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Control of Growth and Development of Protocorm like Body Derived from Callus by Carbon Sources in *Phalaenopsis*

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Abstract

Protocorm like bodies (PLBs) derived from callus of *Phalaenopsis* utilized sucrose, maltose and sorbitol for their growth *in vitro*. These carbon sources affected differently and could control PLB growth. On sucrose supplemented medium a few PLBs produced plantlets and most others regenerated yellowish or greenish callus like body (CLB). Almost 80% of unrooted and 58% of rooted plantlets developed yellowish green CLB at the base of plantlets. On maltose supplemented medium, PLBs regenerated PLBs and a few plantlets. In subsequent culture, about 44% of unrooted and 24% of rooted plantlets initiated green PLBs at the base of cultured plantlets. On sorbitol supplemented medium, most of the PLBs developed plantlets and a few additional PLBs. Among the carbon sources tested, sorbitol supported plantlet development the best *in vitro* and proved to be the most suitable carbon source for plantlet initiation and development from PLB.

1. Introduction

Phalaenopsis is an important orchid genus from horticultural viewpoint, because of its popularity and the easiness of flowering in a green house. To improve the quality of pot plants and to reduce cultivation period, *in vitro* propagation of *Phalaenopsis* is increasing recently. Several micropropagation methods have been reported using different tissues and organs including shoot tip [1], flower stalk nodes [2-5], excised axillary buds of flower stalk [6], leaves of *in vitro* grown plantlets [7, 8], the internodal section of flower stalks [9, 10] and lateral buds from young flower stalks [11]. Recently the multiplication of PLB in a liquid culture system was improved [12]. However, none of these methods has become well established, because of the high frequency of contamination, the length of time for PLB formation, the low rate of PLB production, the differences in PLB formation, and the risk of mutation.

Phalaenopsis callus has the potential for rapid mass clonal production [13, 14]. We developed culture methods which improve callus growth and differentiation by introducing different carbon sources and organic additives (unpublished data). This method might be effective in overcoming some of these barriers. However, for practical application of this method, efficient plantlet regeneration from PLBs derived from callus is required.

In the present study, we report the effects of different carbon sources on the growth and plantlet initiation of PLBs derived from callus.

2. Materials and Methods

Embryogenic callus derived from the lateral bud culture of young flower stalks of *Phalaenopsis* Wedding Promenade (P4) and *Phal.* Hanaboushi × *Phal. equestris* 'Ilocos'(P5) (given by Mr. Tokuhara, Dogashima Orchid Center) were cultured on New *Phalaenopsis* (NP) medium (**Table 1**) supplemented with $10\,g\,l^{-1}$ of sorbitol for 16 weeks to develop PLBs. Culture was maintained at 25±1°C in a constant illumination of 350 lux provided by Plant Lux florescent lamp(FL40s, Toshiba). PLBs developed from callus were used as materials (**Fig. 1a**). The culture medium was solidified with $3\,g\,l^{-1}$ of Gelrite (Merck & Co., Inc.) and the pH was adjusted to 5.6±0.1. 15 PLBs of equal weight (0.016g/one) and larger than 2 mm were planted on 40 ml of medium supplemented with $20\,g\,l^{-1}$ of sucrose, $20\,g\,l^{-1}$ of maltose or $10\,g\,l^{-1}$ of sorbitol at the same molar concentration of 0.056 M in a 100ml flask and allowed to grow in a room at 25±1°C in about 350 lux of constant fluorescent light (FL40S, Toshiba). Each treatment was replicated 5 times. After 8 weeks of culture, growth and plantlet regeneration potentials of

Table 1. Composition of new Phalaenopsis (NP) medium used as basal medium for PLBs and plantlets development in *Phalaenopsis* callus culture.*

Components		($mg.\ l^{-1}$)
Macroelements		
Ammonium sulphate	$(NH_4)_2SO_4$	303.9
Potassium phosphate	KH_2PO_4	462.7
Ammonium nitrate	NH_4NO_3	32.0
Potassium nitrate	KNO_3	424.6
Calcium nitrate	$Ca(NO_3)_2 \cdot 4H_2O$	637.6
Magnesium nitrate	$Mg(NO_3)_2 \cdot 6H_2O$	256.4
Chelated iron		
Chelating agent	Na_2EDTA	37.3
Ferrous sulphate	$FeSO_4 \cdot 7H_2O$	27.8
Microelements		
Manganese sulphate	$MnSO_4 \cdot 4H_2O$	11.15
Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	4.3
Boric acid	H_3BO_4	3.1
Potassium iodide	KI	0.415
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.125
Cobalt chlorite	$CoCl_2 \cdot 6H_2O$	0.0125
Copper sulphate	$CuSO_4 \cdot 5H_2O$	0.0125
Organics/Vitamins		
Nicotinic acid		0.5
Pyridoxine HCl		0.5
Thiamine HCl		0.1
myo-Inositol		100.0
Glycine		2.0
Carbon source		
Sucrose (optional)		20000.0
Maltose (〃)		20000.0
Sorbitol (〃)		10000.0
Solidifier		
Gelrite		3000.0

