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Effects of Complex Organic Extracts on Callus Growth and PLB Regeneration through Embryogenesis in the *Doritaenopsis* Orchid

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Abstract

Potato extract (PE), corn extract (CE) and papaya extract (PAE) at concentrations of 25, 50 and 100 mL L^{-1}enhanced callus growth on New Phalaenopsis (NP) medium containing sucrose. Among the various concentrations, 100 mL L^{-1} PE or PAE and 200 mL L^{-1} CE significantly promoted callus growth compared to the control. Regeneration of protocorm-like bodies (PLBs) from calli was optimum when NP medium (sometimes referred to as BM) was supplemented with 100 mL L^{-1} CE, followed by PE and PAE at the same concentration. Most plantlets also regenerated from PLBs with one 100 mL L^{-1} CE.

Discipline: Biotechnology
Additional key words: potato extract, corn extract, papaya extract

Introduction

Orchids are popular worldwide as pot plants or cut flowers. In orchid production, 22% of pot plants and 3% of cut flowers consist of *Phalaenopsis* and its intergeneric hybrid *Doritaenopsis*. Their colorful and elegant large flowers remain attached to a long flower stalk for a comparatively long time and attract viewers. Commercially, both *Doritaenopsis* and *Phalaenopsis* are attractive and highly priced flowers. Many countries around the world, including Thailand, Singapore, Korea, Malaysia and India produce orchids commercially and earn a large amount of foreign currency.

In vitro techniques for micropropagation of orchids have been widely used for commercial trade purposes in recent years in the developed countries[2–4,11]. Although there have been several reports on successful micropropagation of *Doritaenopsis* using different explant sources[1,4,12], clonal micropropagation has not yet become popular for this orchid, as it is difficult to apply these methods to large-scale production of plantlets, due to the low multiplication rates and occurrence of somaclonal variations. Very recently, a few reports on callus-derived plantlet regeneration have indicated the possibility of producing *Phalaenopsis* and *Doritaenopsis*[6–9] on a commercial scale. If calli remain stable, grow quickly without changes in their totipotency, produce protocorm-like bodies (PLBs) easily and consequently develop into plantlets, this method might become commercially successful for the micropropagation of *Doritaenopsis*. Callus-derived plant production depends on the components of culture media; for instance, sucrose is suitable for callus proliferation, while maltose and sorbitol are suitable for PLB proliferation[8]. Medium quality such as balanced nutrient availability, as well as low cost is required for developing sustainable protocols. Complex organic extract supplements in basal media (BM) containing inorganic nutrients have been used by Ichihashi and Islam[5] in *in vitro* plant regeneration. They indicated that the addition of taro extract, potato extract, coconut water, and apple extract to BM enhanced callus growth in *Phalaenopsis, Doritaenopsis* and *Neofinetia* orchids depending on the properties of each compound. There are few reports on the effects of organic extracts on callus growth and plant regeneration and their nutritional value in *Doritaenopsis*. Moreover, local adjustments of complex organic extracts supplemented to BM are necessary

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to overcome variations in these extracts from the nutritional point of view and to develop a process of micropropagation in *Doritaenopsis*. The present study was conducted to evaluate callus growth and regeneration of PLBs from calli following supplementation to BM of some organic extracts that can be easily obtained in tropical Asia. The objectives of the present study were to enhance the growth of callus-derived plantlets of *Doritaenopsis* and to emphasize the use of organic additives in this process.

Materials and methods

1. Preparation of callus

Embryogenic calli of *Doritaenopsis* previously developed from a flower stalk by Islam and Ichihashi (1999)[5,6] were maintained by monthly subcultures on New Phalaenopsis medium (NP)[4] supplemented with 20 g L^{-1} sucrose. At the beginning, 1.0 g callus was subcultured twice on 20 mL NP supplemented with 20 g L^{-1} sucrose in 50 mL flasks at monthly intervals to obtain homogeneous calli. Uniform, friable, translucent and yellowish calli were used as plant materials.

2. Preparation of organic extracts

Potato (cv. Lal pakhri), papaya (cv. Shahi) and corn (cv. Barnaly) were collected from a local market. Potato and papaya were peeled and cut into pieces about 1 cm^3 in size. Corn was ground. Five hundred grams of each freshly diced or ground material were boiled separately for 20 min (potato and papaya) and 30 min (corn) in 500 mL of distilled water and the hot supernatant was filtered through a steel kitchen mesh (55 m/m) and adjusted to 500 mL by the addition of distilled water. These extracts were stored in a plastic bottle at –4ºC and added to the medium as required after thawing.

