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Callus induction and somatic embryogenesis of *Phalaenopsis*

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Abstract Callus induction and plant regeneration through somatic embryogenesis in *Phalaenopsis* Richard Shaffer 'Santa Cruz' were examined. Protocorm-like body (PLB) segments formed calli in Vacin and Went medium with sucrose. The optimal concentration of sucrose was 40 g · l^{-1}. Medium containing 200 ml · l^{-1} coconut water together with 40 g · l^{-1} sucrose was effective for callus induction. Gellan gum was suitable than agar as a gelling agent for callus induction. The calli easily formed PLBs after being transferred to a medium without sucrose. Histological observation suggested that the PLBs were somatic embryos. No variation was observed in the flowering plants regenerated through somatic embryogenesis.

Key words *Phalaenopsis* · Protocorm-like bodies · Callus induction · Somatic embryogenesis

Abbreviations *PLB* Protocorm-like body · *VW* Vacin and Went · *2,4-D* 2,4-dichlorophenoxyacetic acid · *BA* benzyladenine · *CW* coconut water

Introduction

Phalaenopsis is a monopodial orchid which is difficult to propagate vegetatively. The characteristics of seedlings are not uniform, and propagation through tissue culture has been desired. However, as the in vitro culture of *Phalaenopsis* using shoot-tip may lead to the loss of the mother plant, alternative methods have studied by many workers (Arditti and Ernst 1993).

Callus formation in *Phalaenopsis* was first reported by Sagawa (1990a, b), who presented it as an available method for micropropagation. Since then, growth and development of the callus (Ichihashi and Hiraiwa 1996) and plant regeneration from protoplasts derived from the callus (Kobayashi et al. 1993) have been studied. However, there are few reports on the induction of callus in *Phalaenopsis*.

We therefore chose to investigate callus induction and plant regeneration from calli in *Phalaenopsis* Richard Shaffer 'Santa Cruz'. The effects of sucrose, plant growth regulators, coconut water and gelling agent on callus induction were examined as were the effects of sucrose on PLB formation from the callus and variation of plants through somatic embryogenesis.

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Materials and methods

Explants

Protocorm-like bodies (PLBs) of *Phalaenopsis* Richard Shaffer 'Santa Cruz' obtained through leaf segment culture (Tanaka and Sakanishi 1980) were subcultured every 2 months. The PLBs were bisected transversally and the segments used as explants. About 2-cm-long leaves were obtained from shoots derived from flower-stalk cuttings cultured in vitro (Tanaka and Sakanishi 1978) and then cut into six segments. These segments were also used as explants.

Callus induction

Vacin and Went (VW) medium (Vacin and Went 1949) supplemented with 2 g · l^{-1} gellan gum was used as a basal medium, unless otherwise noted.

For investigations on the effects of sucrose and plant growth regulators on callus induction from PLB segments or leaf segments, various combinations of 2,4-dichlorophenoxyacetic acid (2,4-D; 0, 0.1 and 1 mg · l^{-1}) and benzyladenine (BA; 0, 0.01 and 0.1 mg · l^{-1}) were added to the basal media with or without 40 g · l^{-1} sucrose. The effects of coconut water (CW) on callus induction was examined by supplementing 200 ml · l^{-1} CW to the basal media with or without 40 g · l^{-1} sucrose. Coconut water was obtained from green and unripe coconut fruits.

To examine the effects of sucrose concentration and gelling agent on callus induction from PLB segments, we added various concentrations of sucrose (0, 20, 40, 60 and 80 g · l^{-1}) to basal media sup-

Table 1 Effects of sucrose, plant growth regulators and coconut water on callus induction from PLB segments in *Phalaenopsis* Richard Shaffer 'Santa Cruz'

