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Effect of light quality, sucrose and coconut water concentration on the microporpagation of *Calanthe* hybrids ('Bukduseong' × 'Hyesung' and 'Chunkwang' × 'Hyesung')

Md. Abdullahil Baque[1, 2], Yun-Kyong Shin[1], Turkey Elshmari [3], Eun-Jung Lee[1], Kee-Yoeup Paek[1]*

[1]Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju 361-763, Republic of Korea
[2]Department of Agronomy, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh
[3]Center of Excellence in Biotechnology Research, king Saud University, Riyadh 11451, Kingdom of Saudi Arabia

*Corresponding author: Paekky@cbnu.ac.kr

Abstract

An immensely useful technique was established to elucidate suitable culture conditions for *in vitro* micropropagation of *Calanthe* hybrids by using modified Hyponex media. Among the light emitting diodes (LEDs), the mixture of blue plus red light efficiently enhanced *in vitro* growth of the plantlets, whereas inhibitory effect on the plantlets growth was observed under the mixture of red plus far-red light. However, the effect of light quality on the growth of plantlets was more pronounced in 'Bukduseong' × 'Hyesung' hybrid compared to 'Chunkwang' × 'Hyesung'. Of the various gradients of sucrose tested (0, 7.5, 15, 30 and 60 g l^{-1}), 15 g l^{-1} sucrose was proven suitable concentration for enhancing growth and growth attributes of 'Bukduseong' × 'Hyesung', while 15 and 30 g l^{-1} sucrose were regarded as an optimal concentration for *in vitro* growth of the plantlets of 'Chunkwang' × 'Hyesung' hybrid. Although, higher sucrose concentration (60 g l^{-1}) enhanced root growth but induced plantlets abnormality. In addition, as a source of natural product, 50 ml l^{-1} coconut water effectively enhanced plantlets growth of both hybrids compared to the relative control (without coconut water). After 8 weeks of culture, when the plantlets were transferred to the green house, 85% survivability of the plantlets was achieved upon hardening.

Keywords: *Calanthe* hybrids, light quality, micropropagation, natural product, hardening.
Abbreviations: CEC- cation exchange capacity; EC- electrical conductivity; LEDs- light emitting diodes; LOX- lipoxygenase; MDA- malondialdehyde; PPF- photosynthetic photon flux.

Introduction

Modern propagation and production technology have made orchids accessible to much broader section of the society. Development of new hybrids and their commercial cultivation have now become a lucrative industry in many countries of the world. The rising popularity of orchids has created a demand for high quality plant materials for the development of orchid industry. However, cost efficient protocols for mass propagation of rare, threatened and endangered orchids, new hybrids, as well as transgenic orchids have to be developed in order to commercialize and conserve their existence (Chugh et al., 2009). *Calanthe* hybrids ('Bukduseong' × 'Hyesung' and 'Chunkwang' × 'Hyesung'), belongs to the orchidaceae family are widely adapted to temperate, tropical and subtropical regions. Recently, these hybrids are getting popularity in Japan and Korea due to their beautiful big flowers with various attractive colors, pleasing fragrance, and cold hardiness during cultivation in the southern part of Korea (Godo et al., 2010; Kim et al., 2003; Miyoshi and Mii, 1995). To increase the efficiency of *in vitro* techniques, culturing conditions such as light, temperature, and medium composition must be optimized. For instance, light quality (spectral quality), quantity (photon flux) and photoperiod have a profound influence on the morphogenesis of propagules cultured *in vitro*, and also further growth of the organs initiated from the cultures (Smith, 1982; Economou and Read, 1987). In general, fluorescent lamps are main light source for maintaining tissue cultures (Economou and Read, 1987). In the past few years, light emitting diodes (LEDs) have arisen as an alternative light source for plant tissue culture because of their advantages regarding wavelength specificity and narrow bandwidth (Hoenecke et al., 1992). For instance, LEDs have been used for studies on chlorophyll biosynthesis in wheat (Tripathy and Brown, 1995); adventitious root induction from leaf explants of *Morinda citrifolia* (Baque et al., 2010), bulblets growth of *Lilium* (Lian et al., 2002), stem elongation and leaf expansion in lettuce (Hoenecke et al. 1992), induction of callus growth in rapeseed (Afshari et al., 2011), and photosynthesis research with Kudzu (Tennessen et al., 1994). In plant tissue culture, sucrose is considered for being an indisputably important carbon and energy source because sucrose is the most common carbohydrate in phloem sap and involved in controlling developmental processes (Gibson, 2000). It has been conjectured that sucrose increases vascular regeneration in lettuce pith tissue acting as a signal molecule (Warren Wilson et al., 1994), quality and vase life of *Bougainvillea glabra* (Moneruzzaman et al., 2010), root regeneration in apple microcuttings (Pawlicki and Welander, 1995) and some studies confined to a role of carbohydrates as suppliers of energy and building block. The Hyponex medium widely used for propagation of orchids is a simple composition of nitrogen, phosphorous, and potassium (Park et al., 2000). Therefore, addition of coconut water in the culture media has shown to be effective for enhancing the development of cultured cells and tissues because it possesses a wide spectrum of growth factors, and has been successfully used in orchid production (Pyati et al., 2002). Although, micropropagation is a common practice in plant tissue culture and a lot of efforts have been paid in various plant species (Alam et al., 2010; Chugh et al., 2009; Pyati et al., 2002). To the best of our knowledge, *in*