3. Media and callus culture

The first and second cultures were referred to as 8-week culture and second 8-week culture, respectively in the study and in the results and discussion sections.

Two cultures were performed in the present investigation. In the first culture, 0.2 g of yellow, translucent, friable and homogeneous calli were transplanted on 20 mL NP medium (BM) supplemented with 25, 50, 100 and 200 mL L^{-1} each of potato (PE), corn (CE) and papaya (PAE) extracts as treatment as well as in the absence of supplementation (control) and cultured for 8 weeks (referred to as 8-week culture). After 8 weeks, 0.2 g of proliferated calli obtained from the first culture were subcultured on fresh BM with the same supplementation as that described above for another 8 weeks (second 8-week culture). For both cultures, 20 g L^{-1} sucrose (Merck Co., Germany) and 3 g L^{-1} gelrite (Merck Co., Germany), a solidifier, were added and the pH was adjusted to 5.6 prior to autoclaving. The control and treated flasks were autoclaved at 121ºC at 1.16 kg cm^{-2} pressure for 20 min. Cultures were maintained in a growth chamber and allowed to grow at 25 ± 1ºC under a 16-hour photoperiod and photosynthetic photon flux density of 50 μmol m^{-2} s^{-1} provided by fluorescent tubes to evaluate their effects on callus growth and PLB production.

4. Culture evaluation

In both 1st and 2nd cultures, fresh and dry weight of calli, and number and weight of PLBs initiated from calli were recorded for each flask. Fresh weight of callus/ fresh weight of PLB (callus/ PLB) ratio was calculated by dividing the weight of the callus by the weight of the PLB.

Results and discussion

1. Effects of potato extract

For the 8-week culture used to evaluate callus proliferation, calli on PE-containing medium remained friable and were yellow to pale green. PE at 25, 50 and 100 mL L^{-1} in BM (Fig. 1. P1–3) enhanced callus growth compared to the control lacking complex organic additives (Fig. 1. Control) but a higher concentration, 200 mL L^{-1} was found to be inhibitory (Fig. 1. P4). The highest dry weight of the calli was obtained with 100 mL L^{-1} PE, which was significantly higher than that at other concentrations (Fig. 2). Callus proliferation rate was also significantly higher (0.4798 g $d^{-1}g^{-1}$) at 100 mL L^{-1} PE (data not shown). The highest multiplication rate of the calli grown at 100 mL L^{-1} concentration of PE was 26.86 fold (5.371/0.2) for 8 weeks which is equivalent to 20.78 × 10^8 fold per year. This rate of callus proliferation was sufficient for rapid, and abundant year-round production of orchids commercially.

In this experiment, a few PLBs developed from the calli. The highest (5.0 / flask) and the lowest numbers of PLBs (1.4 / flask) were produced at 100 and 200 mL L^{-1} PE, respectively and no PLBs were formed in the control (Fig. 3). Fresh weight of PLBs (0.046 g / flask) and fresh weight per PLB (0.009 g) at 100 mL L^{-1} PE were significantly higher than those at other concentrations (Table 1).

In the second 8-week culture, the growth trend of the calli on PE-containing medium was almost similar to that in the 8-week culture, and the highest number of PLBs (5.8 / flask) was also obtained at 100 mL L^{-1} PE. The highest and the lowest callus/PLB ratios (122.68 and 85.6) were recorded at 50 and 25 mL L^{-1} PE, respectively

