Plant Cell Reports (1997) 16: 831–835

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Micropropagation of *Spathoglottis plicata*

Received: 20 February 1997 / Revision received: 27 May 1997 / Accepted: 16 June 1997

Abstract A rapid and reliable micropropagation method was established for *Spathoglottis plicata*. Nodal and leaf explants dissected from 8-month-old pot-grown seedlings were cultured on charcoal-amended Murashige and Skoog medium supplemented with 16 combinations of α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BA) at concentrations of 0.54–10.74 μM. Regeneration of protocorm-like bodies (PLBs) and subsequent plantlet development were observed from 98.5% of the nodal explants. Only 6.5% of leaf explants and occasionally some root segments (dissected from regenerated plantlets) were able to produce PLBs and then plantlets. The optimum plant growth regulator (PGR) combination for maximal PLB regeneration was 5.37 μM NAA and 0.44 μM BA. The best combination of PGR for plantlet development was 2.69–10.74 μM NAA and 8.88 μM BA. The NAA to BA ratios for maximal PLB induction and plantlet development were 12.2 and 0.3–1.2, respectively. Regenerated PLBs and plantlets, when cut into pieces of less than 1 mm and subcultured onto the above media, regenerated new PLBs and plantlets in another 3 months.

Key words Organogenesis · Micropropagation · Protocorm-like-body · Orchid

Abbreviations BA, 6-benzylaminopurine · *NAA* α-naphthaleneacetic acid · *PLB* protocorm-like body

Communicated by R. N. Trigiano

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Introduction

Spathoglottis plicata Blume is a terrestrial orchid noted for its attractive flowers and long blooming period. The species originated from tropical Asia and its many hybrids are distributed throughout the world. *Spathoglottis* is conventionally propagated through separation of pseudobulbs; however, the proliferation rate is very low. A more efficient approach is in vitro seed culture (Bapat and Narayanaswamy 1977; Beechey 1970; Chennaveeraiah and Patil 1975). Combinations of exogenous growth regulators at suitable concentrations stimulated zygotic embryos to initiate protocorms that develop into plantlets (Singh 1992). For mass propagation, regeneration from tissue-cultured explants is superior to seed culture due to year-round availability of plant materials and an exponential propagation rate. Publications on the in vitro culture of *S. plicata* are very limited. Bapat and Narayanaswamy (1977) reported inducing callus and adventitious roots from leaves and secondary roots of seedlings. Beechey (1970) reported regenerating plantlets from root culture. The available information fails to provide a comprehensive protocol and understanding of micropropagation of *S. plicata*. We, thus report an easy, fast and reliable in vitro regeneration system for the propagation of this species.

Material and methods

Eight-month-old pot-grown seedlings of *S. plicata*, native to Taiwan (donated by Dr. Fure-Chyi Chen at the Department of Plant Industry of National Pingtung Polytechnic Institute, Taiwan), were washed thoroughly in water, sonicated in 1% NaOCl for 20 min and soaked in a solution of 1000 mg/l of both penicillin-G and streptomycin for 3 h. The antibiotic solution was prepared by adding 1 g penicillin-G and 1 g streptomycin to 20 ml of distilled water, which was then filtered through a 0.22-μm Millipore filter. The sterile antibiotic solution was diluted to 1 l and ten drops of sterile Tween 20 were added into the solution (i.e., one drop per 100 ml of solution). The final concentration of each antibiotic was 1000 mg/l.

The antibiotic solution was removed from the seedlings using a 400-μm polyester screen filter. The seedlings were then rinsed

thoroughly with sterile distilled water. To remove any residuals of antibiotic, the seedlings were left in constant stirring water for 5 min and then filtered off with water; the above process was conducted three times. Seedlings were then separated into nodes (sections one-half of the internodal portion above and below the node), and leaves (2- to 3-mm explants), and placed horizontally on regeneration medium. The regeneration medium consisted of one-half MS basal salts (Murashige and Skoog 1962) supplemented with the following (in mg/l): 0.5 thiamine-HCl, 0.5 pyridoxine-HCl, 0.5 nicotinic acid, 100 myo-inositol, 2 glycine, plus 0.8% (wt/vol) Difco Bacto agar, 3% sucrose, 0.2% charcoal and 5.37 μM α-naphthaleneacetic acid (NAA) and 4.44 μM 6-benzylaminopurine (BA). The pH of the medium was adjusted to 5.5 before autoclaving at 121°C and 124 kPa for 20 min.

