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Research Article

Sodium hypochlorite treatment and light-emitting diode (LED) irradiation effect on *in vitro* germination of *Oreorchis patens* (Lindl.) Lindl

Kee Hwa Bae · Kyoung Hee Oh · Soo-Young Kim

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Abstract In this study, we investigated the effects of sodium hypochlorite (NaOCl) and red or blue light-emitting diode (LED) light on embryo swelling and germination of *Oreorchis patens* (Lindl.) Lindl. A method for determining the swelling and protocorm formation in *O. patens* seeds through *in vitro* examination of immature seeds was established. NaOCl treatment of immature seeds was found to significantly enhance the extent of embryo swelling and protocorm formation in immature zygote embryos compared to those in the untreated controls. Additionally, the effects of white fluorescent light, and red and blue LED lights on embryo swelling and protocorm formation in *in vitro* cultured seeds were examined and compared to the conditions with or without NaOCl treatment. The most suitable light for embryo swelling and protocorm formation was the red LED light.

Keywords Germination, Medium, Orchid, *Oreorchis patens*

Introduction

Oreorchis patens (Lindl.) Lindl. belongs to the Orchidaceae and is distributed across East Russia (Amur, Kamchatka, Sakhalin, and Ussuri), China, Japan, and Korea (Lee and Choi 2006). It commonly grows in temperate deciduous forest margins, thickets, grassy places, shaded places, and along valleys in the main ranges of Baek-du-dae-gan (Lee and Choi 2006). This plant produces scapes (30-50 cm tall) from May to July, each bearing 20-35 whitish-yellow flowers (7-15 mm long; 1.5-3 mm diameter) (Lee 2006). *In vitro* seed culture techniques of various orchid species have been widely used for its conservation and propagation (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 2013). Although this approach has been used for a long time, reports on *in vitro* germination of orchid species are limited, despite its large number of species.

It has been previously reported that sodium hypochlorite (NaOCl) treatment, a common method for disinfecting plant materials, is highly effective in stimulating *in vitro* germination of *Oreochis coreana* seeds (Bae et al. 2013). The effectiveness of seed disinfection using NaOCl and calcium hypochlorite ($Ca(ClO)_2$) solution to stimulate orchid seed germination has also been reported in other species (Malmgren 1996; Miyoshi and Mii 1998; Bae et al. 2009; Bae et al. 2010; Bae and Choi 2013). The mechanism underlying this stimulatory effect on orchid seed germination is not well understood (Harvais and Hadley 1967).

Light-emitting diodes (LEDs) have recently developed as an alternative light source for plant culture systems. They have several advantages compared to conventional light sources, including their wavelength specificity and narrow bandwidth (Bula et al. 1991; Hoenecke et al. 1992). LEDs have been used for studies on chlorophyll biosynthesis in wheat (Tripathy and Brown 1995), stem elongation and leaf expansion in lettuce (Hoenecke et al. 1992), disease development in pepper and cucumber (Schuerger and Brown 1994), and photosynthesis in kudzu (Tennessen et al. 1994).

However, to date, no effort has been made to develop a protocol for *in vitro* propagation or conservation of *O. patens*. This study aimed to describe seed germination and embryo development in *O. patens* and evaluate and establish a method for *in vitro* culture of immature seeds of *O. patens*.

K. H. Bae · K. H. Oh · S.-Y. Kim (✉)
National Institute of Biological Resources, 42 Hwangyeong-ro, Seo-gu, Incheon, 404-708, Republic of Korea
e-mail: sy7540@korea.kr

Materials and methods

Plant materials and culture conditions

The fruits of *Oreochis patens* were collected immediately before dehiscence from a single population near the Hambaek Mountain (Kangwon Province) in late September 2013 (voucher specimen, VP0000288327 and seed number, GR0000255167). Following collection, the seeds were immersed in deionized sterile water and agitated for 30 min. The seeds were then treated with 30 ml of 1% NaOCl in deionized water (v/v) for 30 min, followed by three 30s rinses in deionized sterile water. All media were supplemented with 20 g/L sucrose and the pH was adjusted to 5.5 with 0.1 M KOH before the addition of 3.0 g/L gelrite. The media were autoclaved at 117.7 kPa at 121 °C for 15 min. The cultures were maintained in a growth room at 20 ± 2°C.

Effects of NaOCl and culture medium on embryo swelling and seed germination

Mature capsules were sterilized in 1% NaOCl for 30 min and then rinsed three times with sterile water. The seed capsules were then cleaved using a scalpel blade and the seeds were scraped. The seeds were treated with 1% NaOCl for 30 min. The seeds were then left in the final rinse water until their transferred to Phytomax Orchid Maintenance (POM) medium (P6668, Sigma Co. Ltd., USA) and Seed Germination Maintenance (SGM) medium (P6543, Sigma Co. Ltd., USA). Two basal media were used in this study: POM and SGM media without plant growth regulators. Embryo swelling was defined as an increase in size of at least twice the original size and the formation of a protocorm. The number of swollen embryos and the diameters of the embryos were recorded at 2 week intervals by examining under a microscope.

Effects of NaOCl and light emitting diodes (LEDs) on embryo swelling and seed germination

The seeds were treated with 1% NaOCl for 30 min. and were then left in the final rinse water until these were transferred to the POM medium. Cultures were incubated in growth chambers (Sejong Scientific, Korea) at 25°C and 60% relative humidity. The seeds were subjected to three different cultures treatments by using the following sources of illumination : (1) fluorescent light, (2) red LED (peak wavelength: 660 nm), (3) blue LED (peak wavelength: 450 mm). The duration of lighting for all treatments was 16 h per day. Embryo swelling was defined as an increase in size of at least twice its original size and the formation of a protocorm. The numbers of swollen embryos and the diameters of the embryos were recorded at 4-week intervals by examining under a microscope.

Statistical analysis

All data were expressed as means ± standard error (SE) and subjected to analysis of variance (ANOVA). Each experiment was conducted in triplicate, with at least 200 seeds per replicate. Significant differences among the treatments were determined using Duncan's multiple range test at $P < 0.05$ (SAS, 2003).

Results and discussions

NaOCl treatment and culture medium condition for seed germination

The effect of NaOCl pre-treatment on seed germination was examined. After 30 min of 1% NaOCl treatment, the seed coat was completely bleached and the zygotic embryos inside of seed coats could be clearly seen (Fig. 1A). Swollen embryos were first scored 4 weeks after sowing (Fig. 1B). After NaOCl pretreatment, the frequency of embryo swelling was observed after 8 weeks of culture. The highest rate of swelling of zygotic embryos was observed in 30 min with 1% NaOCl in POM medium (Table 1). The rate of embryo swelling in POM medium (90.7%) was significantly greater than that in SGM medium (60.4%). When untreated (without 1% NaOCl) immature seeds of *O. patens,* were cultured in SGM and POM medium, the rate of swelling was 38% and 29%, respectively, after 4 weeks of culture (Table 1). After 8 weeks of culture of the mature seeds in POM medium without NaOCl treatment, the rate of protocorm formation was very low compared to the seeds treated with NaOCl (Table 1). In contrast, immature seeds subjected to NaOCl pre-treatment showed signs of swelling within 8 weeks of culture (Fig. 1C). NaOCl pretreatment of seeds in POM medium showed a significantly higher rate of protocorm formation compared to that of the immature seeds. The rate of protocorm formation in immature seeds was 58.8% after 8 weeks of culture (Table 1). The maximum rate of protocorm formation with a pretreatment of 1% NaOCl for 30 min and culturing in POM medium was 87.5%. The morphological development from seed to protocorm in *O. patents* was documented

