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Original Research Article

Effect of Cold Stratification and Gibberellin Treatment on *Androsace septentrionalis* L. Seed Germination

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Abstract - *Androsace septentrionalis* is a grass species restricted to North Korea; however, it is at the brink of extinction due to habitat loss and environmental changes caused by natural disasters and anthropogenic activities. This study was conducted to characterize the dormancy conditions of *A. septentrionalis* in an effort to conserve this North Korean plant resource. For this purpose, the morphological characteristics and vigor of *A. septentrionalis* seeds were examined, and its germination characteristics under different temperature conditions (15/6℃ and 25/15℃), low-temperature stratification, and gibberellin (GA_3) treatment were determined. The results revealed that *A. septentrionalis* exhibits non-deep type morpho-physiological dormancy, and low-temperature stratification treatment was not effective in breaking the dormancy of *A. septentrionalis* seeds. Meanwhile, GA_3 treatment significantly increased the mean germination time, rate, and speed of the seeds. The optimal conditions for the germination of *A. septentrionalis* seeds were 25/15℃ fluctuating temperature and 500 ㎎·L^{-1} GA_3 treatment. The results of this study are useful for the mass propagation of *A. septentrionalis*.

Key words – North Korean plant, Plant propagation, Seed dormancy, Underdeveloped embryo

Introduction

The genus *Androsace* of the Primulaceae family (KNA, 2020) consists of approximately 100 species worldwide and is native to the temperate and boreal regions of the Northern Hemisphere (Lee, 2003). Five taxa of *Androsace* native to the Korean Peninsula have been reported, including *A. septentrionalis* L., *A. umbellata* (Lour.) Merr., *A. lehmanniana* Spreng., *A. cortusifolia* Nakai, and *A. filiformis* Retz. Of these, *A. lehmanniana* and *A. septentrionalis* are restricted to North Korea; hence, they are not distributed in South Korea (Park and Ko, 2018).

Androsace septentrionalis is an annual grass species with rhizomatous and spatulate-shaped leaves that have few or no hairs on the abaxial side and with a shallowly lobed tip. Its white flowers are borne in seven umbellate inflorescences, that bloom in July. It has rhomboid-shaped sepals and linear bracts. Its fruit is a drupe, which is approximately 4 ㎜ long, and its brown seeds have three ridges (Lee, 2003).

Androsace septentrionalis is native to subalpine and alpine regions, steppes, steppe grasslands (Stevanović *et al.*, 2005; Käsermann, 1999) and to the boreal regions of Asia, Europe, North America, and the Korean Peninsula, specifically in Mt. Chilbo and Hamgyongbuk-do (Park and Ko, 2018). Mt. Chilbo in North Korea is the southern limit of the Asian distribution of *A. septentrionalis* (Stevanović *et al.*, 2005).

As an Ice Age relict and a northern lineage plant with a restricted distribution in the alpine regions of North Korea, *A. septentrionalis* is at risk of extinction due to environmental changes and habitat loss (Gantsetseg *et al.*, 2020) caused by indiscriminate agricultural land expansion and deforestation due to food and fuel shortages (Park and Yoo, 2009). Moreover, North Korea suffers from widespread forest degradation and fragmentation due to frequent natural disasters, such as large-scale forest fires and floods (Kim and Park, 2001). Consequently, there is a serious threat of forest resource

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degradation and biodiversity loss in North Korea; hence, there is an urgent need to conserve and restore North Korean forest genetic resources through *ex situ* seed and tissue culture (O'Donnell and Sharrock, 2017). Due to their safety, ease of storage, and versatility, seeds are primarily used for conserving forest genetic resources. Information on seed dormancy and germination conditions is essential for the utilization of stored seeds (Hay and Probert, 2013; Kim *et al.*, 2023; Suh *et al.*, 2022).

