

# Improvement of asymbiotic seed germination and seedling development of *Cypripedium macranthos* Sw. with organic additives

Yoon Sun Huh · Jung Kwan Lee · Sang Young Nam · Kee Yoeup Paek · Gang Uk Suh

Received: 11 February 2016 / Revised: 10 March 2016 / Accepted: 15 March 2016

© Korean Society for Plant Biotechnology

**Abstract** To find the optimal propagation condition for endangered *Cypripedium macranthos* Sw., also known as lady's slipper orchid, the effect of various organic additives on *in vitro* germination, protocorm formation and seedling growth was investigated during asymbiotic seed culture. When 100 ml·L<sup>-1</sup> coconut water was added to the basal medium, the highest germination rate and protocorm formation rate were achieved, with 70.8% and 74.2% respectively. Supplementation of phloem sap from birch tree or maple tree also showed a facilitating effect to improve the germination and protocorm development. With 100 ml·L<sup>-1</sup> birch sap or maple sap, both the germination and protocorm formation rates were roughly more than 65% and 68%. The roots and buds of the seedlings grew vigorously in the medium containing 100 ml·L<sup>-1</sup> coconut water or phloem sap, in particular, their bud formation rates increased by more than 70%. Addition of banana powder and peptone could not create a more significantly favorable culture condition, and non-addition had the worst results. Our results demonstrated that proper organic amendments such as coconut water and phloem sap might be preferred to *in vitro* germination and the growth of seedlings developed from the protocorm of *C. macranthos* Sw. during asymbiotic seed culture.

**Keywords** Lady's slipper orchid, Asymbiotic seed culture, Organic amendments, Germination, Seedling growth

Y. S. Huh (✉) · J. K. Lee · S. Y. Nam  
Horticultural Research Division, Chungcheongbuk-do  
Agricultural Research and Extension Services, Cheongju, 28130,  
Korea  
e-mail: [yshuh2@korea.kr](mailto:yshuh2@korea.kr)

K. Y. Paek  
Department of Horticultural Science, Chungbuk National  
University, Cheongju, 28644, Korea

G. U. Suh  
Plant Conservation Division, Korea National Arboretum of the  
Korea Forest Service, Pocheon, 11186, Korea

## Introduction

Members of the *Cypripedium* genus are commonly called lady's slippers because of the slipper-like appearance of their flowers. *Cypripedium macranthos* Sw. is one of the most attractive species among the lady's slipper orchids and widely distributed from Eastern Russia, Northern China, Japan, Korea and Taiwan (Cribb 1997). Unfortunately they are becoming extinct due to the destruction of native habitats and illegal collection (Cribb and Sandison 1998). The most commonly recommended propagation method for conservation and commercialization of *Cypripedium* species is micropropagation, however, many cultural conditions including physical and nutritional factors can affect the germination and regeneration of *Cypripedium* species critically. Numerous studies on efficient micropropagation of orchid plants through callus or protocorm-like bodies (PLBs) have suggested that their proliferation methods are species-specific and the major obstacles to successful micropropagation are involved in *in vitro* germination of seeds (Arditti 1977; Colli and Kerbauy 1993). So it is very important to find the efficient *in vitro* seed germination conditions for each *Cypripedium* species and establish their optimum artificial propagation system.

Orchids produce a number of minute seeds but they have low propagation rate in nature, because ovules are not present or poorly developed at the time of anthesis, and endosperms which contain sufficient nutrient reserves for germination are absent from mature seeds. Therefore, asymbiotic germination has been a beneficial and common technique for micropropagation of orchids (Arditti 1967). However, *in vitro* germination of terrestrial orchids, such as *Cypripedium* (Leroux et al. 1995; Rasmussen 1995), *Spiranthes* (Zelmer and Currah 1997), *Platanthera* (Zettler and MacInnis 1994), *Ophrys* (Kitsaki et al. 2004), *Calanthe* (Lee et al. 2007) and *Epipactis* (Rasmussen 1992) was found to be more complicated as compared to the other epiphytic orchids in tropical areas. Because they have some unfavorable physiological characteristics resulting in

poor germination. The mature seeds of terrestrial orchids contain commonly the rigid and hard seed coats which have strong hydrophobicity, besides, inhibitory substances such as abscisic acid are accumulated in mature seeds (Lee et al. 2007; Van der Kinderen 1987; Van Waes and Debergh 1986). Miyoshi and Mii (1998) also mentioned the similar result that the germination rate of mature seeds was less than 5% in *C. macranthos*. Therefore various strategies have been applied, such as pretreating seeds with hypochlorite solution (Vujanovic et al. 2000), pre-chilling treatment (Masanori and Tomita 1997), immature seed culture (De Pauw and Remphery 1993; St-Arnaud et al. 1992) and development of optimum medium composition (Rasmussen 1995). Furthermore, medium types, plant growth regulators, carbohydrates, organic amendments, vitamins and other components have been studied for the improvement of *Cypripedium* seed germination (Deng et al. 2012; De Pauw et al. 1993, 1995; Harvais 1982; Piao et al. 2011; Rasmussen 1995; Yan et al. 2006).

Growth and morphogenesis of plant tissues can be promoted by the addition of various organic supplements and plant extracts (Fonnesbech 1972). Many organic additives including coconut water, banana powder, the bleeding sap of birch trees, peptone, tomato juice, honey, date palm syrup, corn extract, papaya extract and beef extract have been used effectively for enhancing the development of cultured cells and tissues, though they have undefined mixture of organic nutrients and growth factors (Islam et al. 2003; Murdad et al. 2010). Actually a large number of organic amendments were used successfully for orchid production (Arditti 1967; Arditti et al. 1990; Islam et al. 2003; Pyati et al. 2002). Therefore we tried to find the effect of various organic additives on *in vitro* growth of seedling of *C. macranthos* Sw. during asymbiotic seed culture and identify the most suitable organic additives to enhance its proliferation significantly in this study.

## Materials and Methods

### Plant materials

*C. macranthos* Sw. has been cultivated in field and greenhouse at Chungcheongbuk-do Agricultural Research and Extension Services. During blooming season (from April to June), the flowers were cross-pollinated manually by transferring pollinia onto the stigma of the other flower. Seed capsules were harvested at 75 days after cross-pollination. These premature seeds were brought to the laboratory and prepared for inoculation onto media on the same day. In a laminar flow hood, whole seed capsules which were not injured by insects

such as leaf-miners were surface-sterilized in 3% (v/v) sodium hypochlorite solution for 15 min and rinsed three times in sterile distilled water. Capsules were cut and their seeds were scooped out with forceps onto the culture medium. The seeds were incubated in darkness at  $23 \pm 2^\circ\text{C}$  during 5 months.

### Asymbiotic seed culture with media containing various organic additives

Seeds from surface-sterilized capsules were sown on each basal medium with different organic additives including coconut water (0, 50, 100, 200  $\text{ml}\cdot\text{L}^{-1}$ , Sigma-Aldrich, USA), birch sap (0, 50, 100, 200  $\text{ml}\cdot\text{L}^{-1}$ ), maple sap (0, 50, 100, 200  $\text{ml}\cdot\text{L}^{-1}$ ), banana powder (0, 15, 30, 60  $\text{g}\cdot\text{L}^{-1}$ , MBcell, USA) and peptone (0, 1, 2, 4  $\text{g}\cdot\text{L}^{-1}$ , MBcell, USA). Saps were obtained from the birch (*Betula pendula*) and maple (*Acer platanoides*) trees growing in the field of Chungcheongbuk-do Agricultural Research and Extension Services. They were collected in the early spring (from the end of March to the beginning of April) during the intense period of their production. For the sap collection, the trunk was tapped and the plastic spout which had a tube was connected into the taphole. From this tube, sap water ran into the sterile container, and these collected saps were immediately stored at  $-20^\circ\text{C}$ . The basal medium was made with quarter-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) which was supplemented with 10  $\text{g}\cdot\text{L}^{-1}$  sucrose, 7  $\text{g}\cdot\text{L}^{-1}$  agar and each organic additive. Its pH was adjusted to 5.8 before autoclaving (at  $121^\circ\text{C}$  and  $1.2\text{ kgf}\cdot\text{cm}^{-2}$  pressure for 15 min). 100 ml of medium was poured onto each plant culture dish ( $\Phi 100 \times \text{h}40$  mm). This experiment was designed randomly. Each treatment had ten replicates and was conducted three times. Seeds from each capsule were equally distributed into each replicate. After sowing, the seeds were incubated in darkness at  $23 \pm 2^\circ\text{C}$  during 5 months. After 2 months of culture, the germination rate (the percentage of the number of seeds germinated among the total countered number of seeds) was calculated. Germination indicated the emergence of the full embryo from the testa. After 3 months of culture, the protocorm formation rate was also calculated as the percentage of the number of young protocorms with promeristem among the total germinated seeds. After 5 months of culture, the growth characteristics of young seedlings developed from protocorms were measured.

### Determination of constituents in coconut water, birch sap and maple sap

Sugar contents were measured by high performance liquid

chromatography (HPLC) with a refractive index detector (Agilent 1100 series, Agilent, USA) using ZORBAX carbohydrate analysis column (4.6×150 mm, Agilent, USA). A mobile phase consisting of 75% acetonitrile was used at a flow rate of 1.0 ml·min<sup>-1</sup>. Among inorganic ion contents, the cations such as K<sup>+</sup>, Mg<sup>2+</sup> and Ca<sup>2+</sup> were analyzed by HPLC with a conductivity detector (Breeze HPLC, Waters, USA) on IC-Par CM/D column (3.9×50 mm, Waters, USA, eluent: 0.5 mM EDTA / 2 mM HNO<sub>3</sub>), and the anions such as NO<sub>3</sub><sup>-</sup> and SO<sub>4</sub><sup>-</sup> were analyzed using IC-Par anion HR column (4.6×75 mm, Waters, USA, eluent: 1.6 mM NaHCO<sub>3</sub> / 1.4 mM Na<sub>2</sub>CO<sub>3</sub>). According to the published methods (Amin and Reusch 1987), vitamin B and C group were measured by HPLC with diode array detector (Agilent 1100 series, Agilent, USA) using ZORBAX Eclipse Plus C-18 column (4.6×150 mm, Agilent), its mobile phase channel A was 25 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 2.5), and mobile phase channel B was methanol at a flow rate of 1.0 ml·min<sup>-1</sup>. Organic acids were also analyzed by HPLC with refractive index detector (Agilent 1100 series, Agilent, USA) using Hi-Plex H column (7.7×300 mm, Agilent), and its mobile phase was 0.004 M H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.4 mL·min<sup>-1</sup>. Endogenous plant hormones such as indole acetic acid (IAA), trans-zeatin riboside (trans-ZR) and abscisic acid (ABA) were measured according to the methods described by Nakurte et al (2012). They were analyzed with a modular HPLC system with UV and fluorescence detectors (Agilent 1100 series, Agilent, USA) using a reverse-phase Zorbax Eclipse XDB-C8 column (4.6×150 mm, Agilent, USA), its mobile phase was methanol containing 1% acetic acid (60 : 40 v/v) in isocratic mode at a flow rate of 1 ml·min<sup>-1</sup>.