Sucrose ($g \cdot l^{-1}$)	Plant growth regulators ($mg \cdot l^{-1}$)		Coconut water ($ml \cdot l^{-1}$)	Number of segments[a]							
				Cultured	Forming calli and/or PLB[b]					With no response	Died
	2,4-D	BA			C(+++)	C(++)	C(+)	C and PLB	PLB		
0	0	0	0	50	0	0	0	0	23.5 ± 23.5	14.0 ± 11.0	12.5 ± 6.3
0	0.1	0	0	50	0	0	0	0	23.5 ± 23.5	3.5 ± 0.5	23.0 ± 23.0
0	0.1	0.01	0	50	0	0	0	0	23.5 ± 23.5	19.0 ± 16.0	7.5 ± 7.5
0	0.1	0.1	0	50	0	0	0	0	24.5 ± 24.5	18.0 ± 17.0	7.5 ± 7.5
0	1.0	0	0	50	0	0	0	0	0	0	50.0 ± 0.0
0	1.0	0.1	0	50	0	0	0	0	16.0 ± 16.0	15.5 ± 2.5	18.5 ± 18.5
0	1.0	1.0	0	50	0	0	0	0	21.0 ± 21.0	5.5 ± 1.5	23.5 ± 22.5
40	0	0	0	50	0	9.5 ± 3.5	20.5 ± 6.5	12.5 ± 6.5	4.0 ± 2.0	0	3.5 ± 3.5
40	0.1	0	0	50	0	18.0 ± 3.0	9.0 ± 1.0	14.0 ± 9.0	2.5 ± 0.5	0	6.5 ± 6.5
40	0.1	0.01	0	50	4.0 ± 4.0	18.5 ± 3.5	17.5 ± 9.5	4.0 ± 3.0	4.5 ± 4.5	0.5 ± 0.5	1.0 ± 1.0
40	0.1	0.1	0	50	0.5 ± 0.5	5.5 ± 0.5	22.5 ± 4.5	2.0 ± 2.0	2.0 ± 2.0	2.0 ± 2.0	15.5 ± 2.5
40	1.0	0	0	50	2.0 ± 2.0	10.0 ± 10.0	6.0 ± 6.0	4.5 ± 4.5	0	0	27.5 ± 22.5
40	1.0	0.1	0	50	0	13.0 ± 1.0	15.0 ± 1.0	3.5 ± 3.5	4.5 ± 3.5	2.5 ± 2.5	11.5 ± 10.5
40	1.0	1.0	0	50	0	5.0 ± 1.0	14.0 ± 4.0	4.0 ± 0.0	3.0 ± 3.0	2.0 ± 2.0	22.0 ± 3.0
0	0	0	200	50	0	0	0	0	48.0 ± 2.0	0	2.0 ± 2.0
40	0	0	200	50	26.0 ± 4.0	11.5 ± 4.5	4.5 ± 4.5	7.0 ± 5.0	0	0.5 ± 0.5	0.5 ± 0.5

[a] Each value is the average of two independent experiments and represents means ±SE
[b] C(+++), Large yellow callus was induced; C(++), small yellow callus was induced, C(+), yellow callus was observed but very small; C and PLB, C(++) and PLBs were formed in one segment; PLB, PLBs were formed

plemented with 200 $ml \cdot l^{-1}$ CW. Two gelling agents, 2 $g \cdot l^{-1}$ gellan gum (San-ei F. F. I., Japan) or 8 $g \cdot l^{-1}$ agar (Difco Lab, Mich., USA), were tested for each sucrose concentration.

All the media were adjusted to pH 5.3 with 1 *N* NaOH before autoclaving. PLB segments were cultured under 16-h photoperiods (HomoLux, Matsushita Electric Industrial Co, Japan) at 25 °C. After 8 weeks of culture, those PLB segments forming calli were counted. Leaf segments were cultured at 25 °C, in the dark for 2 weeks, followed by 16-h photoperiods for another 2 weeks. After 12 weeks of culture, leaf segments forming calli were recorded.

PLB formation from calli in subculture

Calli induced from PLB segments or leaf segments were transferred to VW media containing 200 $ml \cdot l^{-1}$ CW and 8 $g \cdot l^{-1}$ agar, with or without 20 $g \cdot l^{-1}$ sucrose. After 8 weeks of subculture, the number of callus mass-formed PLBs were counted.