At present, plant propagation research on North Korean forest genetic resources is very limited. Studies on the multiplication of *A. septentrionalis* have not been reported yet; however, some pretreatment studies have been conducted on related species. For example, the germination rate of *A. mathildae* increased with auxin and cytokinin treatments (Frattaroli *et al.*, 2013), while that of *A. villosa* increased with gibberellin (GA_3) application and low temperature stratification (Arslan *et al.*, 2011). *A. graminifolia* showed increased germination at temperatures higher than those in its native habitat, with the highest germination at 25/15℃ (Wang *et al.*, 2020). Since studies on the seed dormancy of *A. septentrionalis* have not been reported, research on the seed dormancy type and germination conditions of *A. septentrionalis* is expected to contribute to the conservation and restoration of the forest genetic resources in North Korea. In this study, we examined the morphological characteristics of *A. septentrionalis* seeds and evaluated the effects of modified temperature conditions, GA_3, and cold stratification on the germination rate of *A. septentrionalis* seeds. The study is significant in that it identifies the germination mechanism of *A. septentrionalis*, providing information for restoration researchers looking to utilize North Korean plants.

Methods

Experiment materials

Androsace septentrionalis seeds were collected in 2021 from individuals maintained at the DMZ Botanic Garden[1]. The seeds were dried at room temperature in a well-ventilated area, then stored at 4℃ and 40% relative humidity for 1 year. Afterwards, the seeds were selected for morphological and physiological characterization and germination tests. Cold stratification was performed at 4℃ for 6 weeks.

Morphological and physiological characteristics of *A. septentrionalis* seeds

The endosperm on the surface and inside the seeds was examined under a scanning electron microscope (DVM6; Leica Microsystems, Wetzlar, Germany). Seed vigor was determined by performing the tetrazolium test, according to the International Seed Testing Association (ISTA, 2014). Using a razor blade, a thin sheath was excised from the apex of the seeds, immersed in distilled water for 18 hours, and then placed in a 1% solution of 2, 3, 5-triphenyl tetrazolium chloride for another 18 hours. Afterwards, longitudinal sections were prepared and stained. The condition of the endosperm inside the seeds was observed under a dissecting microscope (SMZ 1500; Nikon, Tokyo, Japan), and the vigor of the seeds was analyzed using an X-ray machine (EMT-F70; Softex, Tokyo, Japan).

Seed germination

Prior to germination, *A. septentrionalis* seeds were disinfected by soaking them in 500 ㎎·L^{-1} fungicide (Benomyl; Farm-Hannong, Seoul, Korea) for 1 hour and rinsed with distilled water at least four times. A petri dish (90 ㎜ × 15 ㎜), containing 0.8% agar medium, was used for germination, with 30 seeds sown on the agar medium. The seeds were arranged in a completely randomized design with three replicates per treatment. To determine the germination response of *A. septentrionalis* seeds to temperature, the seeds were cultured in a growth chamber (WCC-1000; Daihan Scientific, Wonju, Korea) conditioned at 15/6℃ or 25/15℃ constant temperature regime (12/12 hours). The light intensity in the growth chamber was maintained at 10±2 μmol·m^{-2}·s^{-1}, with a 16-h photoperiod. Seeds were considered germinated when their primary roots had emerged from the seed coat and protruded more than 1 ㎜. The germinating populations were counted every 24 hours for 30 days. Seeds that germinated during the survey period were removed, and seeds that decayed were excluded from the germination rate calculation. The final

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germination rate (FGR), mean germination time (MGT), and germination speed (GS) of the *A. septentrionalis* seeds were calculated using the following equations (Ellis, 1981):

$$FGR = (\frac{N}{S}) \times 100$$

$$MGT = \frac{\sum(Tx \times Nx)}{N}$$

$$GU = \frac{\sum[(MGT - Tx)^2 \times Nx]}{(N-1)}$$

$$GS = \sum(\frac{Nx}{Tx})$$

where N is the total number of germinations, S is the total number of seeds released, Nx is the number of germinations during the survey period, and Tx is the number of days after the survey had ended.