#### Statistical analysis

Data from each experiment were subjected to Two-way ANOVA and Duncan's multiple range test using SAS program (Version 6.21, SAS Institute Inc., Cary, NC, USA).

## Results and Discussion

Table 1 showed the nutrient components of coconut water, birch sap and maple sap. All of them contained around 2.5% sugars which were almost composed of sucrose, glucose and fructose. Sucrose was the main sugar type in coconut water and maple sap, on the other hand, birch sap was mostly composed of fructose and glucose. They possessed variable inorganic ions such as potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg), iron (Fe) and manganese (Mn).

**Table 1** Comparison of nutrient constituents in coconut water, birch and maple sap added to the culture medium

Nutrient constituents		Coconut water (100g)	Birch sap (100g)	Maple sap (100g)
	Total	2.5	2.3	2.8
Sugars (g)	Sucrose	1.2	0.2	2.3
	Glucose	0.8	0.9	0.3
	Fructose	0.5	1.2	0.2
Inorganic ions (mg)	Ca <sup>2+</sup>	24.2	58.6	63.4
	Fe <sup>3+</sup>	0.3	0.1	0.6
	Mg <sup>2+</sup>	25.3	11.3	14.5
	PO <sub>4</sub> <sup>3-</sup>	20.4	6.4	2.7
	K <sup>+</sup>	242.1	120.4	204.2
	Mn <sup>2+</sup>	0.1	1.1	3.3
	Cu <sup>2+</sup>	0.04	0.03	0.7
	Na <sup>+</sup>	36.5	5.3	10.4
	SO <sub>4</sub> <sup>2-</sup>	21.4	31.2	38.8
	NO <sub>3</sub> <sup>-</sup>	2.8	3.1	3.5
Vitamins (mg)	Thiamin (V <sub>B1</sub> )	0.03	-	0.01
	Riboflavin (V <sub>B2</sub> )	0.05	-	0.01
	Niacin (V <sub>B3</sub> )	0.08	-	0.03
	Pantothenic acid (V <sub>B5</sub> )	0.04	-	0.03
	Pyridoxine (V <sub>B6</sub> )	0.03	-	0.002
	Myo-inositol	0.01	-	-
	Ascorbic acid (V <sub>C</sub> )	2.4	0.3	0.9
Organic acids (mg)	Malic	289.3	359.1	141.7
	Citric	23.7	6.8	15.0
	Succinic	8.4	11.5	12.2
Phytohormones (nM)	IAA	25.6	-	-
	trans-ZR	10.2	-	-
	ABA	8.5	-	-

Water-soluble vitamins such as thiamin (V<sub>B1</sub>), riboflavin (V<sub>B2</sub>), niacin (V<sub>B3</sub>), pantothenic acid (V<sub>B5</sub>), pyridoxine (V<sub>B6</sub>), myo-inositol and ascorbic acid (V<sub>C</sub>), which were recommended for orchid growth, were also detected in coconut water and maple sap. But they were not detected in birch sap, except for ascorbic acid. Organic acids including citric, malic and succinic acid were contained in three organic additives. Phytohormones such as IAA, trans-ZR and ABA were detected only in coconut water.

Table 2 explained the different effects of each organic additives on *in vitro* germination and protocorm formation of *C. macranthos* Sw. With 100 ml·L<sup>-1</sup> coconut water, the highest germination rate (70.8%) and protocorm formation rate (74.2%) were obtained, they increased by 6 times as

**Table 2** Effects of organic additives on *in vitro* seed germination and protocorm formation in *C. macranthos* Sw. after 2 and 3 months of culture

