Journal of Plant Biology, December 2004, 47(4) : 396-400 **SHORT COMMUNICATION**

Effects of Various Pretreatments on Seed Germination of *Calystegia soldanella* (Convolvulaceae), a Coastal Sand Dune Plant

Jung-Moon Ko[1], Hye-Jeong Park[1], Byeong-Mee Min[2], and Hyeon-Cheol Cha[1]*
[1]Department of Biology, Dankook University, Cheonan 330-714, Korea
[2]Department of Science Education, Dankook University, Seoul 140-714, Korea

We tested various pretreatments to enhance the germination of 'Sea bells' (*Calystegia soldanella* Roem. et Schult). One experiment, which improved the germination rate by 70%, involved first scarifying the seed coats, then immersing the seeds in 50 ppm GA_3 for 24 h. A GA_3-alone pretreatment did not increase germination. However, the most effective method, with a 100% success rate, included 3 h of acid pretreatment with 98% H_2SO_4. We also used scanning electron microscopy (SEM) to examine the seed-coat surfaces and cross-sections of dry seeds. SEM showed structural differences between seeds that were not treated and those exposed to 98% H_2SO_4. The latter treatment allowed the seed coats to crack and break, thereby disrupting their physical dormancy.

Keyword: Calystegia soldanella, GA_3, H_2SO_4, scarification, seed germination, SEM

The *Calystegia soldanella* plant (Sea bells; Convolvulaceae) is endemic to coastal sand dunes. It blooms from May to June, and the seeds ripen in August. Its subterranean stems are used as a traditional diuretic medicine. Those long stems also allow the plants to retain sandy soils, making this species ideal for the restoration of coastal dunes and erosion control. Despite its great value in ecological preservation, it has been difficult to maintain appropriate habitats for *C. soldanella* because interference from human activities has caused the loss of coastal dunes. To solve these problems, the populations of sand dune species, e.g., *C. soldanella*, *Elymus mollis*, and *Carex kobomugi*, must be perpetuated. For example, several methods are available for propagating *C. soldanella*, including by seed, tissue and cell culture, and cuttings.

Various chemical strategies can improve seed germination rates, such as the application of gibberellic acid (Miyoshi and Sato, 1997; Bhattacharya and Khuspe, 2001; Puppala and Fowler, 2002; Padilla and Encina, 2003), sulfuric acid (Ishikawa et al., 1993; Sozzi and Chiesa, 1995; Demel, 1998), and potassium nitrate (KNO_3) (Bungard et al., 1997; Kang et al., 2001). In addition, one can use hydration-dehydration treatments (Ren and Tao, 2003), scarification (Baes and Viana, 2001), or smoke and heat (Morris et al., 2000; Tieu et al., 2001) to increase success.

Seed dormancy can be caused by either embryo immaturity or poor imbibition of water and gas exchange through the seed coat. In the latter case, such as with *C. soldanella*, dormancy can be broken by treating those hard coverings. The function of the seed coat is to protect the embryo and endosperm from desiccation, mechanical injury, unfavorable temperatures, and attacks by bacteria, fungi, and insects (Bhojwani and Bhatnagar, 1978).

In the present study, we investigated the most effective methods for enhancing *in vivo* germination of *C. soldanella* by pretreating seeds with gibberellic acid, sulfuric acid, or scarification. We also observed the external and cross-sectional structures of the seed coat via scanning electron microscopy.

MATERIALS AND METHODS

Seed Collection

Seeds of *C. soldanella* were collected in August and September of 2002 at Hakampo and Sinduri, two areas situated on the west coast of the Taean peninsula of Korea. The seeds were air-dried at room temperature for 1 week, then refrigerated at 4°C.

Pretreatment

The following pretreatments were tested in three experiments to determine their efficacy in break-

*Corresponding author; fax +82-41-551-9229
e-mail hccha@dankook.ac.kr

ing seed-coat dormancy: control (no pretreatment), applications of gibberellic acid (GA_3) or sulfuric acid (98% H_2SO_4), and scarification.

Experiment 1: Seeds were immersed in GA_3 (50, 100, 200, or 500 ppm) for 6, 12, or 24 h.

Experiment 2: Seeds were soaked in 98% H_2SO_4 for 10 min, or for 0.5, 1, 2, 3, 4, 5, or 6 h, with stirring at 10-min intervals.

Experiment 3: After the seeds were scarified, they were immersed in GA_3 (50, 100, or 200 ppm) for 12 or 24 h.

