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Suitable Conditions for the Induction and Micropropagation of PLBs in Some Monopodial Orchids

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Abstract

Liquid media suitable for the induction and micropropagation of protocorm-like bodies (PLBs) in *Vandofinetia* Nara 'Yumika Pink' were developed by modifying MS basal medium. The best medium obtained for PLBs induction was one-fourth strength MS medium containing 30 g/*l* sucrose and for PLBs micropropagation was one-fourth strength MS medium containing 10 g/*l* sucrose in which FeEDTA was replaced by 6.95 mg/*l* $FeSO_4 7H_2O$. Usefulness of the latter medium was confirmed in other monopodial orchids such as *Renanetia, Darwinara, Ascocentrum* and *Ascofinetia.* After transfer onto 2 g/*l* Hyponex (N:P:K=10:30:20) medium containing 2 g/*l* proteose peptone and 20 g/*l* sucrose for 2 months, the PLBs (both those regenerating and those not regenerating plantlets) were subcultured into MS medium containing 30 g/*l* sucrose at 2 months intervals for 4 months. More than 10,000 plantlets ready for transplanting to pots were obtained efficiently from one floral bud within a year for each orchid cultivar. These results suggest that the methodology could be used on a commercial scale for micropropagation of a wide range of monopodial orchids.

1. Introduction

Monopodial orchids such as *Vanda, Ascocentrum, Rhynchostylis, Aranda, Renanthera* and *Neofinetia,* and intergeneric hybrids of these genera are now widely cultivated in the world. Selected cultivars of these monopodial orchids are expected to be micropropagated by tissue culture because of the low propagation rate by conventional methods.

Although efficient methods of micropropagation have been reported in some monopodial orchids such as *Ascofinetia, Neostylis* and *Vascostylis* [1], *Renantanda* [2], *Vanda* [3], *Aranda* [4], *Mokara* [5], *Renanthera* [6] and *Phalaenopsis* [7], these methods are only applicable to some limited genotypes and the rate of micropropagation by these methods is unclear for commercial scale of micropropagation of most monopodial orchid species and genotypes.

In this study, we describe the optimum medium for the induction of PLBs from floral buds of some recently developed cultivars of monopodial orchids such as *Vandofinetia, Renanetia, Darwinara, Ascocentrum* and *Ascofinetia.*

2. Materials and Methods

2. 1 Plant materials and culture method

Floral buds of *Vandofinetia* Nara 'Yumika Pink' were used for inducing PLBs. Main shoots (10 cm long) having immature floral buds were harvested from plants and the floral buds (3~6 mm long) were excised using a razor blade. After initial washing with 10% benzalkonium chloride solution for 10 minutes, the explants were surface-sterilized with a solution of 70% ethanol for 30 sec. and 0.5% sodium hypochlorite solution containing 0.5 g/*l* Tween 20 for 5 min., successively. Then, the explants were rinsed 3 times with autoclaved (120°C, 1 atm for 15 min.) distilled water. Floral buds were cut into 0.5 mm cubes using forceps under a dissecting microscope and planted in a test tube (W 3 cm×L 20 cm) containing 20 m*l* of the medium described below. The cultures were kept on a drum type rotary shaker (Nihon Ika Kikai Co., Ltd., Tokyo, Japan) at 1 rpm. The slope of the drum disk was set at 15° toward the vertical line. The cultures were maintained at 25±2°C under a photoperiod of 16 hr with fluorescent illumination at 3000±500 lx throughout the culture period. Twenty floral buds were used for each treatment. The survival rate (%)

was evaluated as the number of floral buds that produced green PLBs after 2 months of culture/number of floral buds cultured. Also, fresh weight of PLBs via each floral bud was measured for each study. Floral buds of other 4 monopodial orchids, *Renanetia* Sun Rise, *Darwinara* Pretty Girl, *Ascocentrum ampullaceum* and *Ascofinetia* Cherry Blossom were also used for inducing PLBs by the same method as used for *Vandofinetia* Nara 'Yumika Pink'.

2. 2 *Culture media for inducing PLBs from floral buds*

Three types of media, MS [8], VW [9] and KC [10] were used as basal media, and each contained 30 g/*l* sucrose. For MS medium, all the compositions of MS were reduced to full, 1/2, 1/4 and 1/8-strengths except for sucrose (30 g/*l*). As a chelate iron, commercially available FeEDTA (Wako Pure Chemical Inc., Osaka, Japan) (10.525 mg/*l*) was used instead of $FeSO_4 7H_2O + Na_2EDTA$. All the media tested were adjusted to pH 5.8 before autoclaving (120°C, 1 atm for 15 min.).