Organic additives		Germination (%)	Protocorm formation (%)
Type (A)	Conc. (B)		
Coconut water	0 ml·L <sup>-1</sup>	12.6 g <sup>z</sup>	14.4 e
	50 ml·L <sup>-1</sup>	60.9 b	62.0 b
	100 ml·L <sup>-1</sup>	70.8 a	74.2 a
	200 ml·L <sup>-1</sup>	64.4 ab	60.8 b
Birch sap	0 ml·L <sup>-1</sup>	12.8 g	13.1 e
	50 ml·L <sup>-1</sup>	55.8 c	62.1 b
	100 ml·L <sup>-1</sup>	65.2 ab	68.2 ab
	200 ml·L <sup>-1</sup>	56.9 c	53.4 bc
Maple sap	0 ml·L <sup>-1</sup>	10.9 g	12.7 e
	50 ml·L <sup>-1</sup>	59.1 b	60.5 b
	100 ml·L <sup>-1</sup>	66.4 ab	66.9 ab
	200 ml·L <sup>-1</sup>	61.1 b	61.4 b
Banana powder	0 g·L <sup>-1</sup>	11.1 g	12.2 e
	15 g·L <sup>-1</sup>	25.5 f	21.3 de
	30 g·L <sup>-1</sup>	30.6 ef	35.4 d
	60 g·L <sup>-1</sup>	36.8 e	41.9 c
Peptone	0 g·L <sup>-1</sup>	9.6 g	10.3 e
	1 g·L <sup>-1</sup>	35.2 e	38.2 cd
	2 g·L <sup>-1</sup>	43.3 d	43.7 c
	4 g·L <sup>-1</sup>	40.9 d	35.0 d
Significance <sup>y</sup>			
A		**	**
B		**	**
A×B		**	**

<sup>z</sup>Mean separation by Duncan's multiple range test at  $P \leq 0.05$ .  
<sup>y</sup>NS, \*, \*\* not significant or significant at  $P \leq 0.05$  and 0.01, respectively.

compared with non-treatment. Both birch sap and maple sap also induced the facilitating effect to improve the germination and protocorm development. When 100 ml·L<sup>-1</sup> birch sap and maple sap were added, the germination rate and protocorm formation rate showed more than 65%. But the addition of banana powder and peptone could not make the favorable culture condition significantly. Table 3 and Table 4 also indicated the beneficial effect of organic amendments including not only coconut water but also phloem sap such as birch sap or maple sap. When these additives were included at the level of 100 ml·L<sup>-1</sup>, the fresh and dry biomass of seedling increased significantly by 4 times as compared with non-treatment. Roots and buds of seedlings grew vigorously in the medium containing 100 ml·L<sup>-1</sup> coconut water or phloem sap. In

**Table 3** Effects of organic additives on the fresh and dry weight of seedlings developed from *C. macranthos* Sw. after 5 months of culture

Organic additives		Fresh weight (mg)	Dry weight (mg)
Type (A)	Conc. (B)		
Coconut water	0 ml·L <sup>-1</sup>	76.8 e <sup>z</sup>	7.8 e
	50 ml·L <sup>-1</sup>	228.1 b	24.2 bc
	100 ml·L <sup>-1</sup>	296.4 a	34.1 a
	200 ml·L <sup>-1</sup>	221.9 b	23.7 bc
Birch sap	0 ml·L <sup>-1</sup>	65.3 e	6.4 e
	50 ml·L <sup>-1</sup>	210.3 c	21.9 c
	100 ml·L <sup>-1</sup>	260.5 ab	28.7 ab
	200 ml·L <sup>-1</sup>	205.6 c	22.0 c
Maple sap	0 ml·L <sup>-1</sup>	71.4 e	6.9 e
	50 ml·L <sup>-1</sup>	207.6 c	22.4 c
	100 ml·L <sup>-1</sup>	273.2 ab	30.9 ab
	200 ml·L <sup>-1</sup>	231.7 b	25.5 b
Banana powder	0 g·L <sup>-1</sup>	72.9 e	7.2 e
	15 g·L <sup>-1</sup>	126.7 d	13.3 d
	30 g·L <sup>-1</sup>	178.4 cd	18.6 cd
	60 g·L <sup>-1</sup>	168.6 cd	16.9 cd
Peptone	0 g·L <sup>-1</sup>	68.4 e	6.8 e
	1 g·L <sup>-1</sup>	135.1 d	14.3 d
	2 g·L <sup>-1</sup>	169.9 cd	18.0 cd
	4 g·L <sup>-1</sup>	173.3 cd	18.2 cd
Significance <sup>y</sup>			
A		**	**
B		**	**
A×B		**	**

<sup>z</sup>Mean separation by Duncan's multiple range test at  $P \leq 0.05$ .  
<sup>y</sup>NS, \*, \*\* not significant or significant at  $P \leq 0.05$  and 0.01, respectively.

particular, the bud formation rates were raised up to 70%, and their length and diameter also increased by 2 and 1.5 times respectively. Similarly, the number and length of roots increased by 2 and 3 times individually. It was found that 100 ml·L<sup>-1</sup> of coconut water or phloem sap showed the best response to the germination and protocorm development in the quarter-strength MS basal medium supplemented with 10 g·L<sup>-1</sup> sucrose. The optimal osmotic potential should be maintained in culture medium for the successful *in vitro* germination and seedling growth of *Cypripedium* species (Piao et al. 2011; Van Waes and Debergh 1986). So when coconut water or phloem sap which contain not only high sugars but also various rich minerals are used as organic supplements, they have been recommended to be applied at

**Table 4** Effects of organic additives on the growth of seedlings developed from protocorm of *C. macranthos* Sw. after 5 months of culture

