

## **Induction and morphogenesis of *Phalaenopsis* callus**

(Induksi dan morfogenesis kalus *Phalaenopsis*)

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Key words: *Phalaenopsis* orchid, callus induction, morphogenesis, protocorm-like body

### **Abstract**

Protocorm-like body (PLB) segments of *Phalaenopsis* var. Hawaiian Clouds x *Phalaenopsis* Carmela's Dream, cultured on half strength Murashige and Skoog medium supplemented with different concentrations of sucrose, were maintained in the dark at  $25 \pm 2$  °C. Friable callus, white to brownish-white in colour were successfully induced on medium containing 30 g/litre of sucrose. Induction of PLBs from callus was carried out on medium supplemented with different combinations of sucrose and maltose at concentration levels between 0 and 10 g/litre. New Dogashima medium (NDM) containing 1 mg/litre of thidiazuron (TDZ) and solidified with 2.8 g/litre of *Gelrite* was used as basal medium. Callus formed PLBs on all medium supplemented with 5 and 10 g/litre of maltose, except those on medium with 10 g/litre of sucrose. An average of 52.5% of callus formed PLBs on medium supplemented with 10 g/litre of maltose without sucrose. Formation of PLBs occurred in all the clumps of callus cultured on medium with addition of 10 g/litre of maltose alone. PLBs derived from the callus were able to regenerate into plantlets.

### **Introduction**

*Phalaenopsis* orchid is one of the most important orchids grown for commercial production of cut flowers and potted plants. Genetic improvement of *Phalaenopsis* through sexual hybridisation is restricted by a long growth and reproductive cycle, and limited genetic variability within the germplasm. One alternative procedure is through biolistic bombardment where genes controlling desired characters are directly delivered into the plant cells.

Protocorm-like bodies (PLBs) of *Phalaenopsis* are very sensitive to physical injury which leads to the phenolic oxidation and browning of the tissue. The problem of phenolic oxidation usually occurs in tropical plant species as they contain

high concentration of phenolic substances (George 1993). This makes the PLBs less favourable for biolistic bombardment. Hence, callus has been used as an alternative target cell for transformation work.

There are limited plant cell lines, which are autotrophic when cultured in vitro, resulting in sucrose being usually incorporated into the medium as a carbon source for micropropagation. Studies on the effects of sugar on growth of in vitro plant tissues and the regulatory effects of sugar on plant development of orchids have been reported. The types of sugar used in these studies include sucrose (Intuwong and Sagawa 1974; Cheah and Sagawa 1978; Ishii et al. 1998), fructose (Ernst et al. 1971; Chia et al. 1988) and

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galactose (Ernst et al. 1971). The use of maltose to substitute sucrose was done by Belarmino and Mii (2000) in their work with *Phalaenopsis* orchid. In this study with *Phalaenopsis*, callus was induced by varying the sucrose levels and PLBs were initiated using various concentrations of sucrose and maltose.

Different types of Murashige and Skoog medium (MS) (Murashige and Skoog 1962), New Dogashima medium (NDM) (Tokuhara and Mii 1993) and Vacin and Went medium (VW) (Vacin and Went 1949) were used in various studies of *Phalaenopsis* orchids (Tanaka and Sakanishi 1977; Griesbach 1983; Tokuhara and Mii 1993; Ishii et al. 1998). In this study, half strength MS and NDM were used as culture media.

## Materials and methods

### Callus induction

PLBs of *Phalaenopsis* var. Hawaiian Clouds x *Phalaenopsis* Carmela's Dream used were previously developed from in vitro plantlets which were derived from young flower stalks harvested from Cameron Highlands grown plant. The PLBs were separated into segments of 2–5 mm in diameter. Half strength MS ( $\frac{1}{2}$  MS) supplemented with 9 g/litre *Bacto* agar was used as the basal medium for callus induction. Different concentrations of sucrose (Duchefa) (0, 20, 30 and 40 g/litre) were added to the basal medium.

