoots were scored after 40 d of culture. Five replicates were taken for each treatment, and one explant was planted in each culture tube. For analytical methods see experiment 1.

*(3) Effects of four cytokinins on PLB proliferation.* Four cytokinins including TDZ (0.45–4.54 μ*M*), BA (0.44–44.38 μ*M*), zeatin-riboside (0.28–28.46 μ*M*), and kinetin (0.46–46.47 μ*M*) were used to test their effects on PLB proliferation. About 50 g of callus with young PLBs were used at a time, broken up using a blender (Osterizer Co.) for 15 s. About 0.8 g of homogenized PLB tissues was placed on the medium surface of each tube. The number of PLBs formed from original tissues was scored after 5 wk of culture. In a preliminary test, we found that 0.45 μ*M* TDZ could speed up the subsequent development of PLBs as compared with hormone-free medium. So all cultures were transfer onto the same basal medium supplemented with 0.45 μ*M* TDZ, and the proliferation rate and number of PLBs with shoots were scored after 1 mo. of culture. Six replicates were taken for each treatment. For analytical methods see experiment 1.

*Microscopy Observation on PLB formation.* Cultures were examined and photographed with a stereozoom microscope (SZH, Olympus). Scanning electron micrographs were taken (DSM-950, Carl Zeiss) of tissues fixed in 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.0) for 4 h at 4°C, dehydrated in ethanol (Dawns, 1971), critical point dried (HCP-2, Hitachi), and coated with gold in an ion coater (IB-2, Giko Engineering Co.).

## Results and Discussion

*In vitro* propagation of *Epidendrum* from callus was documented in several early reports, but the regeneration efficiency was low (Churchill et al., 1972, 1973; Stewart and Button, 1976; Kuhn, 1981). Production of healthy plantlets through multiple shoot formation from flower stalk nodes has been demonstrated (Stewart and Button, 1976), but this protocol could not provide large amounts of uniform regenerated plants from a specific genotype in a short period of time. In orchids, somatic cells of cultured tissues can form protocorm-like bodies that could convert into individual plantlets (Arditti and Ernst, 1993). Regeneration via PLB multiplication is an efficient clonal propagation method for orchids (Tanaka et al., 1975; Arditti and Ernst, 1993; Park et al., 1996).

*Induction of PLBs from flower stalk explants.* Initially, small transparent tissues were formed from the cut end of flower stalk explants on a modified 1/2MS medium with or without TDZ after 1–2 wk (Fig. 1*a*). Detail examined by scanning electron microscopy showed that these transparent calluses already contained many PLBs (Fig. 1*b*). After 3–4 wk of culture, these transparent tissues then expanded and turned into green organized masses on 80–100% of explants (Table 1). After 2–3 mo. of culture, 40% of the tissue cultured on the medium containing 0.45 μm TDZ had produced PLBs (Fig. 1*c*, *d*). Some of the PLBs were still fused to each other at their basal portions (data not shown). No PLBs were found in the cultures without TDZ or TDZ in other concentrations (Table 1).

TABLE 1

EFFECT OF TDZ ON DIRECT PLB FORMATION FROM FLOWER STALK INTERNODES OF *EPIDENDRUM RADICANS*

| TDZ (μ*M*) | Percentage forming nodular masses | Percentage forming PLBs |
|---|---|---|
| 0 | 100 a | 0 b |
| 0.45 | 100 a | 40 a |
| 1.36 | 80 a | 0 b |
| 4.54 | 80 a | 0 b |
| 13.62 | 80 a | 0 b |

Five replicates were taken for each treatment, and one explant was planted in each culture tube. Data were scored after 40 d of culture. Differences between means were scored with Duncan's multiple range test (Duncan, 1955).