vitro micropropagation techniques of *Calanthe* hybrids ('Bukduseong' × 'Hyesung' and 'Chunkwang' × 'Hyesung') have yet not been reported. Therefore, this study was undertaken to establish an *in vitro* culture condition by employing a series of experiments iteratively: to compare the nature of *in vitro* seed germination under fluorescent and dark conditions, the effect of different light sources, various concentrations of sucrose and natural product on the *in vitro* growth and growth attributes of *Calanthe* hybrids suitable for commercial exploitation.

Results and discussion

Progress of Calanthe seed germination under a light and dark condition

The prepared seed explants of the *Calanthe* hybrids ('Bukduseong' × 'Hyesung' and 'Chunkwang' × 'Hyesung') were placed on germination medium for six mon to discern the progress of seed germination under a light and dark environment. Stereo microscopic observations depicted in Fig. 1-2 reveled that light environment facilitate germination process with conspicuous developmental stage than dark environment. The total germination process was categorized as embryo formation followed by swelling of embryo, roots and shoot formation and followed by development of plantlets after 10, 12 weeks under dark and light condition, respectively. Under light conditions, healthy plantlets with vigorous roots and shoots formation were observed compared to the dark. Based on these observations we have established *in vitro* micro-propagation technique of *Calanthe* hybrids emphasized on the optimization of sucrose, coconut water and light sources.

Effects of light quality on in vitro plantlets growth of calanthe hybrids

Light quality significantly affected the growth of plantlets and morphological attributes of *calanthe* hybrids 'Bukduseong' × 'Hyesung' and 'Chunkwang' × 'Hyesung'. The maximum shoot length, no. of roots and respective shoot and root dry weight was obtained from the plantlets cultured under the mixture of blue plus red light followed by fluorescent, red, and blue light. The mixture of red plus far-red light strongly inhibited of these growth and morphological attributes (Table 1). By contrast, leaf width and leaf area were enormously maximized under the mixture of blue plus red light in case of 'Bukduseong' × 'Hyesung', while these parameters were observed higher under fluorescent light in 'Chunkwang' × 'Hyesung' hybrid. The mixture of blue plus red light facilitates formation of well-developed and numerous roots in both hybrids compared to other light sources. Meanwhile, plantlets cultured under far-red light were developed very poor root system (Fig. 3). The ratio of shoot/root dry weight reflects the pattern of dry matter allocation in plant, and was recorded higher (2.67 and 1.78 in 'Bukduseong' × 'Hyesung' and 'Chunkwang' × 'Hyesung' hybrid, respectively) under fluorescent light followed by blue light. The monochromic spectrum of red light or mixture of blue plus red and red plus far-red spectra induced higher root/shoot ratio. These results suggest that fluorescent light accelerated photosynthetic efficiency of the 'Bukduseong' × 'Hyesung' hybrid in shoots compared to roots, therefore, the greater allocation of metabolites was occurred towards shoot than root under fluorescent light. Light quality is one of the most important factors regulating plant development through photoreceptors active under specific wavelength of light (Lee et al., 2007). For instance, increases of leaf area, fresh weight, dry weight and chlorophyll content in *Withania* plantlets were observed the greatest under fluorescent and spectral mixture of red plus blue. In contrast, shoot/root dry weight ratio became entirely reversed under blue or red light regimes (Lee et al., 2007). These observations are not only reflection of poor growth of roots but also indicate that root induction is probably also dependent on the spectral quality of light. In case of *Morinda citrifolia*, Baque et al. (2010) have demonstrated that toxic malondialdehyde (MDA) accumulation causes cell injuries that adversely affected the normal induction process of adventitious roots when leaf explants were cultured under far-red, red plus blue, red and blue light. While lower oxidative damage under fluorescent light accelerated the induction process. On the contrary, in case of *Cymbidium* plantlets, the mixture of red plus blue light enhances plant growth and development by increasing the net photosynthesis (Tanaka et al., 1998), because the spectral energy distribution of red and blue light coincided with that of chlorophyll absorption (Goins et al., 1997). Moreover, plant growth as defined by root number, fresh weight and chlorophyll content was maximized under the mixture of red plus blue light (Moon et al., 2006). The authors have concluded that rooting was promoted by red light but inhibited by blue light. Tennessen et al. (1994) also suggested that monochromic red light causes an imbalance for the availability of light energy required for optimal functioning of photosystem I and II, which may consequently, inhibited shoot growth. Apart from this, it is conjectured that red light, the mixture of red plus blue and red plus far-red induced root growth compared to shoot growth in both *Calanthe* hybrids in our current study. Although, shoot and root dry weight, as well as morphological attributes were the greatest under the mixture of red plus blue light; however, higher shoot/root dry weight ratio in 'Bukduseong' × 'Hyesung' compared to 'Chunkwang' × 'Hyesung' hybrid under fluorescent light suggests that 'Bukduseong' × 'Hyesung' hybrid is more efficient and fluorescent light is suitable for enhancing *in vitro* plantlets growth in terms of metabolite allocation.