As PLBs used were previously maintained on  $\frac{1}{2}$  MS containing 20 g/litre of sucrose, medium supplemented with this concentration of sucrose was chosen as the control for the experiment. The pH of the medium was adjusted to 5.6–5.8 before the addition of the agar and prior to autoclaving at 121 °C for 15 min. A total of 20 PLB segments were cultured onto approximately 25 ml of culture medium in a disposable 90-mm petri dish. Each treatment had 10 petri dishes, placed in the dark at  $25 \pm 2$  °C. The PLB segments were subcultured onto fresh medium every 8–10 weeks.

### PLB formation from callus

Callus induced from PLB segments were transferred to PLB formation medium consisting of NDM, 2.8 g/litre of *Gelrite*, 1 mg/litre of thidiazuron (TDZ) and between 0 and 10 mg/litre of sucrose and/or maltose. The concentration of TDZ used was based on the work on *Cymbidium ensifolium* var. *misericors* by Chang and Chang (1998) where production of shoot buds from orchid callus was reported even with single type of plant growth regulator, TDZ.

The medium was adjusted to pH 5.4 before adding the gelling agent and prior to autoclaving at 121 °C for 15 min. NDM was chosen for PLB induction as Tokuhara and Mii (1993) reported a higher percentage of PLB formation on NDM than on  $\frac{1}{2}$  MS. A total of 12 clumps of callus measuring approximately 8 mm in diameter were cultured onto approximately 25 ml of culture medium in a disposable 90-mm petri dish and placed in the dark at  $25 \pm 2$  °C. Each treatment had five replicates and the cultures were transferred to fresh medium every 6–8 weeks.

Number of callus clumps producing PLBs after 6 months of in vitro culture was counted for all the treatments. Proliferation rate of PLBs was visually estimated based on the percentage of callus clumps covered by PLBs after 6 months.

### Plant regeneration

The PLBs formed from the callus were transferred to regeneration medium ( $\frac{1}{2}$  MS medium supplemented with 20 g/litre of sucrose, 1 g/litre of activated charcoal and 2.8 g/litre of *Gelrite*), same as the growth medium where the original source was maintained. The pH of the medium was adjusted to 5.6–5.8 before adding the gelling agent and prior to autoclaving at 121 °C for 15 min. The PLBs were cultured onto approximately 25 ml of growth medium in a disposable 90-mm petri dish under 16-h photoperiods at  $25 \pm 2$  °C. The cultures were transferred to fresh medium every 2 months.

## Results and discussion

### *Callus induction*

No callus was induced from PLB segments on  $\frac{1}{2}$  MS medium containing 0, 20 and 40 g/litre of sucrose except on medium containing 30 g/litre of sucrose. The callus formed was friable and white to brownish-white in colour (*Plate 1*).

The results showed that sucrose concentration affected callus induction of *Phalaenopsis* from PLB segments. Chia et al. (1988) reported that callus tissues of *Aranda* Tay Swee Eng grown in low carbon source medium produce more protocorms and contain more chlorophyll than those grown in higher carbon source medium. Ishii et al. (1998) in their study on *Phalaenopsis* Richard Shaffer 'Santa Cruz' used VW supplemented with sucrose concentration of 0, 20, 40, 60 and 80 g/litre and found that callus of *Phalaenopsis* could be induced from PLB segments on medium containing an optimal concentration of sucrose at 40 g/litre.