Histological observation

After 8 weeks of subculture, PLBs regenerated on media without sucrose were fixed in FAA [formalin : acetic acid : 70% ethanol=1:1:18 (v/v)] solution. These were dehydrated in a butanol series and embedded in paraffin (m.p. 60°–62 °C). The materials embedded in paraffin were cut into 10-μm-thick sections and stained with hematoxylin for microscopic observation.

Plant regeneration

The PLBs formed on media without sucrose were transferred into the growing media [3 $g \cdot l^{-1}$ Hyponex® (N:P:K=6.5:6:19), Nitsch's micro elements (Nitsch and Nitsch 1967), 100 $mg \cdot l^{-1}$ *myo*-inositol, 1 $mg \cdot l^{-1}$ nicotinic acid, 30 $g \cdot l^{-1}$ sucrose, 30 $g \cdot l^{-1}$ potato extract, 1 $g \cdot l^{-1}$ activated charcoal (powder; Wako Pure Chemical Industries, Japan), 8 $g \cdot l^{-1}$ agar, pH 5.3]. Regenerated plantlets were transplanted and grown in a greenhouse.

Results and discussion

Effects of sucrose, plant growth regulators and CW on the callus induction

Many PLB segments formed calli on media containing sucrose (Table 1). The calli varied in size and were classified as: calli (+++), large and bright yellow; calli (++), small and yellow; calli (+),very small and yellow. Calli (+++) were obtained on media containing plant growth regulators or CW. Medium containing 0.1 $mg \cdot l^{-1}$ 2,4-D and 0.01 $mg \cdot l^{-1}$ BA were more effective for callus induction than media containing other combinations of 2,4-D and BA. However, the largest number of calli (+++) were recorded on the media containing CW. On the other hand, no callus was induced on media without sucrose even in media containing plant growth regulators or CW. These results suggest that callus induction was due to the sucrose in the medium and that the frequency of callus induction was enhanced by the addition of plant growth regulators or CW.

Callus induction from leaf segments was observed only on media containing sucrose, 1.0 $mg \cdot l^{-1}$ 2,4-D and 0.1 $mg \cdot l^{-1}$ BA (Table 2). Leaf segments did not form callus on media without sucrose, though most of the segments swelled and remained green. No effect of CW was observed in callus induction from leaf segments, which is in contrast to callus induction from PLB segments. These results may suggest that callus induction from leaf segments was relatively difficult and required higher concentrations of plant growth regulators than callus induction from PLB segments. Almost all of the leaf segments died on sucrose-containing media. The result may also suggest that the su-

Table 2 Effects of sucrose, plant growth regulators and coconut water on callus induction from leaf segments in *Phalaenopsis* Richard Shaffer 'Santa Cruz'

Sucrose ($g \cdot l^{-1}$)	Plant growth regulators ($mg \cdot l^{-1}$)		Coconut water ($ml \cdot l^{-1}$)	Number of segments[a]			
	2,4-D	BA		Cultured	Forming calli[b]	With no response	Died
0	0	0	0	36	0	32.0±2.0	4.0±2.0
0	0.1	0.01	0	36	0	30.0±2.0	6.0±2.0
0	1.0	0.1	0	36	0	16.0±2.0	20.0±2.0
40	0	0	0	36	0	0	36.0±0.0
40	0.1	0.01	0	36	0	17.0±9.0	19.0±9.0
40	1.0	0.1	0	36	2.5±1.5	24.5±2.5	9.0±1.0
0	0	0	200	36	0	26.5±0.5	9.5±0.5
40	0	0	200	36	0	1.0±1.0	35.0±1.0

[a] See Table 1
[b] Small yellow calli were formed

Table 3 Effects of gelling agent and sucrose concentration on the callus induction from PLB segments in *Phalaenopsis* Richard Shaffer 'Santa Cruz'