Effects of sucrose concentration on plantlets growth of Calanthe hybrids

In plant cell and tissue culture, sucrose is considered as an important carbon and energy source, because initial concentration of sucrose can affect growth and biomass accumulation (Desjardins et al., 1995). On the contrary, higher amount of sucrose can retard the development of cultured cells (Wu et al., 2006) by causing a cessation of the cell cycle when nutrients are limited (Gould et al., 1981). In this study, in case of 'Bukduseong' × 'Hyesung' hybrid, cultures supplemented with 15 g l^{-1} sucrose profusely increases *in vitro* plantlets growth as evidence from greater accumulation of biomass (970 mg $plant^{-1}$ and 108.5 mg $plant^{-1}$ shoot fresh and dry weight, respectively), shoot length (9.74 cm), leaf width (1.21 cm) and leaf area (2.48 cm^2) compared to plantlets grown under without sucrose supplementation (Table 2). In contrast, plantlets grown in the medium fed with 15 to 30 g l^{-1} sucrose significantly enhanced growth and growth attributes of 'Chunkwang' × 'Hyesung' hybrid compared to their respective lower and higher sucrose concentrations (Table 2). Additionally, higher initial sucrose supplementation in culture media enhanced root

Table 1. Effects of light quality on growth and growth attributes of *Calanthe* hybrids ('Bukduseong' x 'Hyesung' and 'Chunkwang' x 'Hyesung') after 8weeks of culture

Cross (Female x male)	Light quality	Fresh weight (mg/Plantlet)		Dry weight (mg/Plantlet)		Shoot length (cm)	Leaf width (cm)	No. of roots	Leaf area (cm^2)
		Shoot	Root	Shoot	Root				
'Bukduseong' x 'Hyesung'	FL	482 b	183 d	78 a	29.2 c	5.98 d	1.01 b	2.9 bc	1.15 a
	B	428 b	246 cd	51.7 b	31.1 c	5.67 d	0.87 bc	2.3 c	0.302 b
	R	462 b	596 b	54.8 b	70.8 b	7.95 b	0.68 d	4 b	0.424 b
	B+R	766 a	874 a	86.2 a	95.7 a	10.23 a	1.17 a	5.1 a	1.224 a
	R+Fr	396 b	44 bc	42.8 b	52.5 b	7.02 c	0.78 cd	2.9 bc	0.366 b
'Chunkwang' x 'Hyesung'	FL	384 b	211 b	54.5 ab	30.5 b	5.08 c	1.29 a	2.3 a	1.473 a
	B	321 b	245 b	45.7 bc	32.2 b	5.64 c	0.99 b	2.7 a	0.882 bc
	R	393 b	428 a	43.8 bc	50.7 a	7.31 b	0.8 c	3 a	0.486 d
	B+R	590 a	539 a	67.1 a	61.1 a	8.19 a	1.01 b	3.5 a	1.12 ab
	R+Fr	294 b	473 a	33.9 c	53.9 a	5.41 c	0.91 bc	2.9 a	0.659 cd

Mean separation within columns by Duncan's multiple range test at 5% level
Each treatment consisted of 5 glass container and each container contains 4 plantlets
FL = Fluorescent, B = Blue, R = Red, B+R = Blue + Red, R + Fr = Red + Far-red