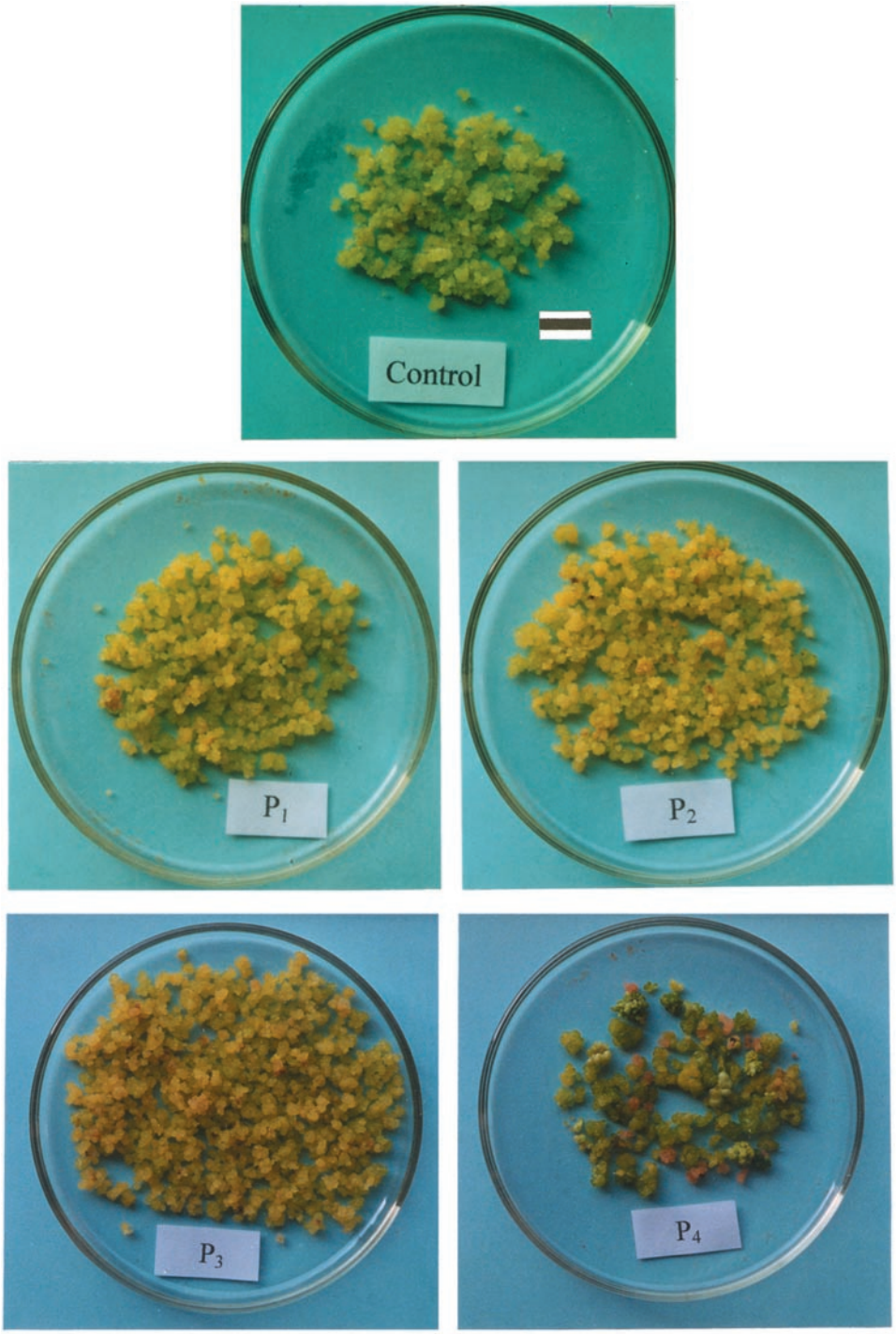

Fig. 1. Proliferation of calli of *Doritaenopsis* on NP medium supplemented with PE
Control, P_1, P_2, P_3 and P_4 show callus growth on medium supplemented with 0, 25, 50, 100 and 200 mL PE, respectively to 1 L NP medium. Scale = 1.0 cm.

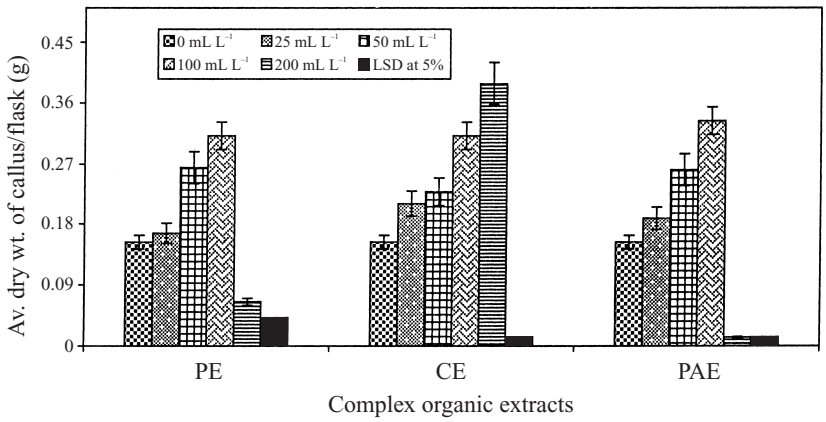

Fig. 2. Effect of supplementation of PE, CE and PAE at different concentrations to NP medium on proliferation of *Doritaenopsis* calli after 8 weeks of culture
Bars indicate SD.