All explants were incubated at 25±2°C under 24 h illumination provided by cool-white fluorescent lamp at 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Regenerated protocorm-like-bodies (PLBs) were cut into pieces ≤1 mm (PLB explants) and recultured to induce more PLBs for the following experiments. To optimize the cultural conditions for PLB induction and subsequent plantlet development, PLB explants (0.2 g fresh weight/90 mm petri dish) and nodal explants (10/petri dish, equivalent to approximately 0.2 g per petri dish) from PLB-developed plantlets were placed onto basal culture medium supplemented with 16 combinations of NAA (0.54, 2.69, 5.37, 10.74 μM) and BA (0.44, 2.22, 4.44, 8.88 μM). As PLBs were not formed synchronously and many of them of various sizes fused together, weight was thus used for evaluation. For plantlet development, PLBs of 0.2 g fresh weight per petri dish were transferred onto the same set of media. The number of plantlets developed from PLBs was calculated. Root sections 5 mm in length dissected from regenerated plantlets were also placed on 5.37 μM NAA and 4.44 μM BA to compare in their efficiencies in PLB induction with nodal and leaf explants.

Regeneration of PLBs and subsequent plantlet development were evaluated every month. For all experiments, three petri dishes of culture were prepared for each treatment and experiments were conducted three times. Experiments were arranged in randomized complete-block designs and data subjected to analysis of variance. Means were separated using Duncan's multiple-range test (MSTAT software, Michigan State University, USA).

Results and discussion

The in vitro regeneration of *S. plicata* first appeared in the form of PLB formation. PLBs were induced directly from nodal sections, leaf explants, and root segments within 1 month. The efficiency of PLB induction was graded as nodal section >> leaf explant > root segment. Almost all nodal sections (98.5%) were capable of forming PLBs. The regeneration started from differentiated growth on the surface of both nodal and internodal areas, and the whole explant later turned into PLBs (Fig. 1a, b). Only 5–8% of leaf explants were able to form PLBs, which occurred at the leaf base (Fig. 1c). The acropetal portion of the leaf explant elongated and eventually turned brown.

Attempts to produce PLBs from roots have not been common in orchids (Arditti and Ernst 1993) and 9–12 months are often required for PLB induction from root explants (Philip and Nainar 1986; Sanchez 1988). In our study, root segments were capable of regenerating PLBs in 1 month and plantlets in 3 months, but the regeneration frequency was low (0.5%, Fig. 1d). Some roots of regenerated plantlets developed PLBs and new plantlets at their tips (Fig. 1e). Similar results were observed in *Mormodes histrio*. The roots, attached to a small remnant of the shoot, developed PLBs and later plantlets at their tips. However, the root systems, attached to shoots with leaves removed, produced only non-shoot-bearing PLBs (Holters and Zimmer 1990). Since roots and leaves of *S. plicata* induced PLBs at relatively low efficiency compared to nodal sections, the following experiments were conducted using nodal-section-induced PLBs.

When PLBs, including those with developed leaves of 1–2 mm, were cut into pieces of less than 1 mm (PLB explants) and placed onto regeneration media, numerous PLBs were induced within 3 months. The induction of PLBs and the subsequent development of plantlets were significantly affected by growth regulators. BA at 0.44 μM in combination with 5.37 μM NAA (auxin to cytokinin ratio 12.2) induced the most PLBs from PLB explants (Table 1). Ratios above or below 12.2 were not as effective in PLB induction. The same ratio also promoted maximal PLB induction from nodal sections of regenerated plantlets (Table 1).

Since many orchid species require auxins and/or cytokinins for PLB formation and plantlet development (Arditti and Ernst 1993), the combinations, concentrations, and the ratio between them are usually critically important. The ratio of auxin to cytokinin for PLB formation varies from species to species. Unlike the high ratio in *S. plicata*, a NAA to BA ratio of 0.12 was employed for mature *Phalaenopsis amabilis* Blume. leaf culture (Tanaka and Sakanishi 1985), 0.42 (NAA/BA) was reported for auxiliary bud explants of *Dendrobium antennatum* Lindley.