Fig 1. Progress of *calanthe* seed germination for 6 months at a 20 μmol m^{-2} s^{-1} PPF with a 16h photoperiod (observations with stereo microscope, sometech): (a) seed capsule, (b) embryo formation, (c) - (f) Swelling of embryo after 10 days, 15 days, 20 days and 25 days of germination, respectively; (g)- (h) root and shoot formation after 8 weeks of germination, (i) plantlet after 12 weeks of germination. Each treatment consisted of five Petri dishes (about 300 seeds/ Petri dish)

growth but induced abnormality in both the hybrids (Fig 4). Considering metabolite partitioning between the hybrids, 15 g l^{-1} sucrose induced maximum (1.31) shoot/root ratio in 'Bukduseong' × 'Hyesung' hybrid compared to 'Chunkwang' × 'Hyesung' hybrid (1.03). These results clearly indicate that 15 g l^{-1} sucrose is suitable to induce shoot growth than root growth through accumulation of metabolites towards shoot than root and the effect was more pronounced in 'Bukduseong' × 'Hyesung' hybrid than 'Chunkwang' × 'Hyesung' hybrid. The present investigation is in consistent with the findings of Yoon et al. (2007), where they demonstrated that 30 g l^{-1} sucrose is associated with greater biomass accumulation compared to sucrose free medium. Whereas, higher concentrations of sucrose (60 and 90 g l^{-1}) linked to reduce biomass production of *Anoectochilus formosanus*. Incase of saga palm, a low concentration of sucrose (22.5 g l^{-1}) in combination with 7.5 g l^{-1} sorbitol was found to be suitable on the growth of explants (Novero et al., 2010), while a comparatively low concentration of sucrose (10 g l^{-1}) as a sole carbon source had a general inhibitory effect on adventitious root regeneration in apple because of the very low osmotic potential (Calamar and Klerk, 2002). These results clearly reflect that the osmotic potential (-0.457 MPa) induced by sucrose supplementation was regarded as an optimal pressure for the initial growth of sago palm explants. Therefore, it can be conjectured that 30 g l^{-1} sucrose might be induced optimal osmotic potential in the culture that facilitates *in vitro* growth of the plantlets in our study. Researchers have claimed that the addition of high concentration of sucrose in the culture media might have inhibitory effect on nutrient uptake by lowering water potential of the medium (Shim et al., 2003) or sucrose is preferentially absorbed by the plantlets for carbohydrate source, which inhibit

Table 2. Effects of sucrose concentration on growth and growth attributes of *Calanthe* hybrids ('Bukduseong' x 'Hyesung' and 'Chunkwang' x 'Hyesung') after 8weeks of culture

Cross (Female x male)	Treat. (%)	Fresh weight (mg/Plantlet)		Dry weight (mg/Plantlet)		Shoot length (cm)	Leaf Width (cm)	No. of roots	Leaf area (cm^2)
		Shoot	Root	Shoot	Root				
'Bukduseong' x 'Hyesung'	0	290 d	84 c	23 c	7.1 c	5.49 c	0.71 b	2.9 b	0.39 c
	0.75	593 c	234 c	50.4 b	23.1 c	7.6 b	1.11 a	4.9 a	1.65 ab
	1.5	970 a	637 b	108.5 a	83 bc	9.74 a	1.21 a	4.7 ab	2.48 a
	3	793 b	711 b	91.8 a	126.9 b	9.04 a	0.97 ab	6 a	1.66 ab
	6	693 bc	1059 a	89.5 a	259.8 a	7.76 b	0.66 b	6.6 a	0.87 bc
'Chunkwang' x 'Hyesung'	0	475 b	78 d	45 b	7.6 c	6.44 c	1.06 c	3 d	1.48 c
	0.75	603 b	430 cd	61.7 b	40.6 bc	8.45 b	1.25 ab	4 cd	2.1 bc
	1.5	979 a	948 b	111.7 a	108.1 b	9.84 a	1.55 a	6.6 ab	3.5 a
	3	955 a	816 bc	113.8 a	136.7 b	9.41 a	1.46 a	5.4 bc	2.89 ab
	6	853 a	1633 a	120.3 a	351.4 a	7.36 c	1.04 b	7.6 a	1.49 c

Mean separation within columns by Duncan's multiple range test at 5% level, Each treatment consisted of 5 glass container and each container contains 4 plantlets.

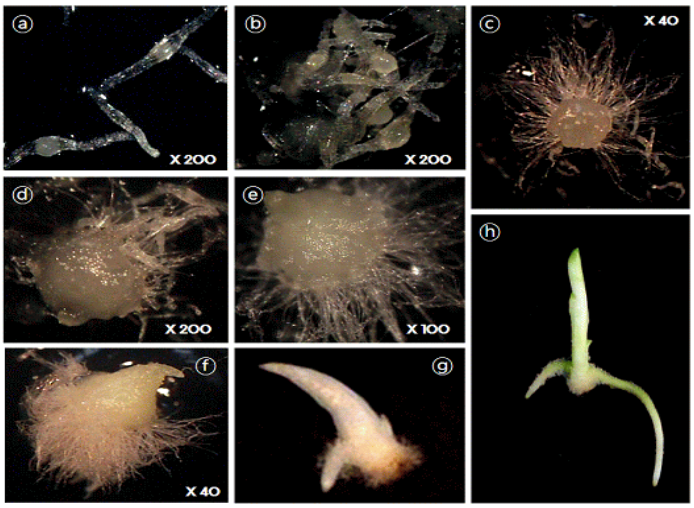

Fig 2. Progress of *calanthe* seed germination for 6 months under darkness (observations with stereo microscope, sometech): (a) seed, (b) embryo formation, (c)–(f) swelling of embryo and shoot formation after 10 days, 20 days, 25 days and 30 days of germination, respectively; (g) root and shoot formation after 6 weeks of germination, (h) plantlet after 10 weeks of germination. Each treatment consisted of five Petri dishes (about 300 seeds/ Petri dish).

photosynthetic activity (Kozai and Sekimoto, 1988). Moreover, a marked increased in malondialdehyde (MDA) content and lipoxygenase (LOX) activity in germinating embryos of *Eleutherococcus sessiliflorus* was also observed in high sucrose (90 g l^{-1}) treatment, which is an effect of osmotic stress arising from a sudden increase in medium osmotic pressure (Shohael et al., 2006). Presumably, higher sucrose concentrations (30 and 60 g l^{-1}) induced maximum root/shoot ratio with abnormal plantlets growth in our current study for both the *Calanthe* hybrids.

Effects of coconut water concentration on plantlets growth of Calanthe hybrids

The Hyponex medium is a simple composition of nitrogen, phosphorous and potassium, and is widely used for *in vitro* seed germination and propagation of orchids (Park et al., 2000). Previous studies have shown that addition of coconut water with Hyponex medium can enhance the development of cultured cells and tissues due to contain a wide spectrum of growth factors (Shantz and Steward, 1952), and thus have been successfully used in orchid production (Murthy and Pyati, 2001; Pyati et al., 2002). In this study, we observed a differential effect of coconut water concentration on the growth and growth attributes of *Calanthe* hybrids ('Bukduseong' × 'Hyesung' and 'Chunkwang' × 'Hyesung'). With increases of coconut water concentration (10 to 50 ml l^{-1}) in the culture media, shoot length, respective shoot and root fresh and dry weight, leaf width, number of roots and leaf area increased in both *Calanthe* hybrids (Table 3). Higher concentration of coconut water (100 ml l^{-1}) decreased all the growth and morphological features, as well as induced abnormal plantlets growth (Fig 5). Of the different concentrations of coconut water tested, 50 ml l^{-1} treated cultures significantly increased shoot and root weight (fresh and dry weight), leaf width and leaf area (Table 3), along with healthy plantlets (Fig 5) compared with other treatments. The beneficial effect of coconut water on orchid production has been reported in different plant species (Yoon et al., 2007; Pyati et al., 2002; Murthy and Pyati, 2001). For instance, in bioreactor cultures of

Table 3. Effects of coconut water concentration on growth and growth attributes of *Calanthe* hybrids ('Bukduseong' x 'Hyesung' and 'Chunkwang' x 'Hyesung') after 8 weeks of culture

Cross (Female x male)	Treat. (ml L^{-1})	Fresh weight (mg/Plantlet)		Dry weight (mg/Plantlet)		Shoot length (cm)	Leaf width (cm)	No. roots	Leaf area (cm^2)
		Shoot	Root	Shoot	Root				
'Bukduseong' x 'Hyesung'	0	478 c	716 b	54.1 b	66.5 b	6.1 c	1 b	3.8 b	1.08 b
	10	558 bc	712 b	78.2 b	69.9b	7.72 b	0.9b	4.8 b	1.7 b
	30	634 b	952 b	68.6 b	97.3 b	9.06 a	1.2 ab	7.2 a	1.77 b
	50	908 a	1248 a	127.6 a	147.8 a	8.52 ab	1.4 a	5.8 ab	3.68 a
	100	534 bc	672 b	64.5 b	70.9 b	7.84 ab	0.9 b	4.8 b	1.51 b
'Chunkwang' x 'Hyesung'	0	538 b	498 b	52.8 a	47.1 b	6.62 b	1.2 a	4.6 a	1.63 b
	10	692 ab	706 b	65.5 a	73 b	7.7 b	1.2 a	5 a	1.92 ab
	30	738 ab	1202 a	81.2 a	115.6 ab	7.88 b	1.4 a	4.4 a	2.99 ab
	50	896 a	1370 a	284.7 a	162.1 a	9.38 a	1.6 a	5.8 a	3.49 a
	100	682 ab	590 b	90.1 a	70.8 b	7.44 b	1.4 a	4.4 a	3 ab