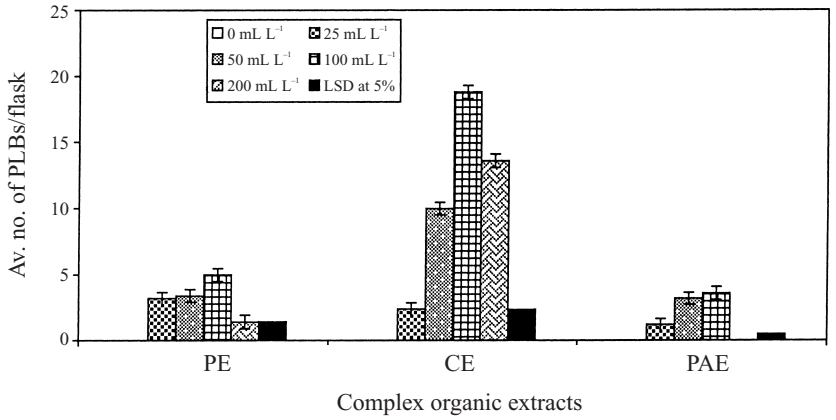

Fig. 3. Effect of supplementation of PE, CE and PAE at different concentrations to NP medium on PLB formation from *Doritaenopsis* calli after 8 weeks of culture
Bars indicate SD.

Table 1. Effects of supplementation of potato extract to NP medium on callus growth and PLB (>2 mm) regeneration from *Doritaenopsis* calli in 8-week and second 8-week cultures

*Organic extract supplemented (mL L^{-1})	Weight of callus explanted (g)	8-week culture (average value)					Second 8-week culture (average value)				
		Fresh weight of callus (g)	No. of PLBs	Fresh weight of PLB (g)	Fresh weight per PLB (g)	Callus/ PLB[a)] ratio	Fresh weight of callus (g)	No. of PLBs	Fresh weight of PLB (g)	Fresh weight per PLB (g)	Callus/ PLB[a)] ratio
PE 0	0.2	2.500	0.0	0.0	0.0	∞	2.611	0.0	0.0	0.0	∞
PE 25	0.2	2.661	3.2	0.0244	0.0077	109.04	2.705	4.0	0.0316	0.0078	85.60
PE 50	0.2	4.162	3.4	0.0301	0.0087	138.26	4.294	4.0	0.0350	0.0088	122.68
PE 100	0.2	5.325	5.0	0.0460	0.0090	115.76	5.542	5.8	0.0580	0.0098	95.55
PE 200	0.2	1.084	1.4	0.0090	0.0064	120.44	1.475	2.0	0.0139	0.0071	106.12
LSD at 5%		0.7035	1.4210	0.0132	NS	105.10	0.8124	2.223	0.0132	0.0042	66.87

*Each treatment had 6 replicates. PE = Potato extract. NS = Not significant.
a: Fresh weight of callus / fresh weight of PLB.

Table 2. Effects of supplementation of corn extract to NP medium on callus growth and PLB (>2 mm) regeneration from *Doritaenopsis* calli in 8-week and second 8-week cultures

*Organic extract supplemented (mL L^{-1})	Weight of callus explanted (g)	8-week culture (average value)					Second 8-week culture (average value)				
		Fresh weight of callus (g)	No. of PLBs	Fresh weight of PLB (g)	Fresh weight per PLB (g)	Callus/ PLB[a)] ratio	Fresh weight of callus (g)	No. of PLBs	Fresh weight of PLB (g)	Fresh weight per PLB (g)	Callus/ PLB[a)] ratio
CE 0	0.2	2.500	0.0	0.0	0.0	∞	2.611	0.0	0.0	0.0	∞
CE 25	0.2	2.957	2.4	0.0263	0.0107	112.42	3.064	3.6	0.0413	0.0115	74.18
CE 50	0.2	4.004	10.0	0.1050	0.0105	38.13	4.107	16.6	0.1785	0.0108	23.00
CE 100	0.2	5.300	18.8	0.2210	0.0118	23.98	5.508	29.0	0.3580	0.0116	18.32
CE 200	0.2	6.738	13.6	0.1464	0.0109	46.02	6.558	22.0	0.2480	0.0114	22.21
LSD at 5%		0.3107	8.2240	0.0933	0.0042	70.62	1.0370	12.95	0.1866	0.0042	20.91

*Each treatment had 6 replicates. CE = Corn extract.
a: Fresh weight of callus / fresh weight of PLB.