2. 3 *Culture media for PLBs micropropagation*

After induction of PLBs of 'Yumika Pink' from floral buds, the PLBs were maintained in 1/4 MS liquid medium by subculturing at 2 weeks intervals. After 4 months of subculture, PLBs were used to study the effects of the macro- and micro-components of 1/4 MS medium, FeEDTA and sucrose concentration on micropropagation.

A 2 g aliquot of PLBs of 'Yumika Pink' was inoculated in a culture tube containing 20 m*l* of medium. The culture vessel and culture conditions used were the same as those described for the floral bud culture. For each treatment, 20 tubes were used throughout the studies.

2. 4 *PLBs micropropagation of other 4 monopodial orchids*

The medium which showed the best result on PLBs micropropagation of 'Yumika Pink' (ACE medium) was applied to other 4 monopodial orchids such as *Renanetia* Sun Rise, *Darwinara* Pretty Girl, *Ascocentrum ampullaceum* and *Ascofinetia* Cherry Blossom. The composition of ACE medium was 1/4 MS medium containing 10 g/*l* sucrose in which FeEDTA was replaced by 6.95 mg/*l* $FeSO_4 7H_2O$ (pH 5.8). The PLBs growth in ACE medium was compared to the medium which consisted of 1/4 MS medium containing 10 g/*l* sucrose (pH 5.8) (Control). Seven grams of PLBs were cultured in 80 m*l* of medium in 300 m*l* flask on rotary shaker (TB-300L, Takasaki Kagaku Kikai Co., Ltd., Kawaguchi, Japan). The rotation speed was 90 rpm with a 70 mm stroke. Twenty flasks were made for this study.

2. 5 *Regeneration of plantlets from PLBs*

Micropropagated PLBs were transplanted into the medium containing 2 g/*l* Hyponex (N: P: K=10: 30: 20), 2 g/*l* proteose peptone, 20 g/*l* sucrose and 8 g/*l* agar and cultured for 2 months. Then, the PLBs both regenerating and non-regenerating plantlets were subcultured into MS medium containing 30 g/*l* sucrose and 2 g/*l* gelrite at 2 months intervals for 4 months to promote PLBs growth and regeneration. The pH of the media was adjusted to 5.4 before autoclaving (120°C, 1 atm for 15 min.). A plant box (Shibata Hario Glass Co., Ltd., Tokyo, Japan; L 7 cm×W 7 cm×H 8 cm) containing 50 m*l* of the medium was used as a container. The planting densities were *ca.* 1 g PLBs/plant box for regeneration of plantlets and *ca.* 50 plantlets/plant box for plantlet growth. The cultures were kept at 25±2°C under a photoperiod of 16 hr with fluorescent illumination at 4000±1000 lx.

2. 6 *Data treatment for PLBs micropropagation*

The degree of PLBs micropropagation was evaluated as the relative growth rate (final fresh weight/initial fresh weight) after one month. Statistical analysis was made by Duncan's multiple range test or T-test at $P=0.05$ for each data.

3. Results

3. 1 *Effect of basal medium on the survival rate of floral buds and subsequent growth of PLBs*

Among the basal media tested, MS gave the highest survival rate (**Table 1**). In this medium, most of the floral buds developed into green compact PLBs and proliferated well. In KC medium, half of the floral buds grew well and developed into PLBs, but the other half turned pale green. In VW medium, most of the floral buds turned brown within 10 days after planting and subsequently died. Fresh weights of PLBs were quite light in every cases. Among the MS concentrations tested, 1/4 MS showed the best induction of green PLBs with high proliferating potential (**Table 1, Fig. 1**). In 1/2 MS medium, most of PLBs turned pale green. In 1/8 MS medium, some small PLBs were obtained, but they showed the least growth. Fresh weight of PLBs was strongly effected by the strength of MS rather than by the basal medium component. In particular, fresh weight of PLBs obtained in 1/4 MS medium had fresh weight 6 times higher than those in MS medium. Therefore, the optimum basal medium for both induction and micropropagation of PLBs was determined to be 1/4 MS. This basal medium was used for the further experiments.

Table 1.
Survival rate of floral buds and subsequent PLBs growth of *Vandofinetia* Nara 'Yumika Pink' cultured in various media.