Organic additives		Root			Bud		
Type (A)	Conc. (B)	No.	Length (cm)	Diameter (mm)	Formation (%)	Length (cm)	Diameter (mm)
Coconut water	0 ml·L <sup>-1</sup>	3.1 d	1.0 d	1.0 a	20.2 g <sup>z</sup>	3.7 e	1.4 b
	50 ml·L <sup>-1</sup>	6.7 ab	2.7 b	0.9 a	62.5 c	6.8 b	2.0 a
	100 ml·L <sup>-1</sup>	7.1 a	2.9 ab	0.9 a	74.6 a	8.0 a	2.1 a
	200 ml·L <sup>-1</sup>	7.0 a	2.8 ab	0.9 a	69.5 abc	8.1 a	2.1 a
Birch sap	0 ml·L <sup>-1</sup>	3.2 d	0.9 d	0.8 a	23.9 g	3.9 e	1.4 b
	50 ml·L <sup>-1</sup>	5.9 b	2.6 b	0.9 a	57.1 cd	6.4 bc	1.7 ab
	100 ml·L <sup>-1</sup>	6.6 ab	3.0 ab	1.0 a	70.4 ab	7.8 a	2.2 a
	200 ml·L <sup>-1</sup>	6.1 b	2.7 b	0.9 a	67.3 b	7.4 ab	2.0 a
Maple sap	0 ml·L <sup>-1</sup>	3.2 d	1.1 d	1.0 a	23.0 g	3.5 e	1.4 b
	50 ml·L <sup>-1</sup>	5.4 c	2.5 b	0.8 a	61.1 c	7.0 b	1.6 ab
	100 ml·L <sup>-1</sup>	6.7 ab	3.3 a	0.9 a	73.5 a	7.9 a	2.1 a
	200 ml·L <sup>-1</sup>	6.6 ab	2.9 ab	0.9 a	68.0 b	7.8 a	2.1 a
Banana powder	0 g·L <sup>-1</sup>	3.0 d	1.4 cd	0.8 a	21.3 g	3.4 e	1.3 b
	15 g·L <sup>-1</sup>	4.0 cd	1.8 c	1.0 a	31.5 f	4.2 de	1.5 ab
	30 g·L <sup>-1</sup>	5.2 c	2.1 bc	1.0 a	36.9 ef	4.4 de	1.6 ab
	60 g·L <sup>-1</sup>	4.6 cd	1.7 c	0.9 a	29.0 f	5.3 d	1.5 ab
Peptone	0 g·L <sup>-1</sup>	2.8 d	1.2 d	1.0 a	21.0 g	3.7 e	1.3 b
	1 g·L <sup>-1</sup>	4.4 cd	1.9 c	0.9 a	44.2 e	5.2 d	1.6 ab
	2 g·L <sup>-1</sup>	5.1 c	2.3 bc	0.8 a	56.8 cd	6.0 c	2.0 a
	4 g·L <sup>-1</sup>	4.5 cd	1.9 c	0.8 a	52.6 d	5.3 d	1.9 a
Significance <sup>y</sup>							
A		**	**	NS	**	**	*
B		*	**	NS	**	**	*
A×B		*	*	NS	**	**	NS

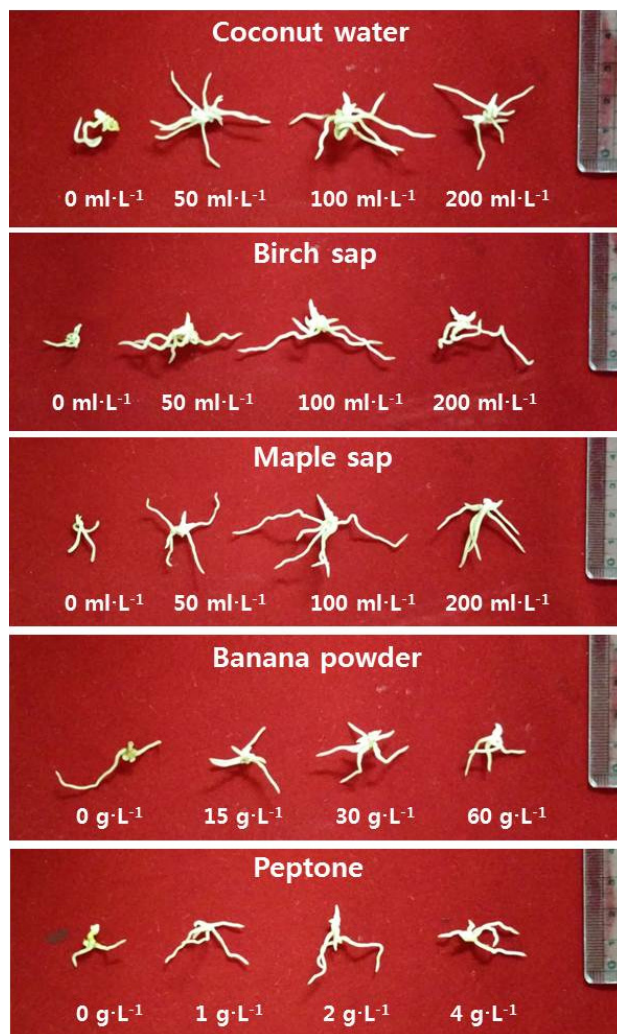
<sup>z</sup>Mean separation by Duncan's multiple range test at  $P \leq 0.05$ .