Table 1 The effect of NAA and BA concentrations and ratios on PLB regeneration (g) of *Spathoglottis plicata* from PLB explants and nodal sections cultured for 1 and 3 months, respectively. Each value is the mean of three experiments, with three replications per experiments

NAA (μM)	BA (μM)							
	0.44		2.22		4.44		8.88	
	Nodal	PLB	Nodal	PLB	Nodal	PLB	Nodal	PLB
0.54	0.27e	0.57e	0.30e	0.63e	0.81cd	1.03cd	0.32e	0.90de
2.69	0.29e	0.54e	0.86cd	0.90de	1.58b	0.69de	0.95c	0.60e
5.37	2.85a	1.98a	0.29e	0.56e	0.58de	1.50b	0.95c	1.32bc
10.74	0.97c	0.90de	1.89b	0.81de	0.29e	0.91de	0.32e	0.66de
Significance (nodal, PLB)		BA*	NAA*	NAA×BA*				

* $P \leq 0.05$; mean separations within "nodal" and "PLB" among main effect means by Duncan's multiple-range test, $P=0.05$

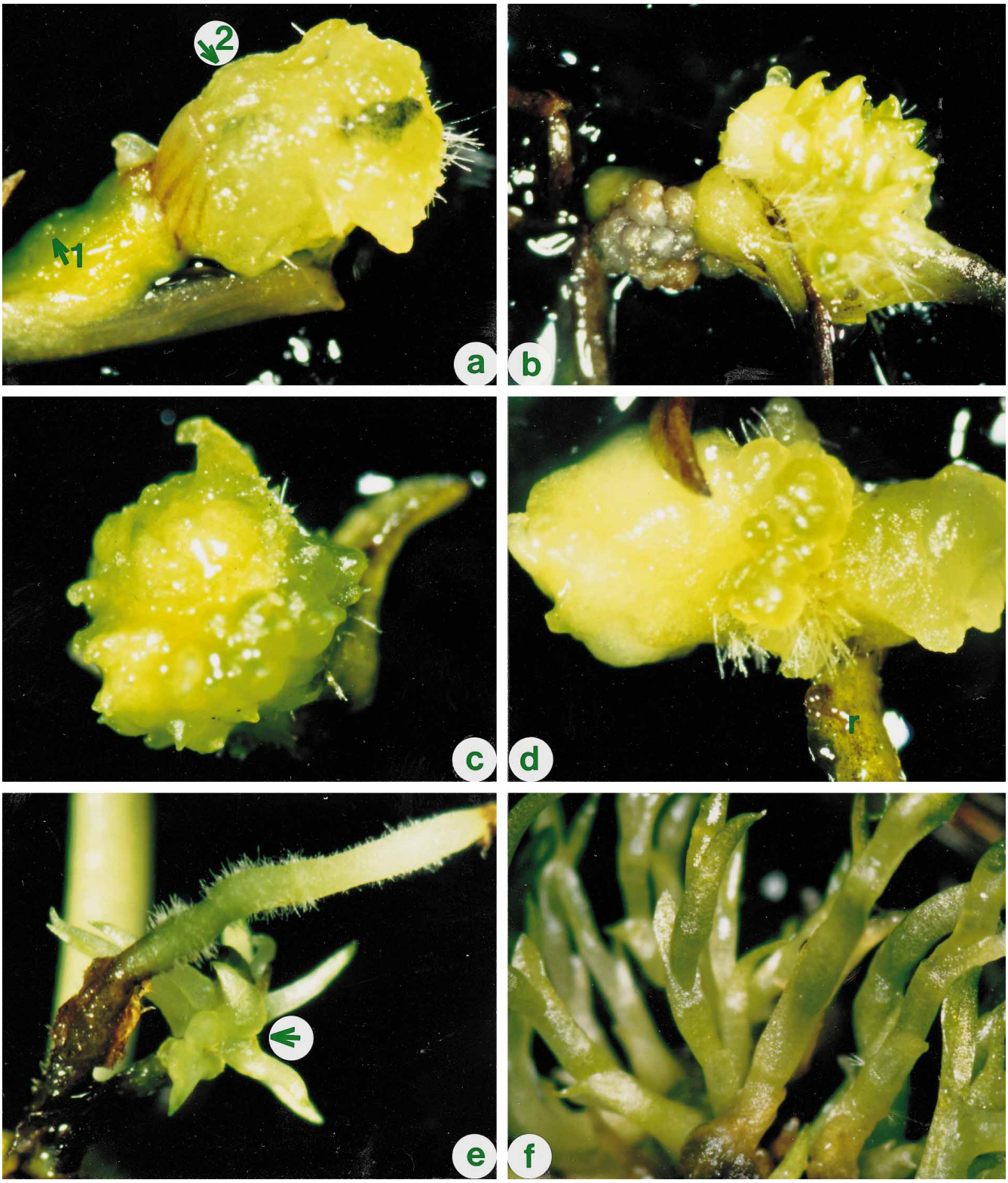

Fig. 1a–f Formation of PLBs and plantlets from various parts of *Spathoglottis plicata*. **a** PLBs induced from internodal and nodal area (*arrow 1* initial development of PLBs from the surface of internodal portion, *arrow 2* nodal and internodal area that has been converted to PLB forming tissue). **b** The surface of nodal and internodal tissue forming PLBs. **c** PLB formation from a leaf base. **d** Regeneration of PLBs from a root segment (r). **e** Regeneration of PLBs and adventitious shoots from root tip (*arrow*). **f** Plantlets developed from PLBs

(Kukulczanka and Wojciechowska 1983), and 1.23 (NAA/BA) was effective in several hybrid species of *Aranda* (Khaw et al. 1978).