* pH of the medium was adjusted to 5.6 with addition of 0.1 N HCl or KOH prior to autoclave. Ionic ratio of this medium is, NH_4^+: Ca^{++}: Mg^{-+}=25: 38: 27: 10, and NO_3^-: $H_2PO_4^-$: SO_4^{--}=60: 17: 23.

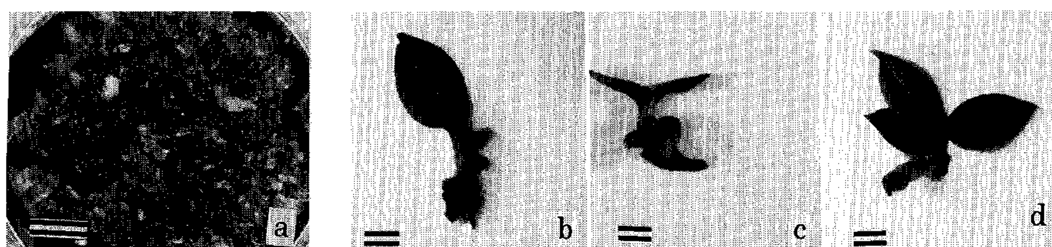

Fig. 1 Morphological features of PLB derived from callus culture (a) and plantlets grown on the NP medium supplemented with sucrose (b), maltose (c) and sorbitol (d) in *Phalaenopsis*. Scale bar=0.5cm.

PLBs were evaluated. Induced plantlets on the medium supplemented with sorbitol were allowed to grow subsequently for a month under the same conditions until about 50% of the plantlets developed roots. Rooted and unrooted plantlets were separated into two groups to evaluate the differences in their growth potential. For each treatment, 15 plantlets were transplanted onto the medium supplemented with 20 gl^{-1} of sucrose, 20 gl^{-1} of maltose or 10 gl^{-1} of sorbitol in polycarbonate culture vessels ($9.5^2 \times 12$cm) equipped with a membrane filter. The culture condition was the same as mentioned above and the number of replications was also five. After 3 months of culture, growth of plantlets was investigated.

3. Results and Discussion

3. 1 PLB growth

Transplanted PLBs on NP medium supplemented with sucrose, maltose or sorbitol showed different growth and development. On sucrose supplemented medium, only 8% of PLB in cultivar P4 and 24% in cultivar P5 initiated plantlets (**Fig. 2**). They developed a few new PLBs and yellowish callus like body (CLB; callus-like masses consist of small granular surface) at the base of newly initiated plantlets (**Table 2**). The total weight of CLB per flask was significantly higher on sucrose supplemented medium than those on maltose and sorbitol containing medium. In *Neofinetia falcata*, similar results have been reported [15]. These results suggested that sucrose promotes the multiplication of PLBs and CLB.

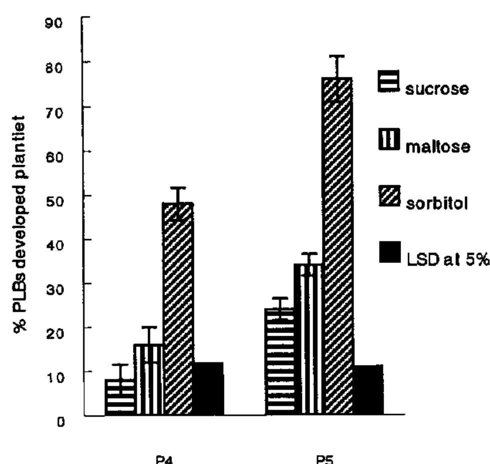

Fig. 2 Plantlet development from callus lead PLBs on the medium supplemented with different carbon sources. Bars indicate standard error.
P4: *Phal.* Wedding Promenade
P5: *Phal.* Hanaboushi × *Phal. equestris* 'Ilocos'

On maltose containing medium 16% of PLBs of cultivar P4 and 34% of PLBs of cultivar P5 developed plantlets (**Fig. 2**). They also developed 21. 6 and 53. 0 new PLBs per flask respectively and their color was green (**Table 2**). PLB regeneration rate was significantly higher on maltose than the other treatments, while CLB development was poorer than that on sucrose.