Mean separation within columns by Duncan's multiple range test at 5% level, Each treatment consisted of 5 glass container and each container contains 4 plantlets .

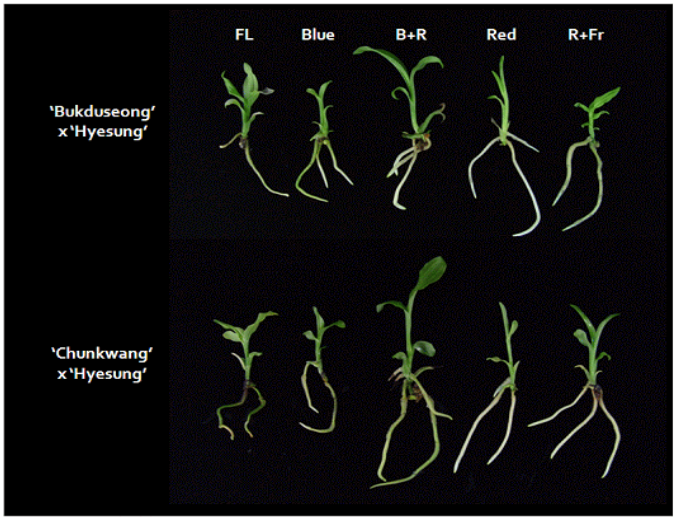

Fig 3. Effects of light quality on plantlets growth of *Calanthe* hybrids ('Bukduseong' x 'Hyesung' and 'Chunkwang' x 'Hyesung') after 8 weeks of culture. FL = Fluorescent, B = Blue, R = Red, B+R = Blue + Red, R + Fr = Red + Far-red.

Anoectochilus formosanus, fresh and dry biomass increased significantly when 50 ml l^{-1} coconut water was added in the culture in combination with 0.5 g l^{-1} activated charcoal (Yoon et al., 2007). In this study, Hyponex medium supplemented with 50 ml l^{-1} coconut water proved as the best concentration for the enhancement of fresh and dry biomass, number of roots, leaf area as well as development of healthy plantlets of *Calanthe* hybrids. Thus, supplementation of Hyponex media with 50 ml l^{-1} coconut water might be beneficial for *in vitro* propagation of *Calanthe* hybrid.

Hardening of in vitro grown plantlets under green house condition

Transplantation stage continues to be a major bottleneck in the micro-propagation of many orchids. A substantial numbers of micro-propagated plants do not survive after transfer from *in vitro* conditions to green house or field environment. The problems are associated with substantially lower relative humidity, higher light level and septic environment under green house or field condition that are stressful to micro-propagated plants compared to *in vitro* conditions. The benefit of any micro-propagation system can be fully realized by successful transfer of plantlets from tissue culture vessels to the ambient conditions (Hazarika, 2003). Therefore, to evaluate the survivability of the *in vitro grown* plantlets of *Calanthe* hybrids under green house conditions, 8 weeks old *in vitro* grown plantlets were transferred into the plastic tray containing horticultural substrate (Shinsungmineral, Korea), which is a well balanced mixture of nutrient and soil, as well as efficient for root development. The plantlets transplanted on the plastic tray were shaded by black cotton net (75% shading). After 4 weeks of shading followed by 2nd transplantation into plastic pot for another 4 weeks (without shading), more than 85% survived plantlets were achieved in case of *Calanthe* hybrid 'Bukduseong' × 'Hyesung', while more than 75% survived plantlets were achieved in 'Chunkwang' × 'Hyesung' hybrid (Fig 6 A-B). A good growing medium having properties such as maximum water holding capacity, porosity and drainage is essential for proper growth and development of *in vitro* raised seedlings of orchids. The survival percentage and growth performance of the seedlings were found to be higher (80%) in