Exp. No.	Basal medium*	Survival rate (%)	Fresh weight (g)
1	MS	75	0.3a
	KC	45	0.3a
	VW	15	0.2a
2	MS	70	0.3a
	1/2MS	85	1.2b
	1/4MS	100	2.0c
	1/8MS	55	0.3a

Values within the column followed by different letters are significantly different at $P=0.05$ by Duncan's multiple range test.
* MS; Murashige and Skoog (1962), KC; Knudson (1946), VW; Vacin and Went (1946). 1/2, 1/4 and 1/8 MS show that the macro- and micro-components of MS were reduced to 1/2, 1/4 and 1/8, respectively.
$n=20$

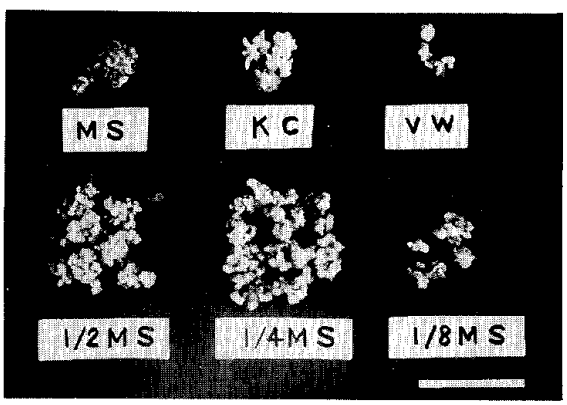

Fig. 1 PLBs obtained from floral buds by culturing on various types of basal media in *Vandofinetia* Nara 'Yumika Pink'., The bar=1cm.
Media components used were written in **Table 1.**

3. 2 *Effect of media components on PLBs micropropagation*

Removal of individual inorganic compounds, except for FeEDTA, from the basal medium (1/4 MS medium) resulted in little or no reduced growth of PLBs (**Table 2**). Removal of organic compounds such as *myo*-inositol, nicotinic acid, pyridoxine hydrochloride, thiamine hydrochloride and glycine had no appreciable effect on PLBs micropropagation. However, removal of FeEDTA significantly increased the rate of PLBs proliferation.

The promotive effect of removal of FeEDTA from the medium for PLBs micropropagation was cancelled by the addition of Na_2EDTA (**Table 3**). Replacement of FeEDTA by $FeSO_4 7H_2O$ resulted in the highest growth rate of PLBs.

3. 3 *Effect of sucrose concentration on PLBs micropropagation*

Among the sucrose concentrations tested, 1/4 MS-D+Fe containing 10 g/*l* gave the highest PLBs micropropagation rate which was more than 3 times higher than that in control medium (**Table 4**). PLBs growth rate gradually decreased as the concentration of sucrose increased. Without sucrose, PLBs turned light green or brown and showed little growth. According to these results, optimum medium for the PLBs micropropagation was determined to be 1/4 MS medium containing 10 g/*l* sucrose in which FeEDTA was replaced by $FeSO_4 7H_2O$. This medium was designated as ACE medium.

Table 2.
Effect of the removal of each macro- and micro-element of 1/4 MS medium on PLBs micropropagation of *Vandofinetia* Nara 'Yumika Pink'.

Basal medium component	Relative growth rate
1/4 MS	2.3b
1/4 MS minus Aa	1.1a
1/4 MS minus Ab	1.1a
1/4 MS minus B	1.4ab
1/4 MS minus C	1.7ab
1/4 MS minus D	4.7c
1/4 MS minus E	2.3b
1/4 MS minus F	2.0ab

Values within the column followed by different letters are significantly different at $P=0.05$ by Duncan's multiple range test.
All the concentrations of MS listed below were reduced to 1/4-strength from the original MS prescription.
Aa; $MgSO_4 7H_2O$, Ab; KH_2PO_4, B; NH_4NO_3, KNO_3, $CaCl_2 2H_2O$, C; $MnSO_4 5H_2O$, $ZnSO_4 7H_2O$, $CuSO_4 5H_2O$, H_3BO_3, $Na_2MoO_4 2H_2O$, $CoCl_2 6H_2O$, KI, D; FeEDTA, E; *myo*-inositol, F; nicotinic acid, pyridoxine hydrochloride, thiamine hydrochloride, glycine
$n=20$

Table 3.
Effect of FeEDTA (D), Na_2EDTA (NE) and $FeSO_47H_2O$ (Fe) on PLBs micropropagation of *Vandofinetia* Nara 'Yumika Pink'.

Basal medium component	Relative growth rate
1/4 MS	2.2a
1/4 MS minus D	4.6b
1/4 MS minus D plus NE	2.3a
1/4 MS minus D plus Fe	5.4b

Values within the column followed by different letters are significantly different at $P=0.05$ by Duncan's multiple range test.
$n=20$

Table 4.
Effect of sucrose concentration on PLBs micropropagation of *Vandofinetia* Nara 'Yumika Pink'.

Concentration of sucrose (g/*l*)	Relative growth rate
0	1.6a
10	8.6b
20	3.8a
30 (Control)	2.4a

1/4 MS containing 6.95 mg/*l* $FeSO_47H_2O$ (pH 5.8) was used for each treatment. Values within the column followed by different letters are significantly different at $P=0.05$ by Duncan's multiple range test.
$n=20$

Table 5.
Effect of modified 1/4 MS medium developed for 'Yumika Pink' (denoted ACE) on PLBs micropropagation of other 4 monopodial orchids.

Cultivar	Medium	Relative growth rate
Renanetia Sun Rise	Control[a]	14.3
	ACE[b]	23.7*
Darwinara Pretty Girl	Control	15.1
	ACE	18.1
Ascocentrum ampullaceum	Control	11.6
	ACE	14.5
Ascofinetia Cherry Blossom	Control	17.0
	ACE	21.6*

[a] Control; 1/4 MS containing 10 g/*l* sucrose.
[b] ACE; 1/4 MS containing 10 g/*l* sucrose in which FeEDTA was replaced by 6.95 mg/*l* $FeSO_47H_2O$.
* Significantly different within each cultivar by T-test ($P=0.05$).
$n=20$

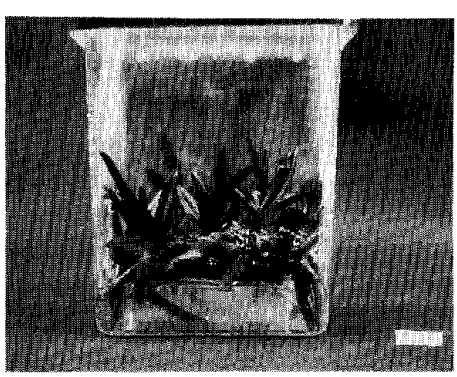

Fig. 2 Micropropagated plantlets ready for transplanting to pots in *Vandofinetia* Nara 'Yumika Pink'., The bar=1 cm.

3. 4 Effect of ACE medium on PLBs micropropagation of other 4 monopodial orchids

The ACE medium developed for 'Yumika Pink' was also tested on other 4 monopodial orchids (**Table 5**). The results showed that ACE medium was better than the original 1/4 MS medium (Control), especially, for two genotypes (*Renanetia* Sun Rise, *Ascofinetia* Cherry Blossom).

3. 5 Plantlet regeneration

More than 10,000 plantlets ready for transplanting to pots were obtained efficiently from one floral bud within a year after the initiation of floral bud culture for each orchid cultivar. Micropropagated plantlets of *Vandofinetia* Nara 'Yumika Pink' ready for transplanting to pots are shown in **Fig. 2**.

4. Discussion

Our results showed that PLBs of monopodial orchids such as *Vandofinetia* Nara 'Yumika Pink', *Renanetia* Sun Rise, *Darwinara* Pretty Girl, *Ascocentrum ampullaceum* and *Ascofinetia* Cherry Blossom could be efficiently micropropagated using a modified MS medium (ACE). The medium was expected to be widely used for commercial micropropagation of monopodial orchids.

Reduction of the strength of MS medium has been shown to be useful in many other tissue cultures [11]. Usefulness of 1/4 MS medium for the monopodial orchids suggests that full-strength MS medium con-

tains excessively high concentrations of some compounds that inhibit PLBs induction and micropropagation. Further investigations will be needed to clarify the compounds that inhibit PLBs induction and micropropagation.

In this study, EDTA was revealed to inhibit PLBs micropropagation. It has been reported that free type of EDTA in liquid medium inhibits pyruvate dehy drogenase action in TCA cycle [12] and S1/mung-bean-type nuclease activity of plant cells [13]. Therefore, it is possible that inhibitory effect of EDTA on PLBs micropropagation is attributed to their inhibitory effect on these enzyme systems. On the other hand, removal of FeEDTA arrested plantlet formation of *Nicotiana tabacum* L. severely [14] and rooting of *Arabidopsis thaliana* econotype Columbia [15]. Thus, it is supposed that FeEDTA has promotive effect at regeneration stage but FeEDTA has negative effect at micropropagation stage.

A sucrose concentration of 20 g/*l* has been used commonly in the tissue culture of a wide range of monopodial orchids such as *Vascostylis* Blue Fairy, *Neostylis* Lou Sneary and *Ascofinetia* Cherry Blossom [1], *Renantanda* Ammani [2], *Aranda* 'Deborah' [4] and *Renanthera imshootiana* Rolfe [6]. However, our results showed that optimum concentration of sucrose for the PLBs micropropagation was 10 g/*l* rather than 20 g/*l* (**Table 4**). It is supposed that relatively long-term (for 4 months subculture) culture using the medium containing 30 g/*l* sucrose prior to use resulted in the lower requirement of sucrose for the subsequent test of sucrose concentration (0, 10, 20, 30 g/*l*).

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