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Effects of activated charcoal, plant growth regulators and ultrasonic pre-treatments on *in vitro* germination and protocorm formation of *Calanthe* hybrids

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Abstract

Many Asian orchids are threatened by extinction because of over-collection, habitat destruction and poor germination percentage (2%) in nature. In an attempt to develop a suitable protocol for the asymbiotic germination of *Calanthe* hybrid seeds, modified Hyponex medium supplemented with different concentrations of activated charcoal (0, 0.01, 0.1 g l^{-1}), α-napthaleneacetic acid (NAA) and 6-benzylaminopurine (BA) (0.1, 0.5, 1.0 mg l^{-1}) were tested. Addition of 0.1 g l^{-1} activated charcoal without NAA or BA in the modified Hyponex medium significantly accelerated the rate of seed germination. Whereas, the effect of NAA or BA without activated charcoal on the rate of seed germination was shown to be non-effective. However, addition of 0.1 mg l^{-1} NAA or 0.5 mg l^{-1} BA in combination with 0.1 g l^{-1} activated charcoal in the germination medium was proven effective to enhance rate of seed germination compared to other treatments. Meanwhile, the rate of seed germination and number of protocorm formation increased with increasing ultrasonic pre-treatment time (up to 10 min) without destroying embryos, showing significant effect especially for the cultivars characterized as low germination capability in nature.

Keywords: asymbiotic germination, activated charcoal, "Hyesung" x "Jeongmong", "Hwagung" x "Heysung", protocorm, ultrasonic pre-treatment.
Abbreviations: BA- 6-benzylaminopurine; IAA- indole-3- acetic acid; IBA- indole butyric acid; NAA- α-napthaleneacetic acid; PGR- plant growth regulator; PPF- photosynthetic photon flux .

Introduction

Calanthe hybrids ("Hyesung" x "Jeongmong", "Hwagung" x "Heysung") belonging to the *Orchidaceae* family are widely adapted to temperate, tropical and subtropical regions. Because of their beautiful big flowers with various attractive colors that are arranged in racemose inflorescence, these hybrids are getting popularity in Japan and Korea (Miyoshi and Mii, 1995a). In Korea, due to pleasing fragrance and big flowers with bright color, as well as cold hardiness during garden cultivation in the southern part, *Calanthe* hybrids rank among the most important orchids. However, despite of high reproductive value, it is very difficult to provide high quality products in a short time with uniform and steady state. On the other hand, the germination rate of *Calanthe* is very low (2%) in nature and plants that have been collected from natural habitats usually become quiescent and eventually disappear within 3-4 years. Therefore, to overcome the problem, it is crucial to establish an efficient *in vitro* culture protocol not only for propagation but also for breeding programs. It is plausible that seeds of the epiphytic and some terrestrial orchids can germinate asymbiotically on defined culture media. Some orchids, mostly terrestrial species from temperate regions, germinate poorly or not at all (Arditti, 1979). Aseptic cultures of orchid seeds on artificial media supplemented with various nutrients can increase germination rate, as well as promote flowering within 3-4 weeks from sowing provide advantages of yields. The potential advantages of propagation of *Calanthe* hybrids by asymbiotic seed germination have previously been reported by several authors (Miyoshi and Mii, 1988; Park *et al*., 2000; Lee *et al*., 2007). Various strategies, such as pre-soaking of seeds in water, organic solvent and phytohormones (Miyoshi and Mii, 1995a), NaOCl solution (Miyoshi and Mii, 1995b), pre-treatment by ultrasonication (Miyoshi and Mii, 1988) and addition of polyphenol absorbent to the medium (Miyoshi and Mii, 1995b) have been employed in efforts to improve the seed germination and /or protocorm formation of temperate species of *Calanthe*. Lee *et al*. (2007) have reported that scarification, for example, by prolonged treatment with chemical solutions and ultrasound stimulated the rate of germination of fully matured seeds of *Calanthe* from 32% to 39%. The successful stimulation on the rate of orchid seed germination by cytokinins has previously been reported for some members of the genus *Cypripedium* (Godo *et al*., 2010). Pre-soaking of *Calanthe discolor* seeds in a solution of 6-benzylaminopurine (BA) for 7 days was reported to have stimulatory effect on seed germination (Miyoshi and Mii, 1995a). However, to our knowledge, no reports are available for the germination of *Calanthe* hybrids seeds ("Hyesung" x "Jeongmong", "Hwagung" x "Heysung") *in vitro*. In this study, the significant role of activated charcoal, plant growth regulators and ultrasonic pre-treatment time on the asymbiotic seed germination and protocorm formation of *Calanthe* hybrids ("Hyesung" x "Jeongmong", "Hwagung" x "Heysung") has been discussed.