Sorbitol affected plantlet development most intensively from PLBs *in vitro*. Among transplanted PLBs, 48% and 67% developed plantlets in cultivar P4 and P5 respectively (**Fig. 2**). The rates of plantlet production on sorbitol medium were significantly higher than those on sucrose and maltose media. Average plantlet weight on the sorbitol supplemented medium was also significantly higher (**Table 2**). A few additional PLBs were initiated at the basal portion of newly regenerated plantlets and their color was green.

No PLB showed necrosis on sorbitol and maltose medium, but some showed necrosis on sucrose. Although the reason why CLB proliferation took place from PLBs on sucrose containing medium is not clear, these results indicate that sorbitol is suitable for plantlet regeneration, maltose for PLB proliferation from PLBs and sucrose for callus like body proliferation *in vitro*.

3. 2 Subsequent plantlet growth

All sugars investigated supported plantlet growth.

Table 2. Growth and development of PLB on sucrose, maltose and sorbitol supplemented NP medium after 8 weeks culture.

Carbon source (0.056mol l^{-1})	Percent PLB developed plantlet per flask	Ave. weight of a plantlet (g)	Newly developed: CLB weight per flask (g)	Newly developed: PLB weight smaller than 2 mm per flask (g)	Newly developed: PLB weight larger than 2 mm per flask (g)	Newly developed: PLB number larger than 2mm	Ave. weight of a new PLB*1 (g)	Necrotic PLB per flask (%)	Color*2 of CLB or PLB
Phal. Wedding Promenade									
sucrose	90	0.03	1. 55	0. 16	0. 33	8. 2	0. 04	10	GY
maltose	100	0. 04	0. 22	0. 39	0. 65	21. 6	0. 03	0	G
sorbitol	100	0. 06	0. 05	0. 35	0. 38	12. 8	0. 03	0	G
LSD at 5%		0. 02	0. 16	0. 06	0. 11	5. 4	0. 01		
Phal. Hanaboushi × *Phal. equestris* 'Ilocos'									
sucrose	94	0. 04	1. 22	0. 35	0. 64	16. 0	0. 04	6	GY
maltose	100	0. 06	0. 16	0. 47	1. 59	53. 0	0. 03	0	G
sorbitol	100	0. 07	0. 03	0. 44	0. 27	9. 0	0. 03	0	G
LSD at 5%		0. 02	0. 22	0. 01	0. 36	5. 7	0. 01		

*1 Only PLBs larger than 2mm were counted.
*2 GY=Greenish yellow, G = Green.

On sucrose supplemented medium, about 80% of unrooted and 58% of rooted plantlets developed yellowish green CLB at the base of growing plantlets (**Fig. 1b, 3**). Roots and shoot growth and number of leaves per plantlet were less on sucrose containing medium in both cultivars. But new additional plantlet regeneration on sucrose medium was higher than that on maltose and sorbitol supplemented media in both cultivars (**Table 3**).

Maltose supported root growth better in unrooted plantlets than did sucrose and sorbitol. The number of roots per plant and the length of the roots were significantly higher than those on sucrose containing medium. About 44% of unrooted and 24% of rooted plantlets developed PLB at the base of the plantlets (**Fig. 1c, 3**). Moreover, 30% of plantlets in both varieties developed additional new shoots from the lower leaf axil on maltose supplemented medium.

On sorbitol supplemented medium root growth of rooted plantlets was greater than that on sucrose and maltose. Shoot length and number of leaves per plant were the highest on sorbitol containing medium (**Table 3**). Initiation of new shoots from the lower leaf axil and new PLB at the base of plantlets was significantly lower on sorbitol supplemented medium (**Table 3, Fig. 3**). All plantlets grew normally on sorbitol supplemented medium (**Fig. 1d**).

No significant difference in plantlet weight was observed between rooted and unrooted plantlets. These results indicate that PLB derived plantlets can utilize all carbohydrates investigated, and show the difference in growth and development for each. Sorbitol was suitable for plantlet growth *in vitro* as a carbon source in the medium.