Before pretreatment commenced, all the seeds were washed in tap water, then placed on vermiculite in three Petri dishes (ϕ = 9 cm) per experiment. The vermiculite was moistened with distilled water and the dishes were kept at 25±5°C in a germination room, under a 16-h photoperiod from fluorescent lights. Germination rates were recorded for up to 4 weeks. Seeds were considered germinated when their radicles had protruded. Each experiment included 60 seeds, and three replications were made for each testing period. To analyze variance, each pretreatment effect was evaluated by one-way ANOVA (Duncan's multiple range test), with a 5% confidence level.

Observation of Seed Structures

Both untreated seeds and those pretreated with 98% H_2SO_4 for 3 h were fixed with FAA solution (100 ml L^{-1} of 40% formalin, 50 ml L^{-1} of glacial acetic acid, 500 ml L^{-1} of 95% ethanol, and 350 ml L^{-1} of distilled water). Afterward, the seeds were dehydrated through an ethanol series (50, 70, 80, 90, 95, and 100%, twice). Ethanol was transferred to hexamethyldisilazane (HMDS) for rapid drying. The seeds were then sectioned and specimens were coated with 20-nm gold particles. The surface and structure of the seed coat was observed with a scanning electron microscope (SEM; Hitachi S-3000, Japan). Images were recorded digitally, and photos were processed with Adobe Photoshop v. 7.0.

RESULTS

Among the three pretreatment types, pre-soaking in 98% H_2SO_4 was most effective for improving the germination of *C. soldanella*. Treatment periods of 0.0 to 0.5 h resulted in germination rates of 40%, while times of >1 h were associated with rates of 80 to 100%. Pre-soaking in 98% H_2SO_4 for 3 h led to 100% germination after 2 weeks of incubation (Fig. 1),

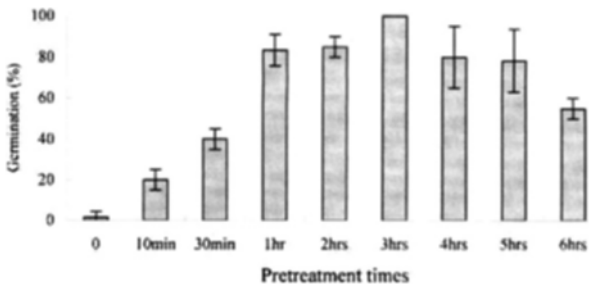

Figure 1. Effect of 98% H_2SO_4 on dark-germination of *C. soldanella*. Nine treatment levels were used. Seeds were incubated for 4 weeks. Data points are significantly different according to Duncans multiple range test at $p < 0.05$. Error bars indicate s.d.

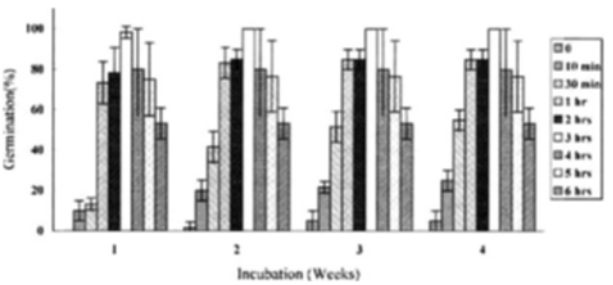

Figure 2. Weekly germination rates as influenced by pretreatment with 98% H_2SO_4. Note that germination was completed within 2 weeks, and rate was 100% when seeds were first soaked for 3 h. Data points are significantly different according to Duncans multiple range test at $p < 0.05$. Error bars indicate s.d.

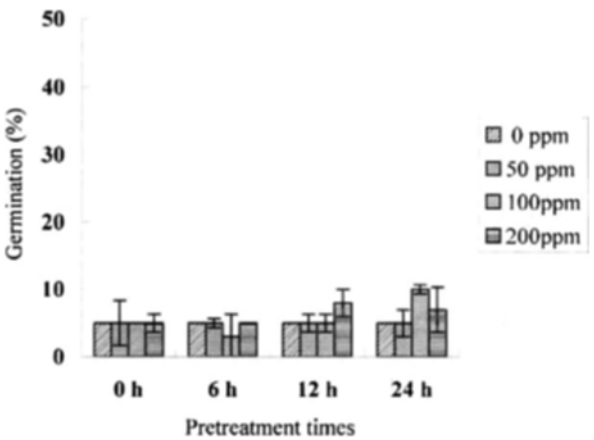

Figure 3. Effect of GA_3 pretreatment on dark-germination of *C. soldanella*, by concentration and duration. GA_3 did not increase germination by more than 20%. Error bars indicate s.d.

although data were recorded for up to 4 weeks (Fig. 2). The variation among treatments was statistically different (P <0.05) for the experiment with 98% H_2SO_4.