Table 1. Effects of different concentrations of activated charcoal, BA and NAA on seed germination of *Calanthe* hybrid 'Hyesung' × 'Jeongmong' after 120 days in culture

Cross (Female × male)	Treatment			No. of germinated seeds/culture
	PGR (mg l^{-1})		Activated charcoal(g l^{-1})	
'Hyesung' × 'Jeongmong'	Control		0	28±0.012[z]
			0.01	42±0.145
			0.1	54±0.244
	NAA	0.1	0	11±0.542
			0.01	18±1.21
			0.1	56±0.854
		0.5	0	1±0.124
			0.01	2±0.744
			0.1	12±1.22
		1.0	0	0
			0.01	0
			0.1	6±1.114
	BA	0.1	0	4±0.755
			0.01	37±0.552
			0.1	53±0.355
		0.5	0	46±0.981
			0.01	47±0.331
			0.1	56±0.681
		1.0	0	14±0.221
			0.01	7±0.447
			0.1	46±0.331

Seeds were germinated on modified Hyponex medium (Hyponex N: P: K = 20: 20: 20 = 1 g l^{1}; N: P: K = 6.5 : 4.5 : 19 = 1 g l^{-1}); Control- without plant growth regulators Each culture consisted of 10 Petri dish and each Petri dish contains 30 seeds, [z] – Values are mean of 3 replicates ± standard error (n=3).

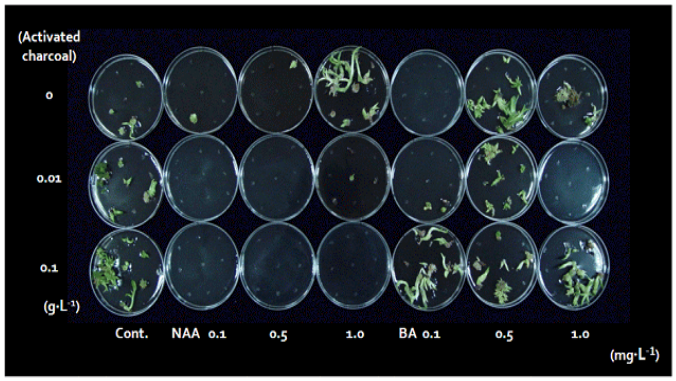

Fig 1. Effects of different concentrations of activated charcoal, BA and NAA on seed germination of *Calanthe* hybrid 'Hyesung' × 'Jeongmong' after 120 days in culture. (Control- without plant growth regulators).

Materials and methods

Plant materials and preparation of explants

Mature pods (120 days) of *Calanthe* hybrids were collected from the Society for the research of *Calanthe*, Korea. After 30 s immersion in 70% ethanol, surface impurities of the capsules were removed by cotton wool. These capsules were surface sterilized in 99% ethanol under aseptic conditions, then washed several times with sterile distilled water. Afterward, the capsules were excised and seeds were removed with a sterile scalpel.