Gelling agents	Sucrose ($g \cdot l^{-1}$)	Number of segments[a]							
		Cultured	Survived	Forming calli[b]	Forming calli and/or PLB[a]				Died
					C(+++)	C(++)	C(+)	C and PLB	PLB	
Agar	0	50	45.5±3.5	0.5±0.5	0	0	0	0.5±0.5	45.0±4.0	4.5±3.5
	20	50	47.0±3.0	30.0±5.0	0	7.0±0.0	18.5±0.5	4.5±4.5	17.0±8.0	3.0±3.0
	40	50	46.0±4.0	38.5±0.5	13.5±8.5	10.0±1.0	10.0±0.5	5.0±1.0	7.5±4.5	4.0±4.0
	60	50	43.5±1.5	44.0±2.0	4.0±3.0	17.5±1.5	20.0±4.0	1.5±0.5	0.5±0.5	6.5±1.5
	80	50	43.5±1.5	43.5±1.5	3.0±0.0	11.5±1.5	28.0±2.0	1.0±1.0	0	6.5±1.5
Gellan gum	0	50	49.5±0.5	1.0±1.0	0	0	0	1.0±1.0	48.5±0.5	0.5±0.5
	20	50	50.0±0.0	50.0±0.0	24.0±1.0	14.5±1.5	3.5±1.5	8.0±2.0	0	0
	40	50	49.5±0.5	49.5±0.5	28.5±3.5	14.0±3.0	3.5±1.5	3.5±2.5	0	0.5±0.5
	60	50	49.5±0.5	49.5±0.5	21.0±0.0	15.0±2.0	11.0±1.0	2.5±1.5	0	0.5±0.5
	80	50	49.0±1.0	49.0±1.0	11.5±4.5	18.5±3.5	17.0±1.0	2.0±1.0	0	1.0±1.0

[a] See Table 1
[b] C(+++), C(++), C(+), and C and PLB were included

crose concentration should be improved. Tanaka and Sakanishi (1980) reported that media containing 20 $g \cdot l^{-1}$ sucrose was suitable for leaf-segment culture in *Phalaenopsis*.

Effects of sucrose and gelling agent on callus induction from PLB segments

The sucrose concentration in media affected callus induction from PLB segments in both media containing gellan gum or agar (Table 3). The optimal concentration of sucrose was 40 $g \cdot l^{-1}$, as it was at this concentration that the largest number of PLB segments formed calli (+++). Zimmarman and Robacker (1988) reported that callus initiation from the seed hypocotyls of *Gossypium* was promoted significantly on media solidified with gellan gum. More PLB segments induced calli on media using gellan gum as a gelling agent than on media using agar. Moreover, many segments cultured on media using gellan gum formed fewer PLBs than those cultured on media using agar. These results suggest that the use of gellan gum as a gelling agent is suitable for callus induction in *Phalaenopsis*. Ichihashi and Hiraiwa (1996) reported that *Phalaenopsis* callus grew better on media solidified with gerlite. The subsequent growth of calli induced in the present study will also be promoted on media with gellan gum.

Table 4 Effects of sucrose on the PLB formation from the calli of *Phalaenopsis* Richard Shaffer 'Santa Cruz' in subculture

Sucrose ($g \cdot l^{-1}$)	Number of callus masses[a]		
	Cultured	Proliferating	Forming PLB
0	45	0	45.0±0.0
20	45	45.0±0.0	0

[a] See Table 1

PLB formation and histological observation

Calli induced on media containing 0.1 $mg \cdot l^{-1}$ 2,4-D and 0.01 $mg \cdot l^{-1}$ BA continued to proliferate after being transferred to media containing sucrose (Table 4, Fig. 1). On the other hand, calli subcultured on media without sucrose turned green and then produced a large number of PLBs. The same results were obtained in calli induced from PLB