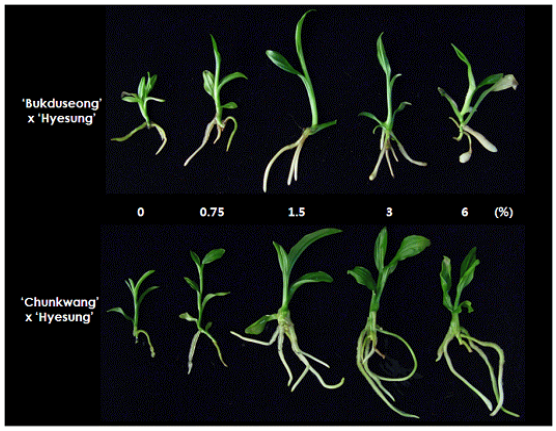

Fig 4. Effects of sucrose concentration on plantlets growth of *Calanthe* hybrids ('Bukduseong' x 'Hyesung' and 'Chunkwang' x 'Hyesung') after 8 weeks of culture.

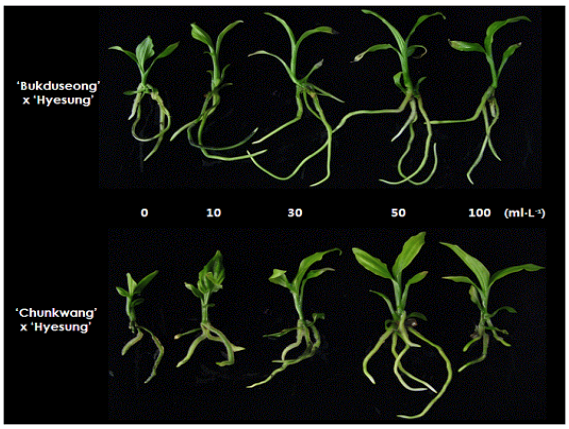

Fig 5. Effects of coconut water concentration on plantlets growth of *Calanthe* hybrids ('Bukduseong' x 'Hyesung' and 'Chunkwang' x 'Hyesung') after 8 weeks of culture.

potting substrate comprised of brick: charcoal at a ratio of 2:1, and additionally mulched with moss (Kishor et al., 2006). The authors have concluded that mulching of the medium with moss increases the water holding capacity of the medium. In our study, the maximum percentage (85%) of survived plantlets was achieved in the culture media composed of organic materials such as coir dust and peat moss, as well as inorganic materials including perlite, vermiculite and zeolite (Shinsungmineral, Korea). A good number of survivability of the plantlets under green house environment in our study might be due to well balanced nutrient, suitable physical chemical properties for root development and maximum water holding capacity (70%±10) of the potting substrates, as well as development of healthy plantlets under *in vitro* conditions.

Materials and methods

Plant materials, preparation of explants and in vitro seed germination

Mature pods (120 days) of *Calanthe* hybrids were collected from the society for the research of *Calanthe*, Korea, and explants were prepared as described by Shin et al. (2011). To understand about the germination nature under light and dark conditions, prepared seed explants (about 300 seeds/ Petri dish) were placed on plastic Petri dish containing modified Hyponex media (Kano, 1965) (Hyponex N: P: K = 20: 20: 20 = 1 g l^{-1}, N: P: K = 6.5 : 4.5 : 19 = 1 g l^{-1}) supplemented with 0.5 mg l^{-1} activated charcoal, 2 g l^{-1} peptone, 50 ml l^{-1} coconut water, 15 g l^{-1} sucrose and 6.5 g l^{-1} agar. The pH of the medium was adjusted to 5.6 before adding agar. Cultures were maintained at the temperature of 25±2 ^{o} C, under light (20 µmol m^{-2} s^{-1} photosynthetic photon flux, PPF) and dark (0 PPF) conditions for 12 weeks.

Effects of light quality

To elucidate the impact of different light sources on the *in vitro* plantlets growth of *Calanthe* hybrids ('Bukduseong' × 'Hyesung' and 'Chunkwang' × 'Hyesung'), light quality treatments were imposed by light emitting diodes (LEDs) such as blue, red, mixture of red plus blue (R + B, 0.7:1), and mixture of red plus far-red (R + Fr, 1:1.1) using LED system (GF-320s, Good Feeling, Sungnam, South Korea). Fluorescent (FL) lamps were used as control. The spectral distribution of the LEDs emitting blue LED had a peak emission at 450 nm, red LED at 660nm, far-red 730 nm (Lian et al., 2002). Modified Hyponex media (Hyponex N: P: K = 20: 20: 20 = 1 g l^{-1}, N: P: K = 6.5 : 4.5 : 19 = 1 g l^{-1}) supplemented with 0.5 mg l^{-1} activated charcoal, 2 g l^{-1} peptone, 50 ml l^{-1} coconut water, 15 g l^{-1} sucrose and 6.5 g l^{-1} agar was utilized for the plantlets growth. The pH of the medium was adjusted to 5.6 before adding agar. Cultures were maintained at the temperature of 25±2 ^{o} C and 16 h of photoperiod for 8 weeks.