Treatment procedure and culture media

The modified Hyponex medium (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 different concentrations of activated charcoal (0, 0.01, 0.1 g l^{-1}), NAA and BA (0, 0.1, 0.5, 1.0 mg l^{-1}), 2 g l^{-1} peptone, 50 ml l^{-1} coconut water, 15 g l^{-1} sucrose and 6.5 g l^{-1} agar was used in this study. The pH of the medium was adjusted to 5.6 before adding agar. The prepared seed explants were placed on plastic Petri dish containing 30 ml of medium according to the designated treatments. Cultures were maintained at the temperature of 25±2 ^{o}C, under a 20 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF) and 16 h photoperiod for 120 days.

Table 2. Effects of different concentrations of activated charcoal, BA and NAA on seed germination of *Calanthe* hybrid 'Hwagung' × 'Hyesung' after 120 days in culture

Cross (Female × male)	Treatment			No. of germinated seeds/culture
	PGR (mg l^{-1})		Activated charcoal (g l^{-1})	
'Hwagung' × 'Hyesung'	Control		0	9±0.447[z]
			0.01	4±0.221
			0.1	5±0.881
	NAA	0.1	0	1±0.078
			0.01	0
			0.1	2±0.991
		0.5	0	0
			0.01	0
			0.1	0
		1.0	0	0
			0.01	0
			0.1	2±0.224
	BA	0.1	0	0
			0.01	2±0.339
			0.1	8±0.841
		0.5	0	0
			0.01	3±0.961
			0.1	2±1.22
		1.0	0	0
			0.01	1±0.587
			0.1	0

Seeds were germinated on modified Hyponex medium (Hyponex N: P: K = 20: 20: 20 = 1 g l^{1}; N: P: K = 6.5 : 4.5 : 19 = 1 g l^{-1}); Control- without plant growth regulators Each culture consisted of 10 Petri dish and each Petri dish contains 30 seeds[z] – Values are mean of 3 replicates ± standard error (n=3).

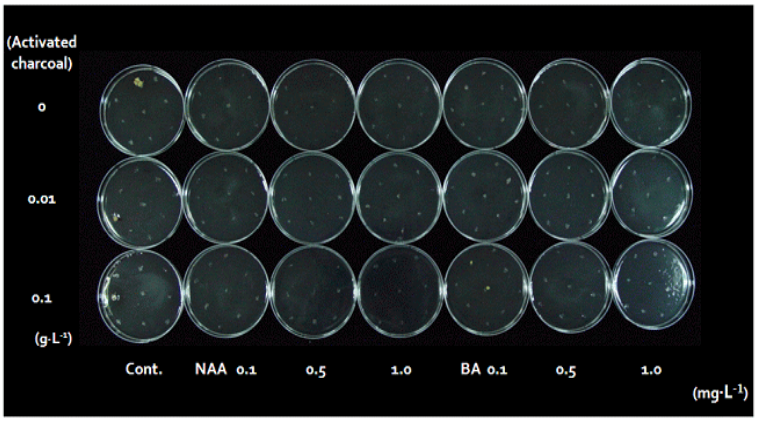

Fig 2. Effects of different concentrations of activated charcoal, BA and NAA on seed germination of *Calanthe* hybrid 'Hyesung' × 'Jeongmong' after 120 days in culture. (Control- without plant growth regulators).

Sonication to induce seed germination

Seed explants were transferred to a pre-sterilized glass tissue culture tube (2 ml) with plastic cap, comprised of 0.5 ml of seed containing solution inserted into the broth and 1 ml liquid modified Hyponex medium [Hyponex N : P: K = 20 : 20 : 20 = 1 g l^{-1}, N : P : K = 6.5 : 4.5 : 19 = 1 g l^{-1}, 2 g l^{-1} peptone, 50 ml l^{-1} coconut water with 15 g l^{-1} sucrose (pH 5.6)]. Sonication was done by an Ultrasonic Cleaner (5510R-DTH, Bransonic, USA) in ultrasonic bath at 42 kHz (±6%) of the frequency, 135 W per treatment under room temperature for 3-10 min. Afterward, centrifuged at 12000 rpm (Micro 17R, Hanil, Seoul, Korea) for 3 min. Then sonicated seeds (about 300) were transferred to solid modified Hyponex medium (supplemented with 0.1 g l^{-1} activated charcoal and 6.5 g l^{-1} agar) in plastic Petri dish containing 30 ml medium. Cultures were maintained at 25±2 ^{o}C temperature, under a 20 µmol m^{-2} s^{-1} of PPF and 16 h photoperiod for 4 mon.