Cold stratification

To investigate the dormancy-breaking effect at low temperature, a low-temperature layered treatment was conducted on *A. septentrionalis*. First, the seeds were disinfected in a similar manner as in the temperature treatment. Afterwards, the seeds were placed in petri dishes sealed with parafilm to prevent desiccation, and the dishes were wrapped with two layers of aluminum foil. After 6 weeks of treatment in a growth chamber (WIM-RL4; Daihan Scientific, Wonju, Korea) maintained at 4℃, the seeds were cultured in a growth chamber (WCC-1000; Daihan Scientific Co., Wonju, Korea) maintained at 15/6℃ and 25/15℃. The light conditions in the growth chamber were the same as in the temperature treatment.

Pretreatment

To investigate the dormancy-breaking effect of GA_3 treatment, *A. septentrionalis* seeds were pretreated with GA_3. The seeds were disinfected in the same way as in the temperature treatment; then, the disinfected seeds were soaked in 500 and 1,000 ㎎·L^{-1} GA_3 solution for 24 hours under dark conditions. The seeds were washed five times with distilled water and plated on the agar medium in the same way as in the temperature treatment and cultured in a growth chamber (WCC-1000, Daihan Scientific Co., Wonju, Korea) controlled at a constant temperature of 15/6℃ and 25/15℃. The light conditions in the growth chamber were the same as in the temperature treatment.

Statistical analysis

The differences in the germination characteristics of the *A. septentrionalis* seeds under different temperature and GA_3 pretreatment conditions were subjected to one-way ANOVA using the SPSS version 12.0 (IBM, Armonk, NY, USA). The statistical significance of the mean differences of each treatment was tested by performing Scheffe's multiple range test ($p < 0.05$).

Results

Morphological and physiological characteristics of *A. septentrionalis* seeds

The results of the morphological examination revealed that the *A septentrionalis* seeds were obovate and reticulate, and the seed coat was dark brown in color (Figs. 1a and 1b). On average, the seeds were 1.00±0.08 ㎜ long and 0.60±0.05 ㎜ wide; the weight of 1,000 grains was 0.13±0.08 g.

The results of the tetrazolium test revealed that all the seeds were stained red throughout the endosperm (Fig. 1c), and that of the X-ray test showed that the seeds were 100% vigorous (Fig. 1d).

Seed germination characteristics by treatment

The FGR of *A. septentrionalis* seeds by treatment is shown in Fig. 2 and Table 1. The FGR of *A. septentrionalis* seeds in the untreated plots at 25/15℃ was 3.3±0.1%; however, seed germination was not observed at 15/6℃. Cold-stratified *A. septentrionalis* seeds at 25/15℃ had an FGR of 1.1±1.0%; however, seed germination was not observed at 15/6℃. The FGRs of the *A. septentrionalis* seeds in the untreated and cold-stratified plots were not significantly different between the two temperature conditions - 15/6℃ and 25/15℃. In contrast, The FGR of the GA_3-pretreated seeds significantly increased compared with that of the untreated and cold-stratified seeds; however, there was no significant difference

between the FGRs of the seeds treated with two GA_3 concentrations (500 and 1,000 $mg \cdot L^{-1}$) and between the FGRs of the seeds exposed to the two temperature conditions.

At 25/15℃, the FGR of the seeds pretreated with 500 $mg \cdot L^{-1}$ GA_3 was 88.9±9.6%, while that of the seeds pretreated with 1,000 $mg \cdot L^{-1}$ GA_3 was 75.6±6.3%. At 15/6℃, the FGR of the seeds pretreated with 500 $mg \cdot L^{-1}$ GA_3 was 87.8±8.7%, while that of the seeds pretreated with 1,000 $mg \cdot L^{1}$ GA_3 was 66.7±7.2%.