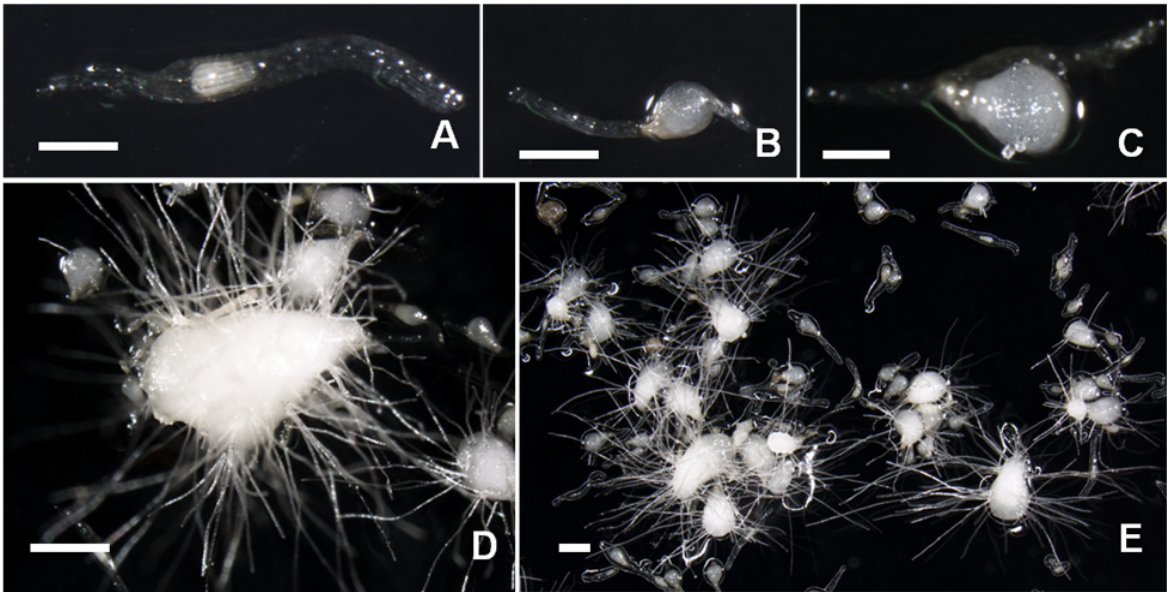

Fig. 1 Developmental stages of seed culture for *O. patens*. A: Hyaline embryo, testa intact (scale bar, 0.1 mm), B: Embryos swollen (scale bar, 0.1 mm), C: Swelled embryos present rhizoids (scale bar, 0.1 mm), D: Closed view appearance of protomeristem and rhizoids elongation (scale bar, 1.0 mm), E: Appearance of protomeristem and rhizoids elongation (scale bar, 1.0 mm)

Table 1 Effect of NaOCl treatment time for embryo swelling and protocorm formation of *O. patens* seeds after 8 weeks culture on medium supplemented with sucrose (20 g/L) and gerlite (3.0 g/L)

NaOCl treatment (min)	Swelled embryos formation (%)		Protocorm formation (%)	
	SGM	POM	SGM	POM
0	38.6 ± 8.1*b	29.1 ± 5.3*b	26.2 ± 4.4b	10.8 ± 3.5*b
30	60.4 ± 6.1a	90.7 ± 3.4a	35.3 ± 4.3a	58.8 ± 2.2a

*Data are the means ± SD, of three experiments. Different alphabetical letters are significantly different according to Duncun's multiple range test at $P < 0.05$. SGM and POM means Seed Germination Medium and Phytomax Orchid Mainterence Medium.

(Figs. 1D-E). The biggest embryo diameters were observed in seeds treated with 1% NaOCl for 30 min and cultured in POM medium (Fig. 2D); these were followed by seeds cultured in SGM (Fig. 2C), and those not treated with NaOCl and cultured in SGM (Fig. 2A). On the other hand, seeds not treated with NaOCl and cultured in POM (Fig. 2B) showed the smallest embryo diameters. 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, and information on the specific requirements for each species is limited (Fast 1982). NaOCl is a disinfecting agent that has been widely used for seed surface sterilization (Bewley and Black 1994; Miyoshi and Mii 1998). This reagent is also known to induce seed germination or overcome seed dormancy in some species (Vujanovic et al. 2000). In *Cypripedium macranthos*, the frequency of germination was 67% after sterilization with NaOCl (Miyoshi and Mii 1998). Yildiz and Celal (2002)

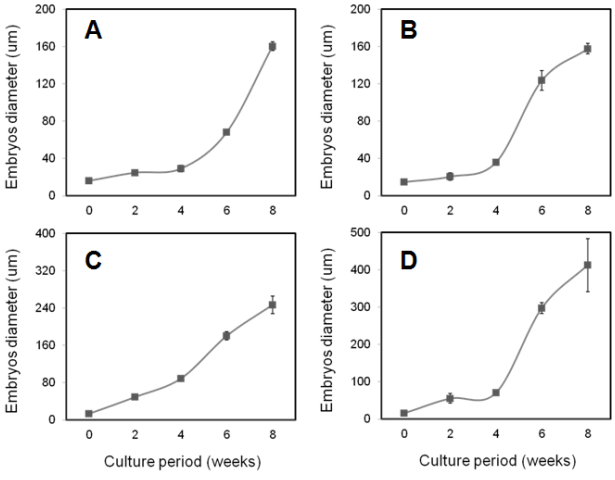

Fig. 2 Effects of culture media and NaOCl pre-treatment on frequency of embryos diameter after 0, 2, 4, 6 and 8 weeks *in vitro* culture. A: Non treated NaOCl for seeds on SGM, B: Non treated NaOCl for seeds on POM, C: NaOCl treated for 30 min on SGM, D: NaOCl treated for 30 min on POM. Vertical bars indicate standard errors (n=3)

previously reported that the pre-treatment of *Linum usitatissimum* seeds with NaOCl for 20 min enhanced its germination. The induction of germination through NaOCl treatment is thought to be due to scarification of the seed coat, which facilitates water and oxygen absorption or enhances oxidative respiration by producing more oxygen through the decomposition of NaOCl (Vujanovic et al. 2000). The effectiveness of disinfection solutions such as NaOCl and $Ca(ClO)_2$ in stimulating the germination for orchid seeds has been reported in other species (Miyoshi and Mii 1998; St-Arnaud et al. 1992; Malmgren 1996), however, its mechanism has not yet been established. Possible mechanisms of action underlying the induction of seed germination or cessation of dormancy by NaOCl might include partial degradation of the seed coat and/or the solubilization and oxidation of certain growth inhibitors. Harvais (1982) interpreted the stimulatory effect of surface sterilization with NaOCl as a physiological effect of washing away the endogenous inhibitor, abscisic acid (ABA), from the seeds.

NaOCl and light source condition for embryo swelling and seed germination

The rate of embryo swelling after 4 weeks of sowing was 88.4, 90.6, and 54.2% under white, red, and blue LED -lights, respectively, in POM medium (Table 2). The 625 nm wavelength (red LED-light) was associated with the highest rate of embryo swelling, although this also strongly inhibited protocorm formation (Table 2, Fig. 3B) after 8 weeks of culture. The highest rate of protocorm formation was observed in seeds treated with 1% NaOCl for 30 min and cultured under red LED -light, followed by white -light (91.8%, Fig. 3A), and blue LED -light (61.7%, Fig. 3C). On the other hand, the treatment without NaOCl in POM medium under white, red, and blue LED -lights was the least effective for protocorm formation (Table 2, Figs. 3D-F). The induction of embryo swelling, seed germination, and protocorm growth by using LEDs has also been previously reported in *Calanthe satsuma* (Fukai et al. 1997). The inhibition of seed germination by lighting has been

Table 2 Effect of NaOCl treatment time and light source (LED) for embryo swelling and protocorm formation of *O. patens* seed after 10 weeks culture on medium supplemented with sucrose (20 g/L) and gerlite (3.0 g/L)

NaOCl treatment (min)	Swelled embryos formation (%)			Protocorm formation (%)		
	White	LED Red	LED blue	White	LED Red	LED Blue
0	8.1 ± 1.6*b	11.9 ± 2.4*b	4.1 ± 2.1*b	14.6 ± 2.1b	12.8 ± 2.8*b	6.4 ± 1.1*b
30	88.4 ± 8.9a	90.9 ± 4.8a	54.2 ± 7.1a	91.8 ± 4.9a	94.1 ± 4.4a	61.7 ± 3.5a

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

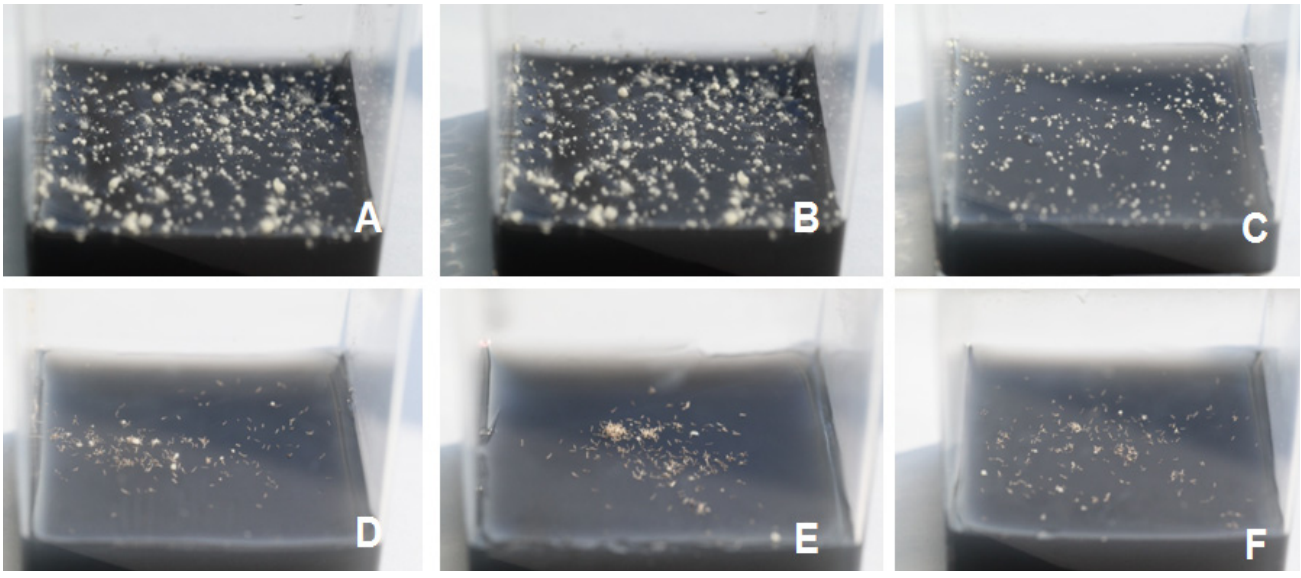

Fig. 3 Effects of light-LEDs for protocorms formation of *O. patens* seeds. A: White light on seed treated by 1% NaOCl 30 min, B: LED Red light on seed treated by 1% NaOCl 30 min, C: LED Blue light on seed treated by 1% NaOCl 30 min, D: White light on seed non-treated by NaOCl, E: LED Red light on seed non-treated by NaOCl, F: LED Blue light on seed non-treated by NaOCl

reported for many terrestrial orchid species, such as *Calanthe tricarinata* (Godo et al. 2010) and *Habenaria macroceratitis* (Stewart and Kane 2006a). LED lighting systems also regulate *in vitro* seed germination and seedling growth. In *Bletilla ochracea,* a maximum seed germination of 74% was obtained using LEDs (Godo et al. 2011).

In conclusion, we demonstrate that embryo swelling occurs after immature seeds are subjected to NaOCl pre-treatment, thus resulting in a higher frequency of seed germination in *O. patens*. This protocol could be very useful for commercial nurseries that conduct large-scale propagation and *ex situ* conservation of *O. patens*.

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