Experimental Design

The experiments were laid out in Completely Randomized Design (CRD) with three replicates and each replicate consisted of a total of 300 seeds to measure the germination capability.

Table 3. Effects of ultrasonic pretreatment time on seed germination and protocorm formation of *Calanthe* hybrids 'Hyesung' × 'Jeongmong' and 'Hwagung' ×'Hyesung' after 120 days in culture

Cross (Female × male)	Treatment time (min)	No. of germinated seeds/culture	No. of protocorms/culture
'Hyesung' ×'Jeongmong'	0	53±0.892	8±0.421
	3	77±0.712[z]	21±0.336
	5	80±0.415	27±0.851
	7	89±0.991	25±0.331
	10	110±0.441	36±0.522
'Hwagung' × 'Hyesung'	0	6±0.662	2±0.421
	3	17±0.325	17±0.651
	5	19±0.585	19±0.055
	7	23±0.776	23±0.284
	10	34±0.359	14±0.745

Seeds were germinated on modified Hyponex medium (Hyponex N: P: K = 20: 20: 20 = 1 g l^{1}; N: P: K = 6.5 : 4.5 : 19 = 1 g l^{-1})
Each culture consisted of 10 Petri dish and each Petri dish contains 30 seeds, [z] – Values are mean of 3 replicates ± standard error

Fig 3. Observation of seed structure of *Calanthe* hybrid 'Hyesung' × 'Jeongmong' after ultrasonic treatment by using a stereo microscope (×200). Arrows indicate the seed coat which was destroyed.

Results and discussion

Activated charcoal and plant growth regulators on seed germination of Calanthe hybrids

A differential effect of activated charcoal and plant growth regulators on seed germination of *Calanthe* hybrids was observed during 120 days of culture period. In case of "Hyesung" x "Jeongmong" hybrid, numbers of germinated seeds were increased with increasing the concentrations of activated charcoal in the culture media without plant growth regulators (Table 1). Combinations of NAA (except 0.1 mg l^{-1}) or BA (except 0.5 mg l^{-1}) with activated charcoal (0.1 g l^{-1}) decreased the number of germinated seeds compared to the relative control (without plant growth regulators). In contrast, 0.5 mg l^{-1} BA was shown to be effective to increase the number of germinated seeds with all the combinations of activated charcoal compared to control. While the similar concentration of NAA showed inhibitory effect on the rate of seed germination (Fig. 1). The maximum numbers of germinated seeds (56) were observed in the cultures treated with 0.5 mg l^{-1} of BA or 0.1 mg l^{-1} of NAA in combination with 0.1 g l^{-1} of activated charcoal compared to the relative control (Table 1). However, the addition of activated charcoal with different concentrations of NAA or BA was proven effective on the rate of seed germination of "Hyesung" x "Jeongmong" hybrid. On the contrary, a reverse phenomenon was observed in case of "Hwagung" x "Heysung" hybrid. Neither activated charcoal nor plant growth regulators were shown to be effective to accelerate the rate of seed germination (Table 2, Fig. 2). Moreover, combinations of NAA and BA with activated charcoal acted as an inhibitory substance on the rate of seed germination of "Hyesung" x "Jeongmong" hybrid. Among the *Calanthe* hybrids, "Hyesung" x "Jeongmong" performed better regardless of germination capability (Fig. 1) compared to "Hwagung" x "Heysung" hybrid (Fig. 2). The asymbiotic germination of mature seeds of orchidaceous plants, such as members of the genera *Calanthe* is a complex phenomenon and numerous reports ascribed the truth. For instance, Godo *et al*. (2010) reported that both NAA and BA had stimulatory effects on the germination of mature seeds of *Calanthe tricarinata*, and BA