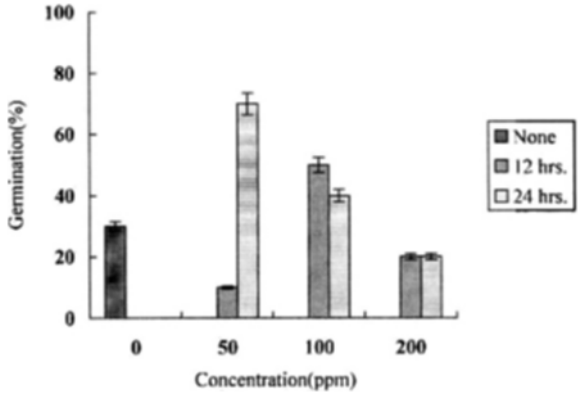

Figure 4. Effect of GA_3 over a 2-week period on germination of *C. soldanella* after seeds were scarified. Pretreatment involved 4 concentrations and 2 periods of darkness. Note that treatment comprising 200 ppm GA_3 for 12 or 24 h reduced the rate to one that was lower than for untreated seed. Error bars indicate s.d.

Incubation periods of greater than 2 weeks did not significantly change germination rates (Fig. 2).

Relatively poor results were obtained with the GA_3 pretreatment (Fig. 3), although scarification beforehand did enhance germination success. For example, applying 50 ppm of GA_3 for 24 h after scarification resulted in 70% germination, whereas a higher gibberellin concentration (200 ppm) was associated with only 30% success (Fig. 4).

SEM procedures revealed that the seed coats from this species have three layers and zones. Cells in the palisade layer are wider and much longer than others (Fig. 5). Pretreatment with 98% H_2SO_4 affected these seed-coat surfaces. For example, the acid removed both the pigmented and the outer colorless zones, and the sponge layer was somewhat hollowed with cavities (Fig. 5). In addition, the original dark brown color of the coat changed to a yellowish hue after the 98% H_2SO_4 application, and the surface was severely shrunken and cracked (Fig. 6).

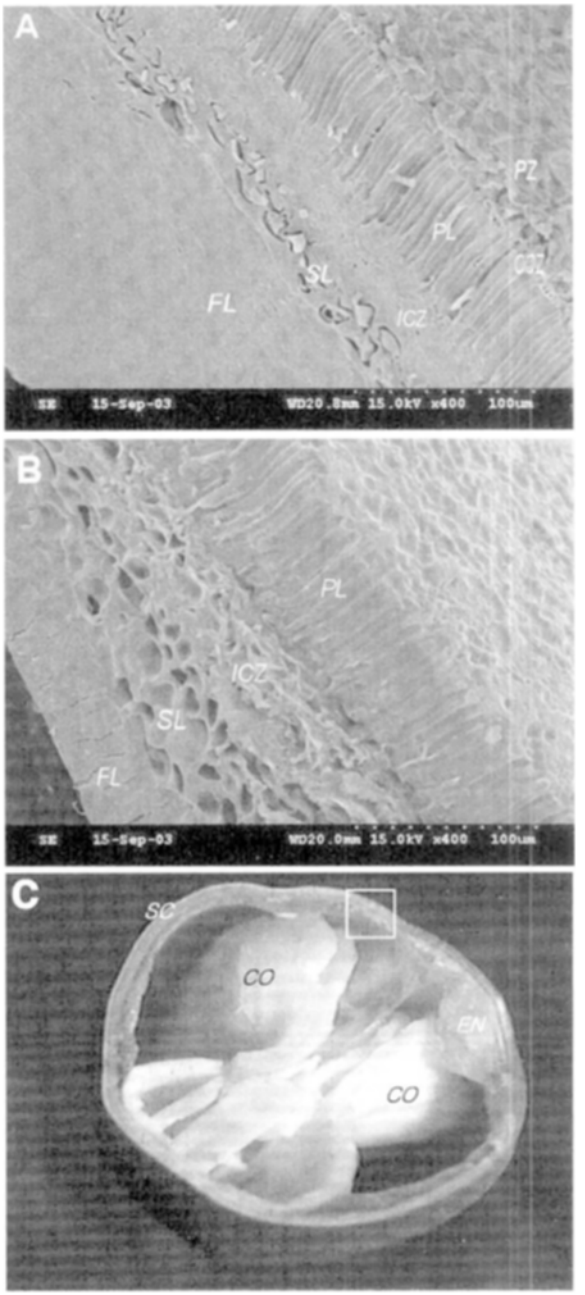

Figure 5. SEM micrographs of untreated (**A**) and H_2SO_4-pretreated (**B**) seed coats, and stereo micrograph of cross-sectioned seed (**C**). Note that *pigmented zone* (PZ) and *outer colorless zone* (OCZ) were removed by acid from pretreated seed. PL, *Palisade Layer*; ICZ, *Inner colorless zone*; SL, *Sponge layer*, FL, *Fringe Layer*; SC, *Seed coat*; EN, *Endosperm*; CO, *Cotyledon*.

DISCUSSION

Over time, hard seed coats have evolved to withstand unfavorable conditions, such as heat from fires, severe drought, and mechanical damage. Therefore, to enhance germination, pretreatments are required to make the coat permeable to water. H_2SO_4, GA_3, and scarification were used in this study to improve permeability by acting on specific weakened spots. Our results demonstrate that such methods were able to enhance germination because seed dormancy in *C. soldanella* is physical in nature. Chemical applications also improve germination in such species as *Capparis spinosa*, where positive effects are seen with pretreatments of either H_2SO_4 for 20 min or GA_{4+7}

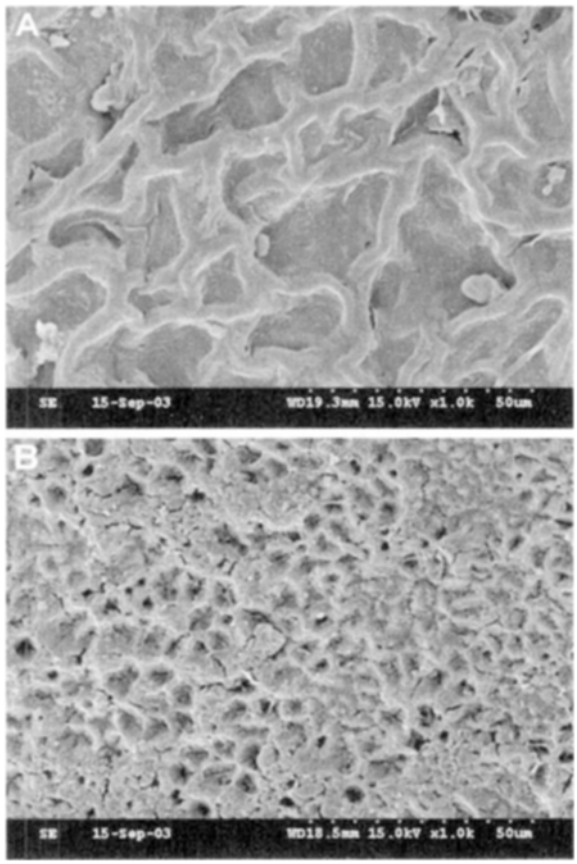

Figure 6. SEM micrographs of surfaces of untreated (**A**) and H_2SO_4-pretreated (**B**) seed coats. Note that seed surface in (**B**) was severely shrunken and cracked.

for 90 min (Sozzi and Chiesa, 1995). Likewise, in *Acacia pilispina* and *Pterolobium stellatum*, pretreatments with H_2SO_4 for 45 and 60 min result in germination rates of 97% and 85%, respectively (Demel, 1998).

One previous study of *C. soldanella* showed that, although pretreatment with sulfuric acid increased germination to 90% (Mariko et al., 1992), the treatment period was too long and the length of the evaluation was not definite. In contrast, our current research demonstrated that a 3-h pretreatment of stirring with H_2SO_4 resulted in 100% success, a rate that was significantly higher than any of our other pretreatments.

However, we also noted that germination declined when soaking time was longer than 3 h.

Gibberellin is an important endogenous growth regulator; one of its specific roles is to induce seed germination (Miyoshi and Sato, 1997; Peng and Harberd, 2002; Padilla and Encina, 2003). Therefore, this growth regulator is used to break dormancy in numerous seed species as well as to accelerate the germination of non-dormant seeds (Bhattacharya and Khuspe, 2001; Puppala and Fowler, 2002). However, in the case of *C. soldanella*, GA_3 pretreatment alone did not improve germination rates but, rather, seeds became more sensitive to this hormone if they were first scarified. Thus, it is likely that the primary control of germination in this species resides in its seed coats, and that the GA_3 effect is probably conditioned by prior seed-coat disruptions. Nevertheless, the act of scarification can damage seed embryos, and the seedlings that result from this pretreatment often grow poorly because their cotyledons cannot properly shed the hard seed coats.

Two types of dormancy exist -- embryo and seed coat-imposed -- and each has its own mechanism. The inability of *C. soldanella* to germinate is unlikely to be caused by embryo dormancy, as was proven when we removed the seed-covering structures with H_2SO_4, thereby enhancing germination. The structure of seed coats have also been analyzed in annual desert plants (Gutterman and Shem-Tov, 1997) and in peas (van Dongen et al., 2003) and the correlation between seed-coat structure and dormancy/germinability has been studied in melon (*Cucumis melo*; Edelstein et al., 1995).

The positive response to our sulfuric acid treatments indicates that the impermeable coat is responsible for very low germination rates from intact seeds, as seen with our experimental, untreated controls, in which imbibition was prevented. By overcoming this physical dormancy with chemical pretreatment, seed coats are softened and water uptake is enabled, both actions being crucial to sustaining the life cycle of hard-seeded species.

ACKNOWLEDGEMENT

We thank the Institute of Environmental Science and Technology (Korea, Ministry of Environment) for providing financial support.

Received August 2, 2004; accepted October 25, 2004.

LITERATURE CITED

Baes PO, Viana ML (2001) Germination in *Prosopis ferox* seeds: Effect of mechanical, chemical and biological scarificators. J Arid Environ 50: 185-189

Bhattacharya J, Khuspe SS (2001) *In vitro* and *in vivo* germination of papaya (*Carica papaya* L.) seeds. Sci Hortic 91: 39-49

Bhojwani SS, Bhatnagar SP (1978) The Embryology of Angiosperms, Ed 2. Vikas Publishing House Pvt Ltd, New Delhi

Bungard RA, McNeil D, Morton JD (1997) Effect of chilling, light, and nitrogen-containing compounds on germination, rate of germination and seed imbibition of *Clematis vitalba* L. Ann Bot 79: 643-650

Demel T (1998) Germination of *Acacia origena, A. pilispina* and *Pterolobium stellatum* in response to different pre-sowing seed treatment, temperature, and light. J Arid Environ 38: 551-560

Edelstein M, Corbineau F, Kigel J, Nerson H (1995) Seed coat structure and oxygen availability control low-temperature germination of melon (*Cucumis melo*) seeds. Physiol Plant 93: 451-456

Gutterman Y, Shem-Tov S (1997) Mucilaginous seed coat structure of *Carrichtera annua* and *Anastatica hierochuntica* from the Negev desert highlands of Israel, and its adhesion to the soil crust. J Arid Environ 35: 695-705

Ishikawa S-I, Furukawa A, Okuda T, Oikawa T (1993) Germination requirements in *Carex kobomugi* (Sea Isle). J Plant Res 136: 245-248

Kang JH, Kim YJ, Jeon BS (2001) Effect of presown cold stratification, GA_3, KNO_3, and acetone treatment on germination of *Crotalaria sessiflora* L. Kor J Medi Crop Sci 9: 124-129

Mariko S, Kachi N, Ishikawa S-I, Furukawa A (1992) Germination ecology of coastal plants in relation to salt environment. Eco Res 7: 225-233

Miyoshi K, Sato T (1997) The effects of kinetin and gibberellin on the germination of dehusked seeds of indica and japonica rice (*Oryza sativa* L.) under anaerobic and aerobic conditions. Ann Bot 80: 479-483

Morris EC, Tieu A, Dixon K (2000) Seed coat dormancy in two species of *Grevillea* (Proteaceae). Ann Bot 86: 771-775

Padilla IMG, Encina CL (2003) *In vitro* germination of cherimoya (*Annona cherimola* Mill.) seeds. Sci Hortic 1864: 1-9

Peng J, Harberd NP (2002) The role of GA-mediated signaling in the control of seed germination. Plant Biol 5: 376-381

Puppala N, Fowler JL (2002) *Lesquerella* seed pretreatment to improve germination. Ind Crop Prod 17: 61-69

Ren J, Tao L (2003) Effect of hydration-dehydration cycles on germination of seven *Calligonum* species. J Arid Environ 55: 111-122

Sozzi GO, Chiesa A (1995) Improvement of caper (*Capparis spinosa* L.) seed germination by breaking seed coat-induced dormancy. Sci Hortic 62: 255-261

Tieu A, Dixon KW, Meney KA, Sivasithamparam K, Barett RL (2001) Spatial and developmental variation in seed dormancy characteristics in the fire-responsive species *Anigozanthos manglesii* (Haemodoraceae) from Western Australia. Ann Bot 88: 19-26

van Dongen JT, Ammerlaan AMH, Wouterlood M (2003) Structure of the developing pea seed coat and the post-phloem transport pathway of nutrients. Ann Bot 91: 729- 737