Effects of sucrose and natural product

In order to determine optimal sucrose concentration on the *in vitro* plantlets growth of *Calanthe* hybrids, selected explants from *in vitro* grown plantlets were cultured in 500 ml glass container containing 120 ml solid modified Hyponex media (Hyponex N: P: K = 20: 20: 20 = 1 g l^{-1}, N: P: K = 6.5 : 4.5 : 19 = 1 g l^{-1}) supplemented with various gradients of sucrose (0, 7.5, 15, 30, 60 g l^{-1}), 0.5 mg l^{-1} activated charcoal, 2 g l^{-1} peptone, 50 ml l^{-1} coconut water, and 6.5 g l^{-1} agar. For coconut water experiment, the same solid modified Hyponex media supplemented with different concentrations of coconut water (0, 10, 30, 50, 100 ml l^{-1}), 0.5 mg l^{-1} activated charcoal, 2 g l^{-1} peptone, 15 g l^{-1} sucrose, and 6.5 g l^{-1} agar was used. The pH of the medium was adjusted to 5.6 before adding agar. All the cultures were maintained at the temperature of 25±2 ^{o}C, under a 20 µmol m^{-2} s^{-1} PPF and 16 h of photoperiod for 8 weeks.

Hardening

After 8 weeks of culture under *in vitro*, well developed plantlets were transplanted into plastic tray filled with horticultural substrates, which was prepared based on favorable physico- chemical properties such as water content (50±10 %), water maintaining capacity (70±10 %), bulk density [0.3±0.1 (mg m^{-3})], pH (5.5-7.0), EC [≤1.2 (dS m^{-1})], NH_4-N [≤ 600 (mg l^{-1})], NO_3-N [≤ 300 (mg l^{-1})], phosphate [≤ 500 (mg l^{-1})], and CEC [20±10 (Cmol l^{-1})] (Shinsungmineral, Korea). To protect the plantlets from high light intensity, plantlets were covered with black cotton net (75% shading) and water irrigation was supplied during the initial first 2 weeks at 3-4 days intervals according to medium saturation. Afterwards, 4 weeks intensive observation, the plantlets were again transferred into plastic pot filled with the same potting

Fig 6. Hardening of the plantlets of *Calanthe* hybrids ('Chunkwang' x 'Hyesung' and 'Bukduseong' x 'Hyesung') in a green house condition. A: Plantlets under shade condition in a green house after 4 weeks transferred from *in vitro* B: Plantlets under a green house after 8 weeks transferred from *in vitro* condition, without shade

substrates under the green house environment (without shading).

Measurement of leaf area

Leaf area of the top second leaves was measured by a leaf area meter (Skye Instruments
Ltd., UK).

Statistical analysis

The experiments were carried out in a completely randomized design and repeated twice with 5 replicates. Data were subjected to an analysis of variance, and to determine the significant differences, Duncan's multiple range test was performed using SAS software (version 6.12; SAS Institute, Cary, NC).

Conclusion

In this study, an efficient *in vitro* micropropagation technique is established for *Calanthe* hybrids by using modified Hyponex media (Hyponex N: P: K = 20: 20: 20 = 1 g L^{-1}, N: P: K = 6.5: 4.5: 19 = 1 g l^{-1}) supplemented with 15 g l^{-1} sucrose, 50 ml l^{-1} coconut water, 2 g l^{-1} peptone, 0.5g l^{-1} activated charcoal, and 6.5 g l^{-1} agar. This newly developed *in vitro* micropropagation technique should be used as an efficient tool for commercial exploitation of *Calanthe* hybrids ('Bukduseong' × 'Hyesung', 'Chunkwang' × 'Hyesung') in biotechnological application. However, the results of our study imply that the effect of light quality and sucrose on *in vitro* growth of the planlets is putative and supported by the lending information. Therefore, much more work is still needed to explore the physiological and molecular mechanism such as light induced antioxidative gene expression for protection of cell constituents against oxidative toxicity, involvement of light on phytohormone synthesis and sucrose regulated gene expression of protein kinases on *in vitro* growth and developmental processes of *Calanthe* hybrids.

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