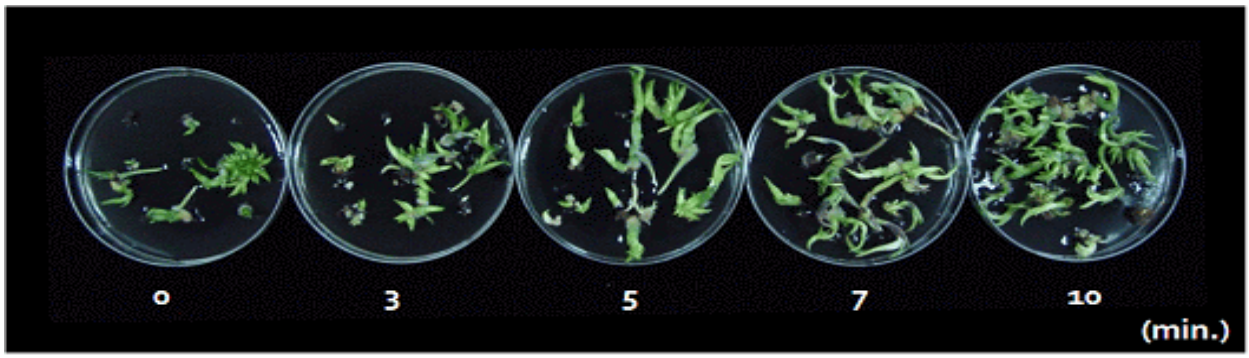

Fig 4. Effects of ultrasonic pretreatment time on seed germination of *Calanthe* hybrid ' Hyesung' × 'Jeongmong' after 120 days in culture.

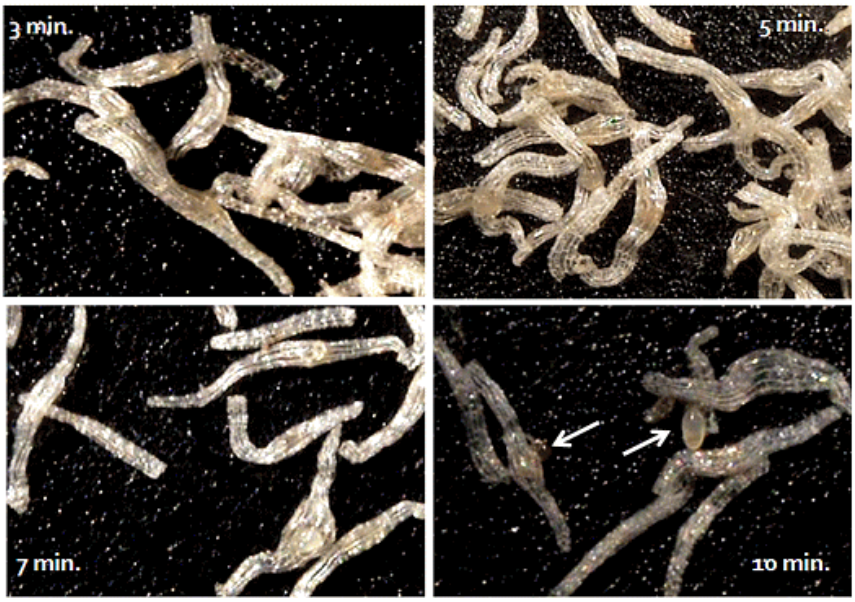

Fig 5. Observation of seed structure of *Calanthe* hybrid 'Hwagung' × 'Hyesung' after ultrasonic treatment by using a stereo microscope (×200). Arrows indicate the embryo get stripped off seed coat

