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Research Article

Effects of NaOCl treatment on *in vitro* germination of seeds of a rare endemic plant, *Oreorchis coreana* Finet

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Abstract *Oreorchis coreana* Finet is threatened globally by over-collection from its natural habitats for horticultural purposes. Its rarity in nature makes this plant one of the most endangered species in Korea. In this study, we investigated the effects of sodium hypochlorite (NaOCl) on orchid seed viability and seed germination. An *in vitro* bioassay swelling test using immature seeds was compared with a standard chemical procedure using triphenyl tetrazolium chloride (TTC) to test seed viability. In general, the bioassay was more appropriate for estimating embryo viability after a prolonged pre-treatment (more than 1 h) in 1% NaOCl, a surface sterilant often used to enhance germination of seeds of terrestrial plants. Therefore, an efficient method for investigating *in vitro* swelling of immature seeds is urgently needed. We established a method for determining the viability and swelling of *O. coreana* seeds *via in vitro* examination of immature seeds. Treatment of immature seeds with 1% NaOCl for 10 min greatly enhanced the extent of swelling of immature zygote embryos when compared to untreated seeds. These data obtained here appear to be comparable to viability and swelling that occurs in *O. coreana* seeds *via* asymbiotic germination.

Keywords Germination, Orchid, *Oreorchis coreana* Finet., Seed viability

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Introduction

Oreorchis coreana Finet (Orchidaceae) was reported as a new species by Finet in 1908. In 1935, however, Maekawa described a new genus, *Dipiolabellum*, based on that species because it lacked the caudicle that is a diagnostic character in *Oreorchis*. Since Maekawa's treatment, this species has been reported by some authors as endemic to Korea (Lee 1996; Lee 1984; Paik 1999), whereas Lee (2006) has either retained it as a species or treated it as a subspecies of *Oreorchis*. The species is classified as VU (Vulnerable) in the IUCN Red List categories (Lee and Choi 2006; IUCN 2009) and *O. coreana* is found in only an extremely small site (Gotchawal province) on Jeju Island. The stems of *O. coreana* are 18 ~ 30 cm in high. The flowers are showy with white or brown coloration on the surface of petals and sepals (Maekawa 1935; Fig. 2A).

In vitro propagation techniques have been widely used for the conservation of threatened orchid species (Stewart and Kane 2006a; Stewart and Kane 2006b; Stewart and Kane 2007; Bae et al. 2009; Dutra et al. 2009; Suzuki et al. 2009; Bae et al. 2010; Bae and Choi 2011). Although this approach has been used for a long time, scientific research on the *in vitro* germination of orchid seeds is limited, especially when considering the large number of species in the family. Moreover, most of the published studies have focused on those species that are partial to terrestrial habits and/or temperate climates.

In a previous paper, we reported that sodium hypochlorite

(NaOCl) treatment, a common method for disinfection of plant materials, was very effective at stimulating *in vitro* germination of *O. coreana* seeds. The effectiveness of disinfection solution such as NaOCl and calcium hypochlorite ($Ca(ClO)_2$) for stimulation of germination of orchid seeds has been reported in other species (Malmgren 1996; Miyoshi and Mii 1998; Bae et al. 2009; Bae et al. 2010, Bae and Choi 2011). The mechanism underlying this stimulating effect on germination of orchid seeds is not well under stood (Harvais and Hadley 1967).

In vitro germination of seeds can be an efficient way to propagate the *O. coreana* species, but micropropagation of *Oreorchis* spp. *via in vitro* culture of immature seeds has not been reported previously. Therefore, the aims of this study were first to describe seed viability and embryo development in this species and then to evaluate and establish a method for *in vitro* culture of immature seeds from *O. coreana*.

Materials and methods

Plant materials and culture conditions

Capsules of *O. coreana* were collected immediately prior to dehiscence from a single population near Aewol Gotchawal province (Fig. 1A, B), Jeju Island, in late October 2009 (immature capsules from six plants, Fig. 2B), and early November 2009 (mature capsules from four plants). All orchids have an obligate relationship with mycorrhizal fungi during seed germination and development (Rasmussen 1992). *O. coreana* also has an association with a mycorrhizal fungus in the root (Fig. 2C, D). Following collection, capsules were placed in paper bags and stored at room temperature for 5 days in a cardboard box, and then transferred to a domestic refrigerator and stored at 5℃ until used for germination trials. Initially, the seeds were immersed in deionized, sterilized water and agitated for 30 min. Seeds were then treated with 30 mL 1% NaOCl in deionized water (v/v) for 10 min, followed by three 30s rinses in deionized, sterilized water. Seeds were left in the final rinse water until transfer to half strength MS medium (Murashige and Skoog 1962). Two basal media were used in this study: 1/2 Phytomax Orchid Maintenance (POM), POM, 1/2 MS, and MS media without plant growth regulators. All media were supplemented with 20 g L^{-1} sucrose and pH was adjusted to 5.5 with 0.1 M KOH before the addition of 3.0 g L^{-1} Gelrite. Media were autoclaved at 117.7 kPa and 121℃ for 15 min. The cultures were maintained in a growth room at 20 ± 2℃.