<sup>y</sup>NS, \*, \*\* not significant or significant at  $P \leq 0.05$  and  $0.01$ , respectively.

the proper concentration. In this study, when these organic additives were adjusted at the level of 100 ml·L<sup>-1</sup>, the most balanced osmolality might be kept during the long culture period, in comparison with their lower or higher concentrations. From Figure 1, it was also confirmed that the roots and buds of seedling grew most vigorously in the culture medium supplemented with 100 ml·L<sup>-1</sup> coconut water or phloem sap. It is one of the advisable methods to use organic additives in orchid culture medium, because they have been reported to be an easy way to improvise the current plant tissue culture media towards commercial production (Ichihashi and Islam, 1999). As organic additives, coconut water, apple homogenate, banana homogenate, potato homogenate, date palm syrup, corn extract, papaya extract, beef extract, casein hydrolysate, pineapple extract, yeast extract, tryptone, peptone or pure

amino acids such as glutamine were used successfully in orchid production (Islam et al. 2003; Murdad et al. 2010; Pyati et al. 2002; Rasmussen 1995). They possess a wide spectrum of growth factors, resulting in the beneficial effects producing more PLBs, shoots and leaves (Akter et al. 2007), increasing the size of somatic embryos (Al-Khateeb 2008) and promoting the development of asymbiotic seeds as well as regeneration of plantlets (Tawaro et al. 2008). *Cypripedium* seed germination and protocorm development are known to be stimulated or inhibited by organic amendments (Chu and Mudge 1994; Deng et al. 2012; DeMarie et al. 1991; Steele 1995; Yan et al. 2006).

Coconut water was reported to promote the development of orchid tubers and roots (McIntyre et al. 1974; Rasmussen 1995). 100 ml·L<sup>-1</sup> coconut water extracted from a fully ripe



**Fig. 1** Morphology of seedlings developed from protocorms of *C. macranthos* Sw. depending on organic additives after 5 months of culture

coconut could induce the germination of *C. reginae*, in particular, quarter-strength MS medium supplemented with  $100 \text{ ml}\cdot\text{L}^{-1}$  coconut water led to synchronous germination, but casein hydrolysate and yeast extract had a deleterious effect (Chu and Mudge 1994). Huang and Hu (2001) reported that 5% coconut water, banana homogenate or potato homogenate in Harvais medium could accelerate the seed germination of *C. flavum*. Similar results have been reported in different plant species. For instance, the fresh and dry biomass of *Anoectochilus formosanus* increased significantly when  $50 \text{ ml}\cdot\text{L}^{-1}$  coconut water and  $0.5 \text{ g}\cdot\text{L}^{-1}$  activated charcoal were added during bioreactor culture (Yoon et al. 2007). Hyponex medium supplemented with  $50 \text{ ml}\cdot\text{L}^{-1}$  coconut water proved for the enhancement of fresh and dry biomass, number of roots, leaf area as well as development of healthy plantlets of *Calanthe* hybrids (Baque et al. 2011). In the proliferation of PLBs of *Dendrobium* Alya Pink, 10% coconut water was selected as

the best organic additive (Nambiar et al. 2012). Due to the difficulty of obtaining coconut endosperm and its expensiveness in Europe, some alternatives were tested. The bleeding sap of birch tress could enhance the growth of *Brassia*, *Cattleya*, *Phalaenopsis* and *Cymbidium* explants instead of coconut water (Pieper and Zimmer 1974; Zimmer and Pieper 1975, 1976, 1978). Natural organic compounds such as bleeding sap of birch trees as well as coconut water have been used as important constituents of culture medium for micropropagation of *Phalaenopsis* (Homa and Asahira 1985).

Coconut water is the colorless liquid endosperm of green coconuts (*Cocos nucifera*) which contain soluble sugars as a natural source of carbon, amino acids, phenols, fiber and vitamins, moreover, it also contains diphenyl urea which functions as cytokinin that can enhance the explant growth and regeneration by inducing cell division (Gnasekaran et al 2010; Texeira da Silva et al. 2006). The faster regeneration of plantlets was observed in PLBs of *Dracaena purplecompacta* L. when coconut water containing cytokinins and auxins was supplied, in fact, IAA extracted from coconut water showed the beneficial effect to facilitate the adventitious root development (Agampodi and Jayawardena 2009). Similarly, Chung et al. (1995) and Kuka et al. (2013) reported that birch and maple saps were rich in bioactive compounds and mineral substances including carbohydrates, proteins, organic acids, B complex vitamin, vitamin C and phenolic compounds, which have been related to the bioactive activities.

In conclusion, our results demonstrated that the organic additives including the phloem sap such as birch and maple sap as well as coconut water could promote the seed germination, increase the protocorm formation and led the vigorous seedling growth of *C. macranthos* Sw. during the culture period. Organic amendments, as a natural source supplying carbohydrates, inorganic ions, vitamins and phytohormones might be one of the most important components in the culture medium and helpful for *in vitro* orchid propagation.

## References

- Agampodi VA, Jayawardena B (2009) Effect of coconut (*Cocos nucifera* L.) water extracts on adventitious root development in vegetative propagation of *Dracaena purplecompacta* L. Acta Physiol Plant 31:279–284
- Akter S, Nasiruddin KM, Khalidun ABM (2007) Organogenesis of *Dendrobium* orchid using traditional media and organic extracts. J Agric Rural Dev 5:30–35
- Al-Khateeb AA (2008) Regulation of *in vitro* bud formation of date palm (*Phoenix dactylifera* L.) cv. Khanezi by different carbon sources. Bioresource Technol 99:6550–6555
- Amin M, Reusch J (1987) High-performance liquid chromatography

- of water-soluble vitamins: II. Simultaneous determinations of vitamins B1, B2, B6 and B12 in pharmaceutical preparations. *J Chrom* 390:448–453
- Arditti J (1967) Factors affecting the germination of orchid seeds. *Bot Rev* 33:1–97
- Arditti J (1977) *Orchid biology, reviews and perspectives I*. Cornell University Press, Ithaca, New York, USA
- Arditti J, Ernst R, Yam TW, Glabe C (1990) The contribution of orchid mycorrhizal fungi to seed germination: a speculative review. *Lindleyana* 5:249–255
- Baque MA, Shin YK, Elshmary T, Lee EJ, Paek KY (2011) Effect of light quality, sucrose and coconut water concentration on the micropropagation of *Calanthe* hybrids ('Bukduseong' x 'Hyesung'). *Aust J Crop Sci* 5:1247–1254
- Chu CC, Mudge KW (1994) Effects of prechilling and liquid suspension culture on seed germination of the Yellow Lady's Slipper orchid, *Cypripedium calceolus* var. *pubescens*. *Lindleyana* 9:153–159
- Chung MJ, Lee SJ, Shin JH, Jo JS, Sung NJ (1995) The components of the sap from birches, bamboos and daeae. *J Kor Soc Food Nutr* 24:727–733
- Colli S, Kerbauy GB (1993) Direct root tip conversion of *Catasetum* into protocorm-like bodies: Effect of auxin and cytokinin. *Plant Cell Tiss Org Cult*. 33:39–44
- Cribb P (1997) The genus *Cypripedium*, p. 301. Timber Press, Portland, USA
- Cribb P, Sandison M (1998) A preliminary assessment of the conservation status of species in the wild. *Bot J Linnean Soc* 126:183–190
- DeMarie E, Weimer M, Mudge W (1991) *In vitro* germination and development of Showy Lady's Slipper orchid (*Cypripedium reginae* Walt.) seeds. *HortScience* 26:727
- Deng L, Zhang Y, Wang MM, Zhao SW (2012) Study on non-asyymbiotic germination of *Cypripedium macranthos* seed. *Seed* 31:31–39
- De Pauw MA, Remphery WR (1993) *In vitro* germination of three *Cypripedium* species in relation to time of collection, media and cold treatment. *Can J Bot* 71:879–885
- De Pauw MA, Remphery WR and Palmer CE (1995) The cytokinin preference for *in vitro* germination and protocorm growth of *Cypripedium candidum*. *Ann Bot* 75:267–275
- Fonnesbech M (1972) Organic nutrients in the media for propagation of *Cymbidium in vitro*. *Physiologia Plantarum* 27:360–364
- Gnasekaran P, Xavier R, Uma Rani S, Sreeramanan S (2010) A study on the use of organic additives on the protocorm-like bodies (PLBs) growth of *Phalaenopsis violacea* orchid. *J Phytol* 2:29–33
- Harvais G (1982) An improved culture medium for growing the orchid *Cypripedium reginae* axenically. *Can J Bot* 60:2547–2555
- Homa Y, Asahira TM (1985) New means of *Phalaenopsis* propagation with intermodal sections of flower stalk. *J Jpn Soc Hortc Sci* 54:379–387
- Huang JL, Hu H (2001). Seed germination requirements of *Cypripedium flavum* in axenic culture. *Acta Bot Yunn* 23:105–108
- Islam MO, Rahman ARMM, Matsui S, Prodhon AKMA (2003) Effects of complex organic extracts on callus growth and PLB regeneration through embryogenesis in the *Doritaenopsis* orchid. *Jpn Agric Res Quart* 4:229–235
- Kitsaki CK, Zygouraki S, Ziobora M, Kintzios S (2004) *In vitro* germination, protocorm formation and platlet development of mature versus immature seeds from several *Ophrys* species (Orchidaceae). *Plant Cell Reports* 23:284–290
- Kūka M, Čakste I, Geršebeka E (2013) Determination of bioactive compounds and mineral substances in latvian birch and maple saps. In *Proceedings of the Latvian Academy of Sciences, Section B, Natural, Exact, and Applied Sciences* 67:437–441
- Lee YI, Lu CF, Chung MC, Yeung EC, Lee N (2007) Developmental changes in endogenous abscisic acid concentrations and asymbiotic seed germination of a terrestrial orchid, *Calanthe tricarinata* Lindl. *J Am Soc Hortc Sci* 132:246–252
- Leroux G, Barabe D, Vieth J (1995) Comparative morphogenesis of *Cypripedium acaule* (Orchidaceae) protocorms cultivated *in vitro* with or without sugar. *Can J Bot* 73:1391–1406
- Masanori T, Tomita M (1997) Effect of culture media and cold treatment on germination in asymbiotic culture of *Cypripedium macranthos* and *Cypripedium japonicum*. *Lindleyana* 12:208–213
- McIntyre DK, Veitch GJ, Wrigley JW (1974) Australian terrestrial orchids from seed. II. Improvements in techniques and further successes. *Amer Orchid Soc Bull* 43:52–53
- Miyoshi K, Mii M (1998) Stimulatory effects of sodium and calcium hypochlorite, pre-chilling and cytokinins on the germination of *Cypripedium macranthos* seed *in vitro*. *Physiol Plant* 102:481–486
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum* 15:473–497
- Murdad R, Latip MA, Aziz ZA, Ripin R (2010) Effects of carbon source and potato homogenate on *in vitro* growth and development of Sabah's endangered orchid: *Phalaenopsis gigantea*. *Asia-Pac J Mol Biol* 18:199–202
- Nakurte I, Keisa A, Rostoks N (2012) Development and validation of a reversed-phase liquid chromatography method for the simultaneous determination of indole-3-acetic acid, indole-3-pyruvic acid, and abscisic acid in barley (*Hordeum vulgare* L.). *J Anal Methods Chem* doi:10.1155/2012/103575
- Nambiar N, Tee CS, Maziah M (2012) Effects of organic additives and different carbohydrate sources on proliferation of protocorm-like bodies in *Dendrobium Alya Pink*. *Plant Omics J* 5:10–18
- Piao RZ, Wang YL, Zhao HY (2011) Preliminary exploration *in vitro* seed culture conditions of *Cypripedium macrnathos* Sw. *J Anhui Agri Sci* 39:18428–18429, 18445
- Pieper W, Zimmer K (1974) Blutungssafte als Zusatz zu Nahrmedien fur Gewebekulturen. *Gartnwelt* 74:461–462
- Pyati AN, Murthy HN, Hahn EJ, Paek KY (2002) *In vitro* propagation of *Dendrobium macrostachyum* Lindle.- A threatened orchid. *Indian J Exp Biol* 40:620–623
- Rasmussen HN (1992) Seed dormancy patterns in *Epipactis*

- palustris* (Orchidaceae): requirements for germination and establishment of mycorrhiza. *Physiol Plant* 86:161-167
- Rasmussen HN (1995) *Terrestrial Orchids: From Seed to Mycotrophic Plant*. Cambridge University Press, New York, USA. pp. 444
- St-Arnaud M, Lauzer D, Barabe D (1992) In vitro germination and early growth of seedling of *Cypripedium acaule* (Orchidaceae). *Lindleyana* 7:22-27
- Steele WK (1995) Growing *Cypripedium reginae* from seed. *Amer Orchid Soc Bull* 64:383-391
- Tawaro S, Suraninpong P, Chanprame S (2008) Germination and regeneration of *Cymbidium findlaysonianum* Lindl. on a medium supplemented with some organic sources. *Walailak J Sci and Tech* 5:125-135
- Teixeira da Silva JA, Chan MT, Chai ML, Tanaka M (2006) Priming abiotic factors for optimal hybrid *Cymbidium* (Orchidaceae) PLB and callus induction, plantlet formation, and their subsequent cytogenetic stability analysis. *Sci Hortic* 109:368-378
- Van der Kinderen G (1987) Abscisic acid in terrestrial orchid seeds: a possible impact on their germination. *Lindleyana* 2:84-87
- Van Waes JM, Debergh PC (1986) *In vitro* germination of some Western European orchids. *Physiologia Plantarum* 67:253-261
- Vujanovic V, St-Arnaud M, Barabe D, Thibeault G (2000) Viability testing of orchid seed and the promotion of colouration and germination. *Ann Bot* 86:79-86
- Yan N, Hu H, Huang JL, Xu K, Wang H, Zhou ZK (2006) Micropropagation of *Cypripedium flavum* through multiple shoots of seedlings derived from mature seeds. *Plant Cell Tiss Org Cult* 84:113-117
- Yoon YJ, Murthy HN, Hahn EJ, Paek KY (2007) Biomass production of *Anoectichilus formosanus* Hayata in a bioreactor system. *J Plant Biol* 50:573-576
- Zelmer CD, Currah RS (1997) Symbiotic germination of *Spiranthes lacera* (Orchidaceae) with a naturally occurring endophyte. *Lindleyana* 12:142-148
- Zettler LW, McInnis TM Jr. (1994) Light enhancement of symbiotic seed germination and development of an endangered terrestrial orchid (*Platanthera intergrilabia*). *Plant Sci* 7:154-161
- Zimmer K, Pieper W (1975) Weitere Untersuchungen zur Kultur *in vitro* con *Aechmea*. *Gartenbauwissenschaft* 40:129-132
- Zimmer K, Pieper W (1976) Zur Wirkung con Blutungssaft der Weissbirke auf das Wachstum von Geweben *in vitro*. *Die Orchidee* 27:267-269
- Zimmer K, Pieper W (1978) Clonal propagation of *Phalaenopsis* by excised buds. *The Orchid Review* 86:223-226