was shown to be more effective than NAA. The chemical as well as the physical scarification of seeds of *Calanthe tricarinata* had a stimulatory effect on seed germination with frequencies reaching close to 40% in some cases (Lee *et al*., 2007). The authors have concluded that initiation of germination was accelerated by the addition of BA to the medium, whereas only a few seeds were germinated in the absence of BA. However, the responses of orchid seeds to cytokinins varied from species to species, and the promotive effects on seed germination of several cytokinins, such as BA, thidiazuron (TDZ), zeatin and kinetin were reported in *Cypripedium macranthos* (Miyoshi and Mii, 1998) and *Habenaria macroceratitis* (Stewart and Kane, 2006). By contrast, the effects of auxins, such as NAA, IBA and IAA in the germination medium have been reported to have smaller promotive effects than those of cytokinins on the germination of seeds of terrestrial orchids (Godo *et al*., 2010). In this present study, either NAA or BA were observed effective to promote germination of *Calanthe* hybrid seeds, while activated charcoal significantly accelerated the rate of seed germination of "Hyesung" x "Jeongmong" *Calanthe* hybrid (Table 1). Furthermore, higher concentration (1.0 mg l^{-1}) of NAA or BA (1.0 mg l^{-1}) in combination with lower concentration of activated charcoal (0.01 g l^{-1}) strongly inhibited seed germination of both *Calanthe* hybrids. Our results are partially in consistent with the findings of several authors (Yam *et al*., 1990; Chu and Mudge, 1994; Michel, 2002) where they reported that plant growth regulators, coconut milk, banana homogenate and agar supported Murashige and Skoog (MS) medium did not promote germination of orchid and *Corchorus olitorius* seeds, rather addition of banana homogenate and agar in the culture medium showed hinder effects on seed germination (Arditti, 1982; Islam *et al*., 2009). Therefore, an exhaustive survey of the effects of auxins and cytokinins is required on the germination of *Calanthe* hybrid seeds in asymbiotic culture *in vitro*.

Ultrasonic pre-treatment time on the seed germination of Calanthe hybrids

Ultrasonic treatment is an interesting physical tool for manipulation of cells and organs. Over the past few decades, various biological effects attributable to ultrasonic treatment have been described in different plant species (Ananthakrishnan *et al*., 2007). In this study, ultrasonic treatment time significantly affected on seed germination and protocorm formation of *Calanthe* hybrids (Table 3). In case of "Hyesung" x "Jeongmong" hybrid, numbers of germinated seeds and formed protocorms were increased with increasing ultrasonic pre-treatment time (Table 3, Fig. 4). In contrast, similar trend was observed in "Hwagung" x "Hyesung" hybrid

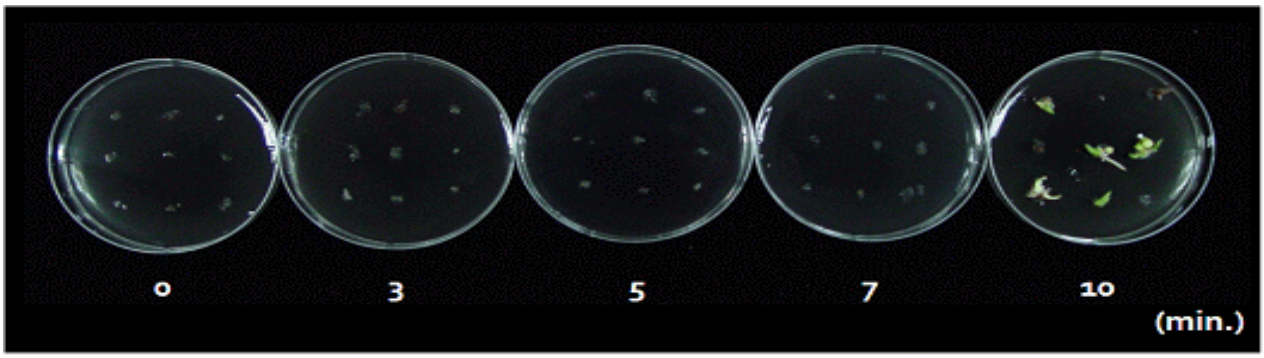

Fig 6. Effects of ultrasonic pretreatment time on seed germination of *Calanthe* hybrid 'Hwagung' × 'Hyesung' after 120 days in culture.