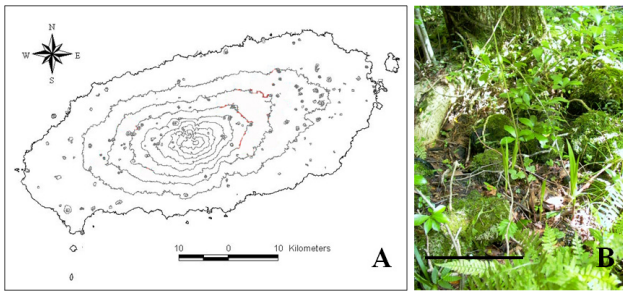

Fig. 1 Collected area and habitate of *O. coreana*. A: Map of Jeju island (Red line was Aewol Gotchawal), B: Habitate of *O. coreana*

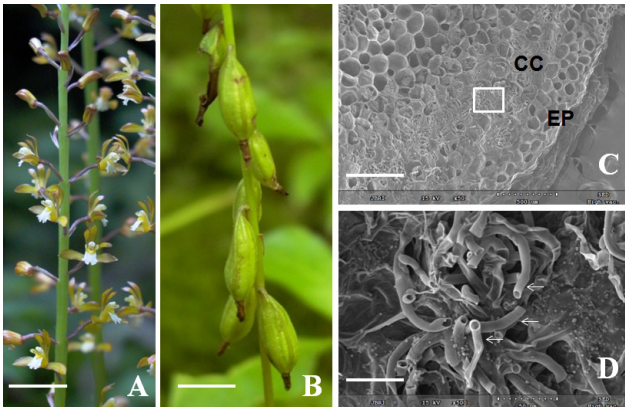

Fig. 2 Morphology and anatomical studies showing association of the fungus in the root of *O. coreana*. A: flowers. B: 4 month old fruits. C: The entry of the fungal hyphae through the cortical cell, Bars indicate 500 um. D: Closed view of white square, Bars indicate 50 um. EP: Epidermis cell, CC: Cortical cell, White arrows indicated fungal hyphae

Viability test for seeds treated with sodium hypochlorite

Immature seeds of *O. coreana* were treated for 0, 10, 30, or 60 min with 1% NaOCl and then rinsed three times with sterile water. Seed viability was evaluated using the Tetrazolium test (Lakon 1949), consisting of a solution of 2,3,5-triphenyltetrazolium chloride (TCC), which stains viable embryos red. Seeds were immersed in 1% TCC and stored in the dark for 24 h at 30℃. Samples of 1,000 seeds per fruit were analyzed under an optical microscope before and after surface sterilization. The percentage of viable seeds was calculated by dividing the number of viable embryos by the total number of embryos analyzed.

Effect of culture medium on seed germination

Immature capsules were sterilized in 1% NaOCl for 15 min, and then rinsed three times with sterile water. The seed capsules were then cleaved with a scalpel blade and

the seeds were scraped off. The seeds were treated with 1% NaOCl for 10 min and rinsed three times with sterile water. The disinfected seeds were then cultured on POM medium (Sigma, USA) modified with MS medium at 20 ± 2℃ in the dark. Embryo swelling was defined as at least a doubling in size and formation of a protocorm. The numbers of swollen embryos were recorded after 12 weeks of culture by examination with a microscope.

Statistical analysis

All data were expressed as means ± standard error (SE) analyzed using analysis of variance (ANOVA). Each experiment was replicated three times with at least 200 seeds per replication. Significant differences among the treatments were determined by performing multiple comparison tests using Duncan's multiple range test at $\alpha \leq 0.05$ (SAS).

Results and discussion

Effect of NaOCl treatment on seed viability and germination

Viable embryos of *O. coreana* seeds turn red with tetrazolium chloride and NaOCl pre-treatment resulted in coloration of the embryos (Fig. 3A, B), while non-surviving embryos remained uncolored (Fig. 3C). Tetrazolium chloride (TTC) viability testing following different treatment times with 1% NaOCl indicated that a 10 min resulted in 74.5% viability compared to the observed percentage of swollen embryos (84.4%) and protocorm formation (81.5%) after 12 weeks of culture (Table 1).

The effects on embryo swelling were first scored at 10 weeks (Fig. 3D) and indicated that this was a useful bioassay for estimating the viability of mature seed of terrestrial orchids, together with TTC staining. Pigment production could be an alternative to the standard chemical methods (Singh 1981; van Waes and Debergh 1986; Lauzer et al. 1994), particularly when the latter are not easily applied. Moreover, the coefficient of the bioassay staining was similar to that of the TTC and AF methods. The test worked well on seeds pretreated for more than 2 h in 10% NaOCl, which generally promotes dormancy release in terrestrial orchid seeds (St-Arnaud et al. 1992; Malmgren 1996). Lauzer et al. (1994) pointed out that the TTC staining assay, based on the activity of dehydrogenases (MacKay 1972), could produce an over-estimation of *Cypripedium acaule* seed viability when seeds were subjected to a prolonged pretreatment. Pigment synthesis by fungi has frequently been reported in the context of seed coloration (Wicklow et al. 1987; McLean and Ray 1988) or fungal taxonomical characterization (Marrasas et al. 1998). In addition, inoculation of red fungi onto the seed

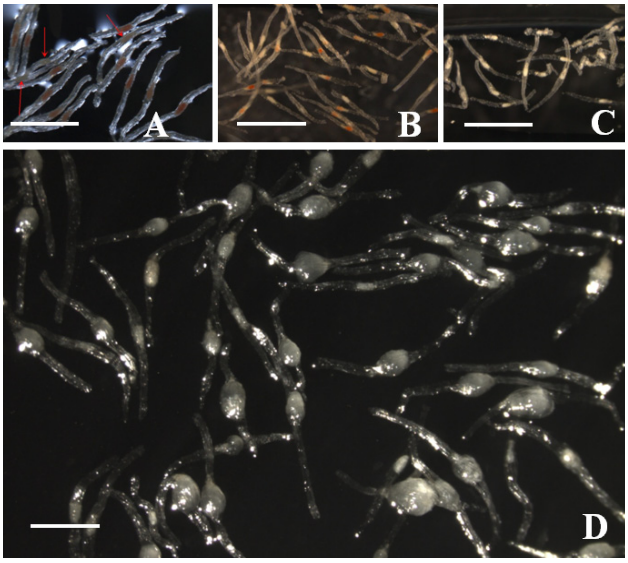

Fig. 3 Tetrazolium test and seed germination of *O. coreana*. Treatment of 1% NaOCl for 0 min (A), 10 min (B), 60 min (C), Red arrows indicated survived embryos after treatment of 1% NaOCl indicated treated time. D: Asymbiotically germinated seeds are white. Bars indicate 50 um

Table 1 Effect of treatment time of 1% NaOCl on swelled embryo formation and protocorm formation from seed of *O. coreana* after 12 weeks of culture on POM medium supplemented with sucrose 20 g/L and gerlite 3.0 g/L

Treatment time (min)	Seed viability (%)	Embryo stage		Protocorm stage	
		Swelled embryos formation (%)	Diameter of embryo (um)	Protocorm formation (%)	Diameter of protocorm (um)
0	98.7 ± 0.9*a	32.8 ± 2.7*b	21.6 ± 6.9d	25.2 ± 2.8b	47.5 ± 7.9d
10	74.5 ± 12.8b	84.4 ± 7.7a	88.4 ± 4.9a	81.5 ± 3.2a	174.2 ± 18.9a
30	14.9 ± 5.4c	15.3 ± 2.6c	67.1 ± 3.9c	13.2 ± 2.4c	142.6 ± 19.9c
60	2.6 ± 0.7d	2.2 ± 0.8d	72.9 ± 8.9b	1.8 ± 0.4d	166.4 ± 17.5b

*Data are the means ± SD, of three experiments. Different alphabetical letters are significantly different according to Duncun's multiple range test at $P < 0.05$.

at sowing has been suggested to extend fungal activity. The effectiveness of disinfection solutions such as NaOCl and $Ca(ClO)_2$ for stimulation of germination of orchid seeds has been reported in some orchid species (Miyoshi and Mii 1998; St-Arnaud e tal. 1992, Malmgren 1996), but its mechanism has not yet been documented. Possible mechanisms of action underlying the induction of seed germination or breaking dormancy by NaOCl have been considered to be partial degradation of the seed coat and/or the solubilization and oxidation of some type of growth inhibitor. Harvais (1982) interpreted the stimulating effect of surface sterilization with NaOCl as a physiological effect of washing away the endogenous inhibitor abscisic acid (ABA) from the seeds.

Effect of culture medium on seed germination

The effect of NaOCl pre-treatment was examined on seed germination. When immature seeds of *O. coreana,* without 1% NaOCl treatment, were cultured on POM medium, they were not responsive at all during 8 weeks of culture (Fig. 4A). After culture for 12 weeks in POM medium, the seed swelling was very low in the untreated seeds compared to the seeds treated with NaOCl (Table 1, Fig. 4C, D). In contrast, immature seeds pre-treated with NaOCl began to swell within 8 weeks (Fig. 4B). Seeds pre-treated with NaOCl on POM medium showed a greatly enhanced frequency of swollen embryos formed from immature seeds (Table 2). The frequency of embryo swelling from immature seeds reached 85.1% after 12 weeks of culture (Table 2). The maximum protocorm formation was recorded for the seeds treated with 1% NaOCl for 10 min and cultured on POM medium (87.5%), followed by 1/2MS (74.6%), and MS (80.5%), whereas 1/2POM (81.2%) was the least effective treatment medium for protocorm formation. The morphological development of *O. coreana* from seed to seedling was documented (Fig. 4D).

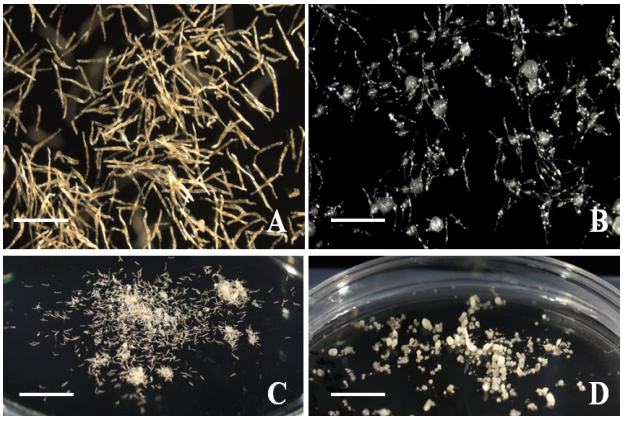

Fig. 4 Germination of mature seed from *O. coreana*. A-B: Treatment without 1% NaOCl (A), with 1% NaOCl (B) for 6 weeks C-D: Treatment without 1% NaOCl (C), with 1% NaOCl (D) for 12 weeks D: 1% NaOCl treated Development of germinated embryos. Bars indicate 20 um.

Seed germination of orchid species is typically very low or nonexistent in *ex vitro* and *in vitro* conditions (Ault and Blackmon 1987; Anderson 1996). Terrestrial orchids have more stringent requirements for germination but little is known about the specific requirements for each species (Fast 1982). NaOCl is a disinfecting agent that is widely used for seed surface sterilization (Bewley and Black 1994; Miyoshi and Mii 1998) and it is also known to favor seed germination or to overcome seed dormancy in some species (Vujanovic et al. 2000). In *C. macranthos*, the frequency of germination was 67% after sterilization with NaOCl (Miyoshi and Mii 1998). Yildiz and Celal (2002) reported that pre-treatment of *Linum usitatissimum* seeds with NaOCl for 20 minute enhanced germination. The promotion of germination by a NaOCl treatment is thought to be due to scarification of the seed coat, which allows more water and oxygen absorption or to the enhancement of oxidative respiration by an extra supply of oxygen arising from decomposition of NaOCl (Vujanovic et al. 2000).

In conclusion, we demonstrated that immature seeds of *O. coreana* showed enhanced viability and embryo swelling

Table 2 Effect of different culture medium on swelled embryo formation and protocorm formation from seed of *O. coreana* treated 1% NaOCl for 10 minute after 12 weeks of culture

Culture medium	Embryo stage		Protocorm stage	
	Swelled embryos formation (%)	Diameter of embryo (um)	Protocorm formation (%)	Diameter of protocorm (um)
1/2MS	78.2 ± 1.9*b	77.8 ± 1.9c	74.6 ± 1.2d	181.6 ± 4.6b
MS	74.6 ± 6.3c	81.4 ± 3.2b	80.5 ± 1.2bc	179.2 ± 4.1c
1/2POM	72.2 ± 1.8d	76.1 ± 2.8cd	81.2 ± 2.6b	178.6 ± 2.8cd
POM	85.1 ± 6.1a	87.1 ± 5.3a	87.5 ± 1.8a	188.4 ± 5.1a

*Data are the means ± SD, of three experiments. Different alphabetical letters are significantly different according to Duncun's multiple range test at $P < 0.05$.

in response to a pre-treatment with NaOCl, which resulted in a high frequency of germination of seeds of this orchid. This protocol offers could be used by commercial nurseries for large-scale propagation as well as for *ex situ* conservation of *O. coreana*.

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