Thidiazuron (TDZ) was first reported to have cytokinin activity by Mok et al. (1982). Since then, TDZ has been used successfully to induce *in vitro* morphogenesis and was especially effective with recalcitrant woody species (Lu, 1993). Recently, TDZ alone was used in several orchid species to induce direct somatic embryogenesis of *Oncidium* (Chen et al., 1999; Chen and Chang, 2001), shoot regeneration of *Phalaenopsis*, *Doritaenopsis*, and ephityic cymbidium (Ernst, 1994; Chen and Piluek, 1995; Nayak et al., 1997), and embryogenic callus of *Cymbidium ensifolium* var. misericors and *Oncidium* (Chang and Chang, 1998; Chen and Chang, 2000a, b). In our experiments, TDZ was found to be effective in induction of PLB formation from flower stalk internodes of *Epidendrum radicans*.

*PLB proliferation.* Different concentrations of sucrose, $NH_4NO_3$, and $KNO_3$ were used to test their effects on PLB proliferation and shooting (Table 2). The lowest response on PLB proliferation was found on a medium without sucrose. In addition, higher concentrations (30 and 40 g $l^{-1}$) gave a higher PLB proliferation rate than lower concentrations (10 and 20 g $l^{-1}$) of sucrose. Sucrose at 20, 30, and 40 g $l^{-1}$ retarded shoot development of PLBs compared with 10 g $l^{-1}$ sucrose. At 20 g $l^{-1}$ sucrose, higher concentration of $NH_4NO_3$ and $KNO_3$ promoted PLB proliferation, but retarded shoot development (Table 2). The lowest average number of PLBs (1.7) was found on a modified basal medium without any nitrogen source, and no PLBs formed shoots with this treatment. After 40 d of culture, a clump of about 9 $mm^2$ formed the highest number of PLBs (23.6) on a modified medium supplemented with the highest concentrations of sucrose, $NH_4NO_3$, and $KNO_3$, 40 g $l^{-1}$, 825 mg $l^{-1}$, and 950 mg $l^{-1}$, respectively (Table 2). At a sucrose concentration of 10 g $l^{-1}$ plus 825 mg $l^{-1}$ $NH_4NO_3$ and 950 mg $l^{-1}$ $KNO_3$, the highest number of shoot buds (8.4 on average) was found (Table 2).

FIG. 1. Plant regeneration through PLB formation on explants of flower stalk internodes of *Epidendrum radicans*. *a*, Callus proliferation from the cut ends of a flower stalk explant on 1/2MS basal medium plus 0.1 mg $l^{-1}$ TDZ for 2 wk (*bar* = 0.26 mm). *b*, Young PLBs protruded from the callus mass shown in (*a*) (*bar* = 51 μm). *c*, PLBs enlarged and young leaves produced on the apex (*bar* = 0.44 μm). *d*, A scanning electron micrograph showing the well-developed PLBs in (*c*) (*bar* = 314 μm). *e*, Proliferated PLBs started forming shoots at 7 wk (*bar* = 0.35 mm). *f*, Well-developed PLB-derived shoots at 10 wk (*bar* = 0.5 mm). *g*, Mass PLB-derived regenerants proliferated in a culture flask at 14 wk (*bar* = 1.53 cm). *h*, Potted plantlets maintained in the greenhouse at 26 wk (*bar* = 1.32 cm).

TABLE 2

EFFECTS OF SUCROSE, $NH_4NO_3$, AND $KNO_3$ ON PLB FORMATION AND SHOOT FORMATION OF *EPIDENDRUM RADICANS*

| Sucrose ($g\,l^{-1}$) | $NH_4NO_3$ ($mg\,l^{-1}$) | $KNO_3$ ($mg\,l^{-1}$) | PLBs per tube | PLBs with shoots per tube |
|---|---|---|---|---|
| 0 | 825 | 950 | 4.4 b | 3.8 b |
| 10 | 825 | 950 | 11.4 a | 8.4 a |
| 20 | 0 | 0 | 1.7 b | 0 b |
| 20 | 165 | 190 | 4.3 ab | 5.3 a |
| 20 | 330 | 380 | 9.6 a | 3.6 ab |
| 20 | 495 | 570 | 7.1 ab | 0.4 b |
| 20 | 660 | 760 | 7.6 ab | 2 ab |
| 20 | 825 | 950 | 10.6 a | 1.6 ab |
| 30 | 825 | 950 | 20.0 a | 3 b |
| 40 | 825 | 950 | 23.6 a | 1.6 b |

One piece of PLB clump ($9\,mm^2$) was placed in each tube. Data were scored after 40 d of culture.

Means of five replicates (tubes) with the same *letters* are not significantly different at $P < 0.05$ (Duncan, 1955).

TABLE 3

EFFECTS OF FOUR CYTOKININS ON PLB PROLIFERATION AND SHOOT FORMATION FROM HOMOGENIZED PLB TISSUES OF *EPIDENDRUM RADICANS*

| Treatments ($\mu M$) | | PLBs per tube | PLB proliferation rate | PLBs with shoots per tube |
|---|---|---|---|---|
| 0 | | 87.7 b | 9.9 b | 88.7 abc |
| TDZ | 0.45 | 91 ab | 9.5 b | 34.2 bc |
| | 1.36 | 93.7 ab | 9.6 b | 72.3 bc |
| | 4.54 | 95.3 ab | 11.6 b | 58.3 bc |
| BA | 0.44 | 93.5 ab | 9.6 b | 98.7 ab |
| | 1.33 | 96 ab | 10.9 b | 94.7 ab |
| | 4.44 | 117 a | 11.7 b | 82.3 abc |
| | 44.38 | 88 b | 2.3 c | 51 bc |
| ZR | 0.28 | 32 d | 30.3 a | 31 bc |
| | 0.85 | 54.3 cd | 10.3 b | 53.7 bc |
| | 2.85 | 73.3 bc | 2.6 c | 8.7 c |
| | 28.46 | 97.3 ab | 11.6 b | 47.3 bc |
| Kinetin | 0.46 | 94 ab | 3.0 c | 16 bc |
| | 1.39 | 98.5 ab | 8.3 b | 154 a |
| | 4.65 | 77.7 bc | 8.4 b | 65 bc |

About 0.8 g homogenized PLB tissue was tested in each tube. The number of PLBs per tube was scored after 5 wk of culture. After another 1 mo. of culture, the proliferation rate and number of shoots per tube were scored. The proliferation rate of homogenized tissues was calculated by dividing the final fresh weight by the initial fresh weight.

Means of six replicates (tubes) with the same *letters* are not significantly different at $P < 0.05$ (Duncan, 1955).

Homogenized PLB tissues produced by blending were used as explants to test the effects of four cytokinins on PLB proliferation and shoot formation. As the results in Table 3 show, homogenized PLB tissues proliferated into more PLBs on a hormone-free basal medium than the average number of PLBs formed from original tissues promoted by 0.45–4.54 $\mu M$ TDZ, 0.44–4.44 $\mu M$ BA, 0.46–1.39 $\mu M$ kinetin, and 28.46 $\mu M$ zeatin-riboside. In addition, the highest number of PLBs was found on a basal medium supplemented with 4.44 $\mu M$ BA, and 0.8 g of original tissue formed an average of 117 PLBs after 5 wk of culture. All the PLBs were transferred onto a basal medium supplemented with 0.45 $\mu M$ TDZ, and the proliferation rate and number of PLBs with shoots were both scored after another 1 mo. of culture. The highest proliferation rate was found at 0.28 $\mu M$ zeatin-riboside, which proliferated 30.3 times their fresh weight in 1 mo. Moreover, kinetin at 1.39 $\mu M$ gave a significantly higher number of 154 PLBs forming shoots than other treatments.

*Plant regeneration.* The PLBs that formed from flower stalk explants developed into normal plantlets with both roots and shoots on the same TDZ-containing medium for PLB induction within 2 mo. (Fig. 1*e–g*). Regenerated plants potted in sphagnum moss acclimatized well in the greenhouse with about a 90% survival rate (Fig. 1*h*).

In conclusion, direct PLB proliferation and subsequent shoot development were achieved by using flower stalk internodes of *Epidendrum radicans*on a simple defined medium supplemented with TDZ. The regeneration system was efficient, and could provide large amounts of clonal plantlets in a short period of time, and is potentially to be considered in mass propagation and transgenic work on the *Epidendrum* orchid.

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