(Table 1).

Potato tubers contain carbohydrates, protein, fat, several vitamins, phenolic compounds and low levels of some amino acids and fatty acids. Any of these or other substances, yet unknown, single or in combination might be factor(s) that enhance(s) callus growth compared to the control. High concentration of PE (200 mL L^{-1}) inhibited callus growth, which may be due to the low C/N ratio (58.7/1) and possible excessive presence of phenolic compounds, sterols and organic acids. The factors particularly responsible for callus growth have not been elucidated and further studies should be carried out. Results on growth promotion of calli by PE in the present study were in agreement with the results obtained in our previous report[5] in which PE extracted from Danshaku potatoes yielded the optimum callus growth at 50 mL L^{-1} and was inhibitory at higher concentrations. Also, PE promoted adequate growth of *Phalaenopsis* and *Neofinetia* compared with banana extract and coconut water, but its effect was less conspicuous than or similar to that of taro extract.

2. Effects of corn extract

After 8 weeks of culture, the texture of the calli on CE-supplemented medium ranged from friable to compact and the calli were yellow to pale green. With a gradual increase of CE in the medium, the growth of the calli was enhanced. Optimum growth was found on BM with 200 mL L^{-1} CE (Fig. 2). The callus proliferation rate at this concentration was significantly higher (0.614 g $d^{-1}g^{-1}$) than that of the control and other treatments. The highest multiplication rate of the calli after 8 weeks with 200 mL L^{-1} CE was 34.42 fold and 10.47×10^9 fold per year.

As for PLB formation in the first culture experiment, the number of PLBs initiated, total fresh weight per flask and fresh weight per PLB were relatively lower than those obtained in the second 8-week culture. However, the values were higher than those for the PE supplementation. The highest and lowest numbers of PLBs initiated were recorded with 100 and 25 mL L^{-1} CE, respectively and PLBs were not produced in the control in the first culture (Fig. 3). Fresh weight of PLBs and fresh weight per PLB were also higher with 100 mL L^{-1} CE (Table 2).

In the second 8-week culture, the trend of growth and development of the calli on CE-containing medium was almost similar to that in the first 8-week culture (Table 2). The highest and lowest numbers of PLBs (29 and 3.6/flask) were obtained with 100 and 25 mL L^{-1} CE, respectively. The fresh weight of a PLB that developed on the medium with 100 mL L^{-1} CE was significantly higher than that under other conditions. The highest and the lowest callus/ PLB ratios (74.18 and 18.32) were observed at concentrations of 25 and 100 mL L^{-1} CE respectively (Table 2).

Corn grains contain carbohydrates, protein, fat, fiber, iron, a few vitamins, organic acids and a small amount of some amino acids. Any of these or other substances, yet unknown, single or in combination might be factor(s) that enhance(s) PLB formation.

3. Effects of papaya extract

In the first culture experiment, calli on PAE-containing medium remained friable after 8 weeks. Color of proliferated calli was yellow to brown. PAE significantly enhanced callus growth over that of the control when BM was supplemented at concentrations of 25, 50 and 100 mL L^{-1} but a higher level (200 mL L^{-1}) was inhibitory. The highest dry weight of the calli was obtained with 100 mL L^{-1} (Fig. 2). Callus proliferation rate was also significantly higher (0.5712 g $d^{-1}g^{-1}$) with 100 mL L^{-1} extract

Table 3. Effects of supplementation of papaya extract to NP medium on callus growth and PLB (>2 mm) regeneration from *Doritaenopsis* calli in 8-week and second 8-week cultures