concerning the seed germination (Fig. 6), while protocorm formation increased up to 7 min of ultrasonic pre treatment time followed by decreased but maintain higher value compared to the control (without sonication) treatment (Table 3). However, maximum numbers of germinated seeds (110/culture) and formed protocorms (36/culture) were observed in "Hyesung" x "Jeongmong" hybrid at 10 min ultrasonic pretreatment. The same treatment time inhibited protocorm formation (2.5-fold) in "Hwagung" x "Hyesung" hybrid compared to "Hyesung" x "Jeongmong" hybrid. It has been reported that plants exposure to non-lethal levels of ultrasound exhibit altered characteristics at different organizational levels. At the organism (plant) level ultrasound enhances the germination of various seeds and the subsequent growth of seedlings (Miyoshi and Mii, 1988; Gaba *et al*., 2006). In *Calanthe discolor*, the rate of seed germination was significantly increased (up to 60%) in the cultures treated by sonication for 4-16 min (Miyoshi and Mii, 1988). The authors have demonstrated that after 4 min of sonication treatment the seed coat was removed from almost all the seeds. Prolonged treatment of more than 8 min increased the percentage of destroyed embryos. However, the effect of ultrasonic treatment appears to vary widely between species and cultivars. In our study, we observed a differential stimulatory effect of ultrasonic pre-treatment time on the number of germinated seeds and protocorm formation. The ultrasonic pretreatment affected differentially both seed germination and protocorm formation in "Hyesung x Jeonmong" and "Hwagung" x "Hyesung" hybrids. Moreover, ultrasonic pretreatment over 7 min strongly inhibited protocorm formation in "Hwagung" x "Hyesung" hybrid. The stereo microscopic observation of seed structure in "Hyesung" x "Jeongmong" hybrid upon ultrasonic pretreatment showed that seeds exposure to less than 10 min of ultrasonic pretreatment, almost all of the seeds coat removed without destroying the embryos (Fig. 3). Therefore, the numbers of germinated seeds and formed protocorms were stimulated in this hybrid. On the other hand, prolonged treatment time (≥ 7 min) increased the percentage of destroyed embryos in "Hwagung" x "Hyesung" hybrid that leads to decrease in number of germinated seeds and formed protocorms compared to "Hyesung" x "Jeongmong" hybrid (Fig. 5). The differential effects of ultrasonic pre-treatment have previously been described by several authors. For instance, mild ultrasonic irradiation significantly stimulated protein synthesis in plant cells and protoplasts (Joersbo and Brunstedt, 1990) and affected plasma membrane permeability (Dong *et al*., 2002). Ultrasonic treatment was reported to cause reversible inhibition of DNA, RNA and protein synthesis in *Pisum sativum* root meristem cells (Miller *et al*., 1976). In *Orchis* papilionacea seeds, a low dose of ultrasound damaged the testa and therefore permitting *in vitro* germination and subsequent protocorm and minituber production (Pedroso and Pais, 1992). Presumably, sonication pretreatment for 3-10 min damaged the testa and therefore permitting seed germination and protocorm formation of "Hyesung" x "Jeongmong" hybrid in this study. In conclusion, the efficient seed germination of *Calanthe* hybrids was achieved with the use of modified Hyponex media supplemented with 0.1 g 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 addition of NAA or BA in the modified Hyponex media did not show promotive effects on seed germination. On the contrary, ultrasonic pre-treatment time up to 10 min regarded as non-lethal at organizational levels and therefore facilitated the rate of seed germination of "Hyesung" x "Jeongmong" *Calanthe* hybrid characterized as low germination capability in nature. These results suggest that the methodology developed here could be a useful tool for restoration of endangered species and commercial exploitation of orchid cultivation in the field of biotechnology.

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