Most nutrient media used in plant tissue culture incorporate sugar(s) as carbon source. In *in vitro* culture of orchid, sucrose is also a common carbon source. However, some reports indicated that elimination of sucrose from a culture medium was beneficial and sometimes essential for some orchid species [16-18]. Our earlier experiment indicated that a carbon source in a medium was indispensable for the growth and proliferation of callus of *Phalaenopsis*, *Doritaenopsis* and *Neofinetea*. Their growth and proliferation were highly dependent on sugar type [19]. It indicated that carbon source in the medium affected plant growth. The present experiment suggests that sugar in culture medium does not only provide nutritional support for the growth of plant tissues *in vitro*, but also affects subsequent plant development. Chia *et al.* [20] indicated that C/N ratio of cultured tissue determined the chlorophyll content of the tissues and the residual C/N ratio of the medium affected protocorm formation in an orchid hybrid *Aranda* Tay Swee Eng. They also reported that a lower concentration of sugar enhanced PLB proliferation and vice versa. In the

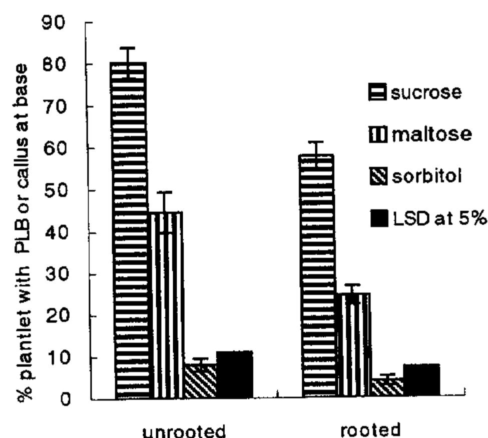

Fig. 3 Effect of carbon source in the medium on plantlet growth of *Phal.* Hanaboushi × *Phal. equestris* 'Ilocos' developed from PLBs. Bars indicate standard error.

Table 3. Effect of sucrose, maltose and sorbitol on plantlet growth of *Phal.* Hanaboushi × *Phal. equestris* 'Ilocos' in NP medium after 3 months culture.

Plantlets used	Carbon source (0.056 mol.)	Ave. values per plantlet*1					Percent of plantlets with new plantlets(%)
		weight (g)	number of root	length of root (cm)	length of shoot (cm)	number of leaf	
Unrooted	sucrose	0.19	0.71	1.06	1.53	3.30	33.0
	maltose	0.27	1.27	1.85	1.93	4.30	23.5
	sorbitol	0.24	1.04	1.10	2.04	4.30	15.5
	LSD at 5%	0.03	0.25	0.38	0.20	0.40	5.9
Rooted	sucrose	0.26	1.10	1.00	1.43	4.00	33.3
	maltose	0.28	1.38	1.50	1.91	4.10	35.3
	sorbitol	0.23	1.47	1.60	1.94	4.60	13.3
	LSD at 5%	0.04	0.30	0.45	0.24	0.35	6.0

*1 For each treatment replicated 5 times in flasks containing 15 plantlets each.

present experiment, sucrose affected color of PLBs and CLB differently from the other two carbohydrates, and induced CLB from cultured PLB and did not increase plantlet growth when compared with sorbitol and maltose. The differentiation of PLB and plantlet from callus or CLB might be due to sucrose/N ratio and the other unknown factor(s).

In this experiment, sorbitol supported plantlet growth the best whereas sucrose induced CLB at the base of plantlet. Effects of carbon sources on plant growth were the same as in PLBs growth. Few reports are available on callus lead plantlet production in *Phalaenopsis*. However direct PLB production, proliferation and plantlet production from different parts of plants in *Phalaenopsis* are available. In most of these experiments [1-8, 10, 15, 21], plant growth regulators (PGR) were used. Tokuhara and Mii [21] considered PGR in a culture medium as indispensable for the induction of PLB from flower stalk bud. It might increase the possibility of somaclonal variations [22]. However, the somaclonal variation rate differed largely from 0% to 100% among cultivars cultured in same condition throughout micropropagation [23]. It is also reported that plants developed from embryogenic callus produced normal flowers which were the same as the parent plant in *Phalaenopsis* [24]. Further investigations are required to confirm the risk of somaclonal variations of callus derived plantlets. In the present study, efficient production of PLBs from callus and plantlets from PLBs were achieved only by changing the carbon source in a medium.

Acknowledgments

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