*Organic extract supplemented (mL L^{-1})	Weight of callus explanted (g)	8-week culture (average value)					Second 8-week culture (average value)				
		Fresh weight of callus (g)	No. of PLBs	Fresh weight of PLB (g)	Fresh weight per PLB (g)	Callus/ PLB[a)] ratio	Fresh weight of callus (g)	No. of PLBs	Fresh weight of PLB (g)	Fresh weight per PLB (g)	Callus/ PLB[a)] ratio
PAE 0	0.2	2.500	0.0	0.0	0.0	∞	2.611	0.0	0.0	0.0	∞
PAE 25	0.2	3.651	1.2	0.0071	0.0060	514.21	3.980	2.2	0.0139	0.0064	286.33
PAE 50	0.2	5.078	3.2	0.0246	0.0061	253.90	5.376	4.0	0.0290	0.0069	185.38
PAE 100	0.2	6.366	3.6	0.0292	0.0081	218.00	6.812	5.2	0.0440	0.0085	154.82
PAE 200	0.2	0.2450	0.0	0.0	0.0	∞	0.2630	0.0	0.0	0.0	∞
LSD at 5%		0.3154	0.4936	0.0132	0.0042	100.30	0.8027	2.729	0.0132	0.0042	132.20

*Each treatment had 6 replicates. PAE = Papaya extract.
a: Fresh weight of callus / fresh weight of PLB.

compared to the other treatments. The multiplication rate of the calli with 100 mL L^{-1} PAE was 64.83×10^8 fold per year. A similar growth trend of calli was observed in the second 8-week culture (Table 3). The present findings indicate that 100 mL L^{-1} PAE was optimum for callus proliferation, possibly due to optimum levels of organic acids, vitamins and other growth substances of which one or all in combination might be responsible for enhanced growth of the calli. On the other hand, high concentrations of PAE may contain high amounts of phenolic compounds, organic acids, sterols, etc. that inhibit callus growth.

In the first culture, the highest and the lowest numbers of PLBs (3.6 and 1.2 /flask) were recorded at concentrations of 100 and 25 mL L^{-1} PAE respectively, and no PLBs were produced at 200 mL L^{-1} (Fig. 3). The highest total fresh weight of PLBs, fresh weight per PLB and the lowest callus growth and callus/PLB ratio were found at 100 mL L^{-1}PAE. Similar results were obtained in the second 8-week culture but the values were slightly higher than those for the first culture (Table 3).

Papaya contains carbohydrates, protein, fat, calcium, iron, nicotinic acid, riboflavin and several vitamins. Any of these or other substances, yet unknown, single or in combination might be factor(s) that enhance(s) PLB formation.

Conclusion

The above findings indicate that supplementation of 100 mL L^{-1} PE and PAE and of 200 mL L^{-1} CE markedly enhanced callus proliferation, but 100 mL L^{-1} of each extract was suitable for PLB regeneration from calli, which may be attributed to carbon source availability and absorption of inorganic nutrients. Studies on *Doritaenopsis* calli by Islam and Ichihashi revealed that sucrose, a sugar that can be easily utilized, is suitable for callus growth, whereas PLB proliferation is promoted by sugars that cannot be utilized easily, such as maltose and sorbitol[8]. Also, these supplementations might enhance inorganic nutrient levels in calli. Our preliminary analyses revealed that in the calli showing the optimum growth, absorption was improved, that is, C/N ratio and contents of phosphorus, potassium and sulfur in these calli were higher (77–79, 0.082–0.097%, 1.99–2.32% and 0.38–0.44%, respectively) than those in the calli grown on the control medium (75, 0.087%, 1.77% and 0.32%, respectively) except for the contents of phosphorus in the PE supplementation (0.082%) and potassium and sulfur in the PAE one (1.56 and 0.30%, respectively).

Among the 3 organic extracts tested, CE was highly suitable for both callus proliferation and PLB formation from calli, followed by PE and PAE. Thus, the addition of organic extracts to culture media is a simple, beneficial and convenient measure to improve the culture media used for commercial production (Ichihashi and Islam, 1999)[5]. The present results indicated that organic extracts enhanced the growth of explants selectively. However, proliferation of PLBs by supplementation of these extracts was less satisfactory than that by supplementation of maltose and sorbitol[8], presumably due to the addition of sucrose to BM. Therefore, it is necessary to study the effect of the removal of sucrose from BM on PLB proliferation, although the incorporation of organic extracts was not always effective and their effects were not always constant. Effects of organic extracts depend on plant sources, cultivars (varieties) and formulation of the materials used and also, BM composition.

Simplification of culture media may enable commercial orchid growers to reduce the labor cost. Use of

organic extracts also reduces the material cost required for BM. Preparation of CE used in the present experiment is simple. Corn is available in *Doritaenopsis*-growing regions of the world. Thus, the present information regarding the use of CE may contribute to the development of simple and economical media. However, local adjustments related to the effects of CE might be necessary for different regions.

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