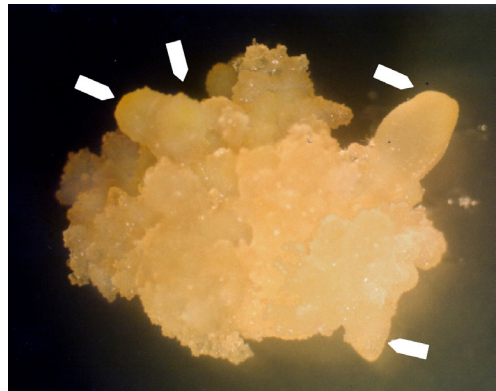
In this study, PLB segments formed callus on medium containing 30 g/litre of sucrose, indicating that carbon source in culture medium could induce callus from *Phalaenopsis* PLB segments and thus has a host of regulatory effects upon the plant development besides serving as a nutrient requirement for the growth of in vitro plant tissue, as what was found by Chia et al. (1988).

### *PLB formation from callus*

PLBs were visible in the midst of the callus clumps after 6 months of in vitro culture (*Plate 2*). All the callus clumps of *Phalaenopsis* produced PLBs on medium supplemented with 10 g/litre of maltose alone, while an average of 84.4% of the callus clumps produced PLBs on medium supplemented with 5 g/litre of maltose. PLBs were formed in all treatments containing maltose except on medium in combination with 10 g/litre of sucrose. The callus clumps also formed PLBs on medium containing sucrose as the sole



*Plate 1. Callus derived from PLB segment on  $\frac{1}{2}$  MS medium containing 30 g/litre of sucrose under dark condition*



*Plate 2. PLBs (indicated by white arrows) derived from callus on NDM medium containing 10 g/litre maltose under dark condition*

carbon source at 5 g/litre of sucrose, but not on medium containing 10 g/litre of sucrose (*Table 1*). These results indicated that induction of PLB from *Phalaenopsis* callus was achievable with medium supplemented with 5 g/litre of sucrose alone, but appeared unfavourable when the sucrose concentration was doubled.

Ishii et al. (1998) reported that callus of *Phalaenopsis* Richard Shaffer 'Santa Cruz' continued to proliferate on medium containing 20 g/litre of sucrose, but recorded no formation of PLB. Similar results were shown in the study by Ricci et al. (2002) on 'Itaboraí' sweet orange (*Citrus sinensis* L. Osbeck.) that sucrose concentration higher

than an optimum level inhibits the formation of embryos from the citrus callus.

Medium containing maltose alone was the best in inducing PLBs from *Phalaenopsis* callus as compared to medium with sucrose alone or a combination of maltose and sucrose (Table 1). Maltose hydrolysis is 20 times slower than sucrose hydrolysis in barley anther cultures (Roberts-Oehlschlager et al. 1990). The beneficial effect of maltose on promoting PLB formation in this study came from its slow hydrolysis, similar to what was found by Blanc et al. (2002). Slower hydrolysis of maltose when compared to that of sucrose result in less supply of hexose thus triggers embryogenic differentiation of callus cells (Blanc et al. 2002). In the presence of both maltose and sucrose in the medium, increasing the concentration of sucrose inhibited the formation of PLB from *Phalaenopsis* callus when the concentration of maltose was fixed (Table 1).

Similarly, medium with 5 g/litre of sucrose together with maltose did not promote PLB formation from *Phalaenopsis* callus as compared to medium supplemented with maltose alone. When combining sucrose and maltose in the medium, the callus cells might have taken the metabolic pathway triggered by faster hydrolysis of sucrose, resulting in cellular growth and continued callus cell proliferation. Hence, the beneficial effect of maltose was not observed in the medium with the combination of sucrose and maltose in this study.

This study showed that no PLB was induced from callus when cultured on medium without both sucrose and maltose (Table 1). Chia et al. (1988) found that callus of *Aranda* grown in liquid VW medium with a low concentration of fructose forms PLBs but not when grown in the medium with only coconut water and without fructose suggesting that PLB formation depends on the residual carbon/nitrogen (C/N) ratio in the medium. Hence, elimination of carbon source completely

Table 1. Mean percentage of clumps of *Phalaenopsis* callus producing PLBs after 6 months of in vitro culture on various combinations of sucrose and maltose in NDM medium