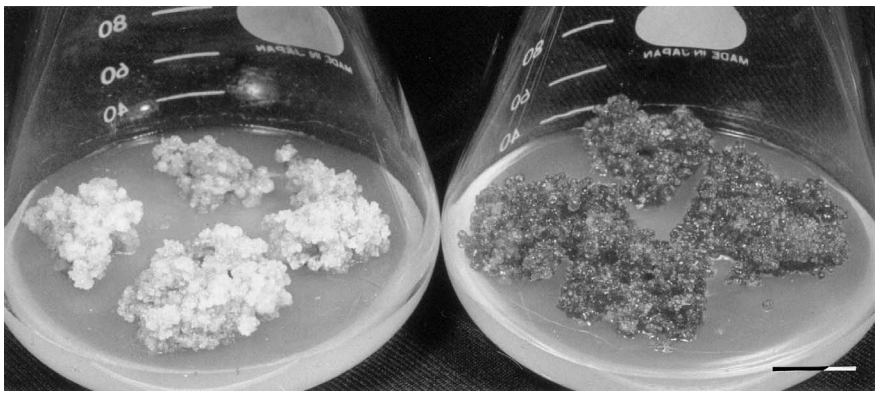

Fig. 1 Callus proliferation on medium with sucrose (*left*) and PLB formation on medium without sucrose (*right*) from calli induced from PLB segments. *Bar*: 1 cm

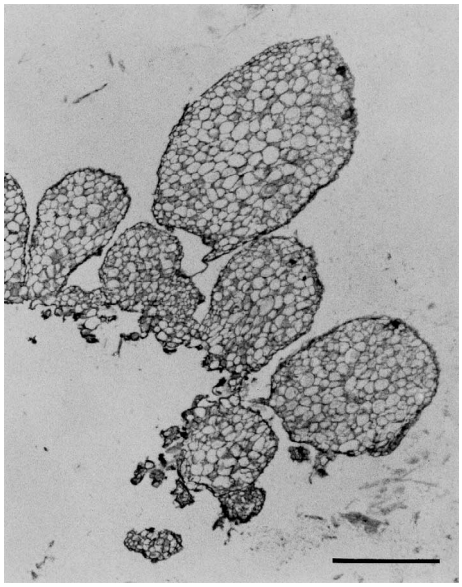

Fig. 2 The section of somatic embryos (infant PLBs) regenerated from calli (10-μm section). *Bar*: 500 μm

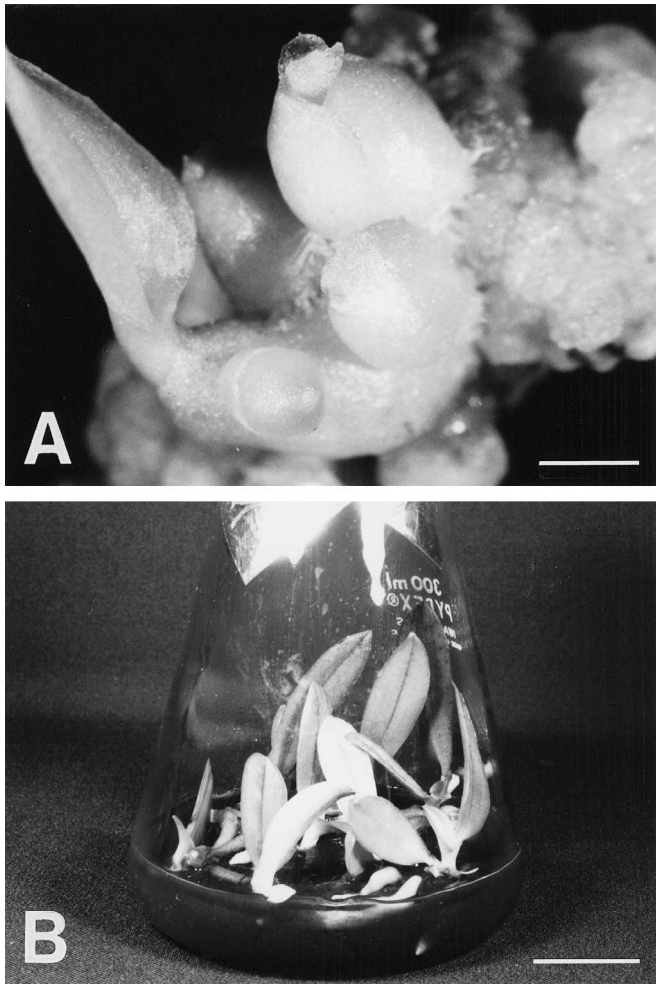

Fig. 3A, B Plant regeneration through somatic embryogenesis. **A** PLBs regenerated from calli. *Bar*: 1.5 mm. **B** Plantlets transferred to the growing medium. *Bar*: 2 cm