The available information on *S. plicata* in vitro shows that the cytokinin requirement for PLB induction from nodal and PLB explants and for protocorm formation from

Table 2 The effect of NAA and BA on plantlet development of *S. plicata* from PLBs, induced from nodal explants or PLB explants after 1 month in culture. Regeneration was evaluated as the number of plantlets per petri dish >3 mm. Each value is the mean of three experiments, with three replications per experiment

NAA (μM)	BA (μM)							
	0.44		2.22		4.44		8.88	
	Nodal	PLB	Nodal	PLB	Nodal	PLB	Nodal	PLB
0.54	64de	48e	89c	80bc	66de	72bcd	64de	60cde
2.69	16hi	27f	30gh	51e	49ef	64bcde	145a	102a
5.37	3i	8g	78cd	75bcd	93c	57de	112b	103
10.74	32fgh	78bc	43fg	70bcde	68d	70bcde	130a	81b
Significance (nodal, PLB)	BA*		NAA*		NAA×BA*			

* $P \leq 0.05$; mean separations within "nodal" and "PLB" among main effect means by Duncan's multiple-range test, $P=0.05$

zygotic embryos is different (Bapat and Narayanaswamy 1977; Beechey 1970; Singh 1992). Singh (1992) showed that 0.44 μM BA had no effect on seed germination, whereas 4.44 μM resulted in healthy protocorms. A much lower BA concentration (0.44 μM) was required for PLB formation from both nodal and PLB explants (Table 1). The cytokinin requirement for PLB production also differs for each species. In various species of *Vanda*, 0.44–4.44 μM BA was applied (Chaturvedi and Sharma 1986; Sharma and Chaturvedi 1988); in *Cymbidium grandiflorum* Griff., 4.44 μM BA (Gu et al. 1987), whereas in *Phalaenopsis*, 44.44 μM BA was employed (Tanaka and Sakanishi 1980).

In contrast to the cytokinin level, the difference in auxin requirement between seed germination in vitro and tissue explant culture of *S. plicata* was not significant, as higher auxin concentrations were used in both. For PLB regeneration, the optimum NAA concentration was 5.37 μM NAA (Table 1). For zygotic embryos, 5.37 μM NAA accelerated initial germination whereas higher concentrations inhibited further growth (Singh 1992). NAA at 26.9 μM or higher resulted in nodular callus or callusing with loss of the ability to regenerate PLBs (Bapat and Narayanaswamy 1977; Singh 1992). However, in *Vanda*, the highest PLB production was obtained with 100 μM indoleacetic acid (Goh and Lie 1978).

PLBs developed plantlets in all media with combinations of 0.44–8.88 μM NAA and 0.54–10.74 μM BA (Table 2, Fig. 1f). However, the process was slower when PLBs were maintained in medium that favored PLB regeneration. The optimum combination for PLB induction, NAA/BA 5.37/0.44 μM, delayed plantlet development for 1–2 months. PLBs quickly developed plantlets when transferred to medium containing 2.69–10.74 μM NAA and 8.88 μM BA. Up to 145 plantlets could be developed from 0.2 g PLBs in 1 month (Table 2). Approximately 15–20 plantlets could be developed from PLBs in a single nodal section (data not shown). The ratio of auxin to cytokinin favoring plantlet development was 0.3–1.2, a value much lower than that for PLB formation, indicating that a high ratio was required for initiating PLBs, whereas lower ones were required for plantlet development.

Plantlets 2 cm in length were individually dissected from the plantlet clusters and placed on either regeneration medium or MS basal medium for rooting. Plantlet growth medium promoted rooting within 1 month. Nevertheless, plantlets were able to root eventually in all NAA and BA combinations. The rooted plantlets were transplanted to pots (peat:perlite:vermiculite 1:1:1) when they were approximately 5 cm in length, and 80% of plantlets were successfully grown in the 25°C greenhouse.

In summary, the developed system offers a promising and reliable propagation method for *S. plicata*. PLBs can be induced by 5.37/0.44 μM NAA/BA from nodal explants, and then transferred to 2.69–10.74/8.88 μM NAA/BA for plantlet development. A plantlet of five nodes has the potential to regenerate 10^6 plantlets in a year.

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