Maltose (g/litre)	Sucrose (g/litre)		
	0	5	10
0	0d	33.4b	0d
5	84.4a	27.8bc	0d
10	100a	9.1cd	0d

Mean values followed by the same letter are not significantly different at  $p = 0.01$

Table 2. Mean percentage of *Phalaenopsis* callus clumps covered by PLBs after 6 months of in vitro culture on various combinations of sucrose and maltose in NDM medium

Maltose (g/litre)	Sucrose (g/litre)		
	0	5	10
0	0c	15cb	0c
5	10cb	30b	0c
10	52.5a	10cb	0c

Mean values followed by the same letter are not significantly different at  $p = 0.01$

from the culture medium may not promote induction of PLB from callus.

An average of 52.5% of *Phalaenopsis* callus clumps on medium supplemented with 10 g/litre of maltose were covered by PLBs after 6 months. Other combinations of sucrose and maltose were less favourable in inducing PLBs from callus (Table 2). The callus formed no PLBs when the level of sucrose was high (10 g/litre). The results showed that PLBs were the most proliferating on the medium supplemented with 10 g/litre of maltose alone. Maltose being more effective than sucrose for embryogenesis induction has been reported in alfalfa (Strickland et al. 1987), conifer (Nirgaard 1997), rubber tree (Blanc et al. 1999) and citrus (Ricci et al. 2002).

#### **Plant regeneration**

An average of 70% of PLBs transferred to the growth medium survived. The PLBs turned green and continued to form more



Plate 3. Plantlets regenerated from PLBs on the growth medium

PLBs. Plantlets were formed from the proliferated PLBs (Plate 3). The result suggested that the callus was embryogenic as it formed PLBs that further regenerated into plantlets.

### Conclusion

It is possible to induce callus from PLB segments of *Phalaenopsis* var. Hawaiian Clouds x Carmela's Dream by regulating the sucrose concentration in the medium. The use of  $1/2$  MS medium supplemented with 30 g/litre of sucrose was effective for callus induction from PLB segments, but a high concentration of sucrose was not favourable for PLB induction from *Phalaenopsis* var. Hawaiian Clouds x Carmela's Dream callus. However, the presence of maltose alone as the carbon source in the medium promoted the formation of PLBs suggesting that morphogenesis of callus was influenced by the type and the level of sugar in the medium. This study indicated that medium with maltose was more suitable for PLB induction than sucrose and the best level of sugar for PLB induction was 10 g/litre of maltose alone. The PLBs derived from the callus regenerated into plantlets suggesting that the callus was embryogenic.

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### Abstrak

Segmen 'protocorm-like body' (PLB) bagi *Phalaenopsis* var. Hawaiian Clouds x *Phalaenopsis* Carmela's Dream dikultur di atas medium separuh kepekatan Murashige dan Skoog yang mengandungi kepekatan sukrosa yang berbeza dan disimpan di dalam bilik gelap pada suhu  $25 \pm 2$  °C. Kalus berwarna putih ke putih keperangan telah berjaya dihasilkan di atas medium yang mengandungi 30 g/liter sukrosa. Pengaruh PLB daripada kalus telah dilakukan pada medium yang mengandungi kombinasi sukrosa dan maltosa yang berbeza pada tahap kepekatan antara 0 dan 10 g/liter. Medium New Dogashima (NDM) dengan tambahan 1 mg/liter thidiazuron (TDZ) dan 2.8 g/liter *Gelrite* digunakan sebagai medium asas. PLB terbentuk pada semua medium yang mengandungi 5 dan 10 g/liter maltosa kecuali pada medium yang mengandungi 10 g/liter sukrosa. Purata kalus yang membentuk PLB ialah 52.5% pada medium yang mengandungi 10 g/liter maltosa tanpa sukrosa. Semua gumpalan kalus menghasilkan PLB pada medium yang mengandungi 10 g/liter maltosa sahaja. PLB yang dihasilkan berupaya membentuk plantlet.