segments under different culture conditions and from leaf segments (data not shown). Sagawa (1990a) reported that the callus of *Phalaenopsis* turned green and produced embryoids upon subculture in medium without sucrose, which agrees with our results. Ichihashi and Hiraiwa (1996) reported that there were varietal differences in the optimal kinds and levels of carbohydrates for the growth of *Phalaenopsis* and *Doritaenopsis* callus. They also showed that almost all of the calli turned green on media without sucrose, though the calli of a strain of *Doritaenopsis* turned green on media with sucrose. In *Aranda*, calli grown in medium with a low concentration of fructose produced many PLBs (Chia et al. 1988). These results suggest that the growth of monopodial orchid callus and PLB formation from callus are influenced by the sugar in medium and that the optimal carbohydrate kinds and levels vary with the genera. In *Phalaenopsis* Richard Shaffer 'Santa Cruz', sucrose also influenced PLB formation from the calli, as shown in the present study.

PLBs formed on media without sucrose were round or oval, and thereby similar in shape to the protocorms of *Phalaenopsis* formed after seed germination (Nishimura 1981). From histological observation, no vascular connection was observed between each PLB and the other tissue (Fig. 2). The protocorm is one structure unique to the orchids, including *Phalaenopsis*, and it is the earliest structure formed during embryo development of the orchid seed. This observation suggests that PLBs derived from calli

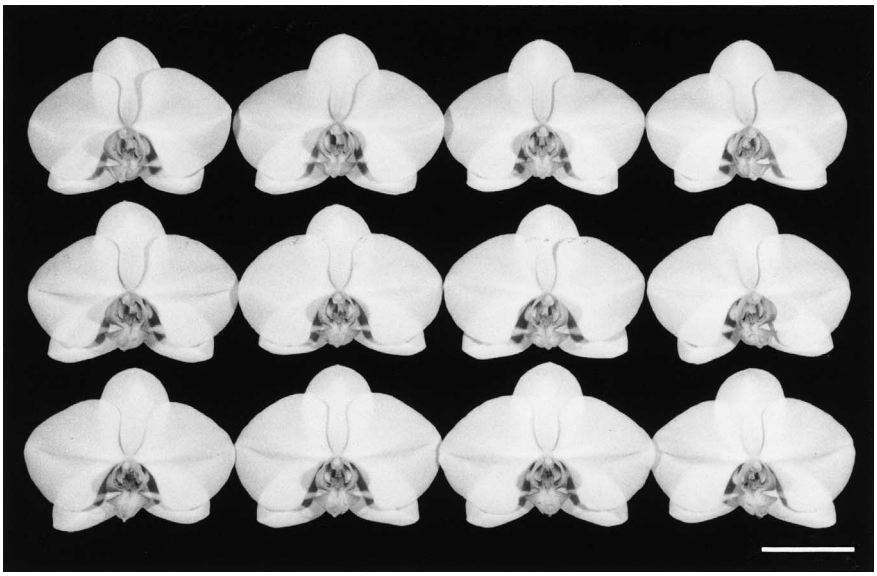

Fig. 4 Flowers of plants regenerated through somatic embryogenesis. *Bar*: 5 cm

could be considered as somatic embryos and that the callus induced in this study was embryogenic.

Plant regeneration

Plantlets regenerated through somatic embryogenesis grew on the growing media (Fig. 3A, B). Among 31 plants that flowered in a greenhouse within 2 years after acclimatization, no difference was observed in flower shapes (Fig. 4). This result suggests the possibility that plant regeneration via somatic embryogenesis would be a new procedure of clonal propagation in *Phalaenopsis*.

Conclusion

There has been no report of the induction of embryogenic callus in *Phalaenopsis*. The present study is the first to show that calli of *Phalaenopsis* can be induced from PLB segments on media containing sucrose. The use of media supplemented with plant growth regulators or CW and those solidified with gellan gum were also effective for callus induction from PLB segments. PLB formation from calli needed subculturing in media without sucrose, whereas callus proliferation required sucrose in medium. In almost all of the flowering plants regenerated through somatic embryogenesis of *Phalaenopsis* Richard Shaffer 'Santa Cruz' there was no visual variation.

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