The MGT of the *A. septentrionalis* seeds by treatment is shown in Fig. 3 and Table 1. The MGT of the seeds in the untreated plots at 25/15℃ was 10.3±0.6 days; however, the MGT at 15/6℃ was not calculated because seed germination was not observed. The MGT of the cold-stratified seeds at 25/15℃ was 2.0±1.5 days; however, the MGT at 15/6℃ was not calculated. The MGT of the seeds treated with 500 $mg \cdot L^{-1}$ GA_3 at 25/15℃ was 6.0±0.0 days, while that at 15/6℃ was 6.6±0.4 days. Meanwhile, the MGT of the seeds treated with 1,000 $mg \cdot L^{-1}$ GA_3 at 25/15℃ was 6.4±0.1 days, while that at 15/6℃ was 8.3±0.9 days. At 25/15℃, the MGT of the cold-stratified seeds was significantly higher than that of the

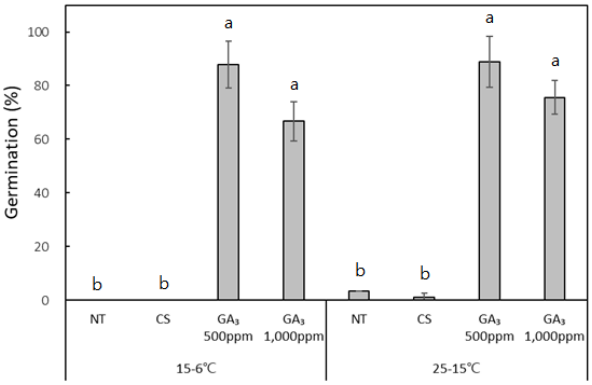

Fig. 2. Germination rate of *Androsace septentrionalis* L. seeds under controlled temperature (25/15℃ and 15/6℃) and pre-treatment conditions. GA_3, gibberellin treatment; NT, non-treatment; CS, cold stratification. Error bar represents the standard deviation. Plots with different letters are significantly different (Scheffe's multiple test, $p < 0.05$).

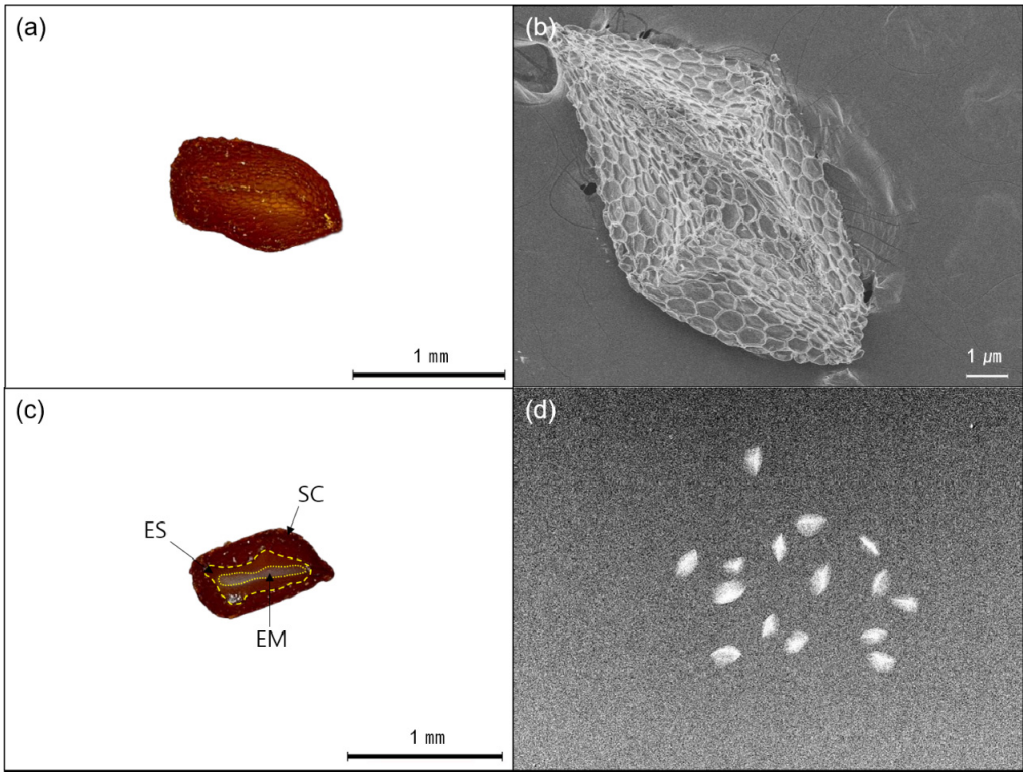

Fig. 1. (a) Seed morphology of *Androsace septentrionalis* L., (b) seed morphology observed under a scanning electron microscope, (c) undeveloped embryos and endosperm stained with tetrazolium are shown in the initial seed at seed coat split. EM, embryo; ES, endosperm; SC, seed coat. (d) Seed fullness of *A. septentrionalis* via X-ray inspection.

untreated seeds. Moreover, the MGT of the GA_3-treated seeds was higher than that of the untreated seeds; however, the MGT of the GA_3-treated seeds at 15/6℃ was not significantly different from that of the untreated seeds.

The GS of *A. septentrionalis* by treatment is shown in Fig. 4 and Table 1. The GS of the untreated seeds at 25/15℃ was 0.1±0.0; however, the GS at 15/6℃ was not calculated because seed germination was not observed. The GS of the cold-stratified seeds at 25/15℃ was 0.1±0.1; however, the GS at 15/6℃ was not calculated. The GS of the seeds pretreated with 500 $mg \cdot L^{-1}$ GA_3 at 25/15℃ was 4.4±0.6, while that at 15/6℃ was 4.1±0.7. Meanwhile, the GS of the seeds pretreated with 1,000 $mg \cdot L^{-1}$ GA_3 at 25/15℃ was 3.7±0.4, while that at 15/6℃ was 2.8±0.5. At 25/15℃, the GS of the cold-stratified seeds was not significantly different from that of the untreated seeds. In contrast, the GS of the GA_3-treated seeds significantly increased compared with that of the untreated seeds. Specifically, the GS of the seeds treated with 1,000 $mg \cdot L^{-1}$ GA_3 at 25/15℃ was significantly higher than that at 15/6℃; the GS of the seeds treated with 500 $mg \cdot L^{-1}$ GA_3 was significantly higher than that of the seeds treated with 1,000 $mg \cdot L^{-1}$ GA_3, with no difference between the

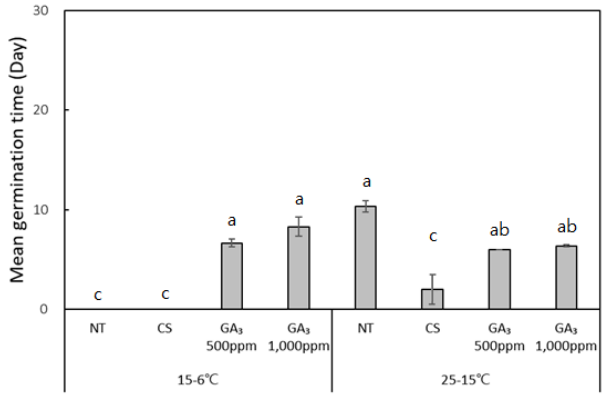

Fig. 3. Mean germination time (MGT) of *Androsace septentrionalis* L. seeds under controlled temperature (25/15℃ and 15/6℃) and pretreatment conditions. GA_3, gibberellin treatment; NT, non-treatment; CS, cold stratification. Error bar represents the standard deviation. Plots with different letters are significantly different (Scheffe's multiple test, $p < 0.05$).

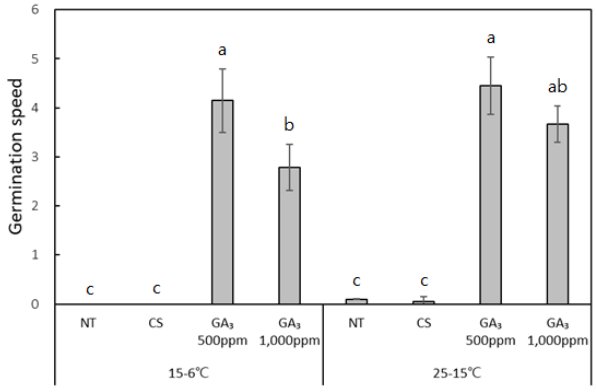

Fig. 4. Germination speed of *Androsace septentrionalis* L. seeds under controlled temperature (25/15℃ and 15/6℃) and pretreatment conditions. GA_3, gibberellin treatment; NT, non-treatment; CS, cold stratification. Error bar represents the standard deviation. Plots with different letters are significantly different (Scheffe's multiple test, $p < 0.05$).

Table 1. Final germination rate (FGR), mean germination time (MGT), and germination speed (GS) of *Androsace septentrionalis* L. seeds under controlled temperature (15/6℃ and 25/15℃) and pretreatment conditions. NT, non-treatment; CS, cold stratification; GA_3, gibberellin.

Treatment	FGR (%)	MGT (day)	GS
15/6℃ NT	0.0 ± 0.0^{bz}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
15/6℃ CS	0.0 ± 0.0^{b}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
15/6℃ GA_3 500 $mg \cdot L^{-1}$	87.8 ± 8.7^{a}	6.6 ± 0.4^{a}	4.1 ± 0.7^{a}
15/6℃ GA_3 1000 $mg \cdot L^{-1}$	66.7 ± 7.2^{a}	8.3 ± 0.9^{a}	2.8 ± 0.5^{b}
25/15℃ NT	3.3 ± 0.1^{b}	10.3 ± 0.6^{a}	0.1 ± 0.0^{c}
25/15℃ CS	1.1 ± 1.0^{b}	2.0 ± 1.5^{c}	0.1 ± 0.1^{c}
25/15℃ GA_3 500 $mg \cdot L^{-1}$	88.9 ± 9.6^{a}	6.0 ± 0.0^{ab}	4.4 ± 0.6^{a}
25/15℃ GA_3 1000 $mg \cdot L^{-1}$	75.6 ± 6.3^{a}	6.4 ± 0.1^{ab}	3.7 ± 0.4^{ab}

[z]Similar superscript letters in each column indicate no significant difference by Scheffe's multiple range test ($p < 0.05$).

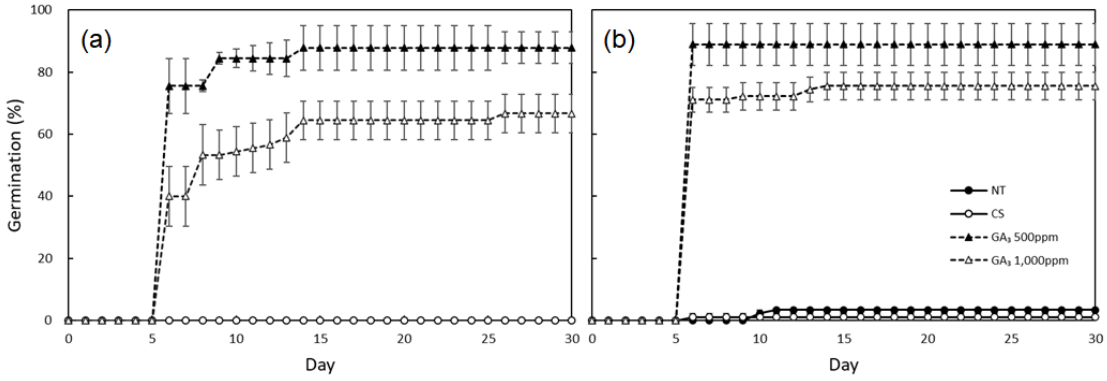

Fig. 5. Cumulative germination rate of *Androsace septentrionalis* L. pretreated with gibberellin (GA_3) and incubated under (a) 15/6℃ and (b) 25/15℃. NT, non-treatment; CS, cold stratification. Error bar represents the standard deviation.

temperature conditions.

The initial and final germination dates by treatment are shown in Fig. 5. At 15/6℃, the untreated and cold-stratified seeds did not germinate. The seeds treated with 500 ㎎·L^{-1} GA_3 germinated from 6.0±0.0 to 10.7±2.4 days, while those treated with 1,000 ㎎·L^{-1} GA_3 germinated from 6.0±0.0 to 12.7±1.9 days. At 25/15℃, the untreated seeds first germinated at 10.3±0.5 days, then no germination was observed thereafter. The cold-stratified seeds germinated at 2.0±2.8 days, and no further germination was observed. The germination of the seeds treated with 500 ㎎·L^{-1} GA_3 occurred at 6.0±0.0 days, and was not observed thereafter, while that of the seeds treated with 1,000 ㎎·L^{-1} GA_3 lasted from 6.0±0.0 to 13.3±0.5 days.

Discussion

Seeds are classified into three types based on the shape of the endosperm inside the seed: basal, peripheral, and axile (Martin, 1946). Linear-type seeds, in which the endosperm is located at the center of the seed, is the most representative seed type (Song *et al.*, 2019). Axile-type seeds vary in size and can be further categorized according to length: dwarf-type seeds are 0.3-2.0 ㎜ long, while micro-type seeds are ≤ 0.2 ㎜ long (Martin, 1946). Based on the results of this study, *A. septentrionalis* embryo is considered axial-miniature (Fig. 1c) and micro type based on shape and size, respectively.

An underdeveloped embryo matures under appropriate temperature conditions to form a radicle, which then undergoes a period of secondary embryo-axis dormancy to form a cotyledon (Lee *et al.*, 2003). Seeds with underdeveloped embryos must grow to a certain size before undergoing germination. If an embryo elongates and germinates within 30 days under appropriate conditions, it is classified as morphologically dormant (MD); however, if it takes longer than 30 days to germinate or requires cold stratification or combined treatment, it is classified as morpho-physiologically dormant (MPD) (Baskin and Baskin, 2004). The genus *Androsace*, to which *A. septentrionalis* belongs, has been reported to have linear-type underdeveloped embryos (Finch-Savage and Leubner-Metzger, 2006), and *A. septentrionalis* appears to be an MPD with underdeveloped embryos based on the size and shape of its embryo. Temperature is a major factor in the after-ripening or dormancy of underdeveloped embryos (Lee *et al.*, 2003). MPD embryos can be categorized into simple-type MPD, which requires 15-20℃ for embryo development, and complex-type MPD, which requires 0-10℃ (Geneve, 2003; Baskin and Baskin, 1998, 2004; Song *et al.*, 2019). *Androsace septentrionalis* was considered a simple-type MPD because it germinated in the untreated plots at 25/15℃; however, it did not germinate at 15/6℃.

MPD is a combination of morphological dormancy (MD) and physiological dormancy (PD). PD can be categorized into deep, intermediate, and non-deep types based on the depth of dormancy (Geneve, 2003; Baskin and Baskin, 1998, 2004). GA treatment is ineffective in breaking the dormancy of deep-type PD because it requires 3-4 months of cold stratification to break dormancy. Intermediate-type PD requires 2-3

months of cold stratification to break dormancy; nonetheless, GA treatment is effective in breaking the dormancy of few species. In contrast, GA treatment is known to easily break the dormancy of and induce an after-ripening effect in non-deep type PD, which requires a low-temperature stratification treatment for a few days to two months to break dormancy (Song *et al.*, 2019). GA_3 is a phytohormone that breaks seed dormancy and promotes germination; its effects are opposite to those of abscisic acid (ABA), which induces dormancy (Jang *et al.*, 2016; Kim and Lee, 2013). The GA/ ABA ratio and sensitivity are known to regulate the balance between dormancy and germination in seeds (Finch-Savage and Leubner-Metzger, 2006); increased GA concentration and decreased sensitivity to ABA can break dormancy and promote germination (Song *et al.*, 2019). In a study of related species, Frattaroli *et al.* (2013) found that GA_3 treatment and cold stratification did not affect the germination of *A. mathildae*; however, the germination rate of the *A. mathildae* seeds cultured on Murashige and Skoog medium supplemented with a germination promoter containing humus extract or auxin and cytokinin increased to 90% (Fasciani and Pace, 2015). Arslan *et al.* (2011) reported that the germination rate of *A. villosa* seeds in untreated plots was 24%; however, it increased to 97% with combined GA_3 treatment and cold stratification. Based on the results of this study, *A. septentrionalis* is considered a non-deep type MPD because its germination rate after the GA_3 treatment significantly increased compared with that in the untreated plots; however, its germination rate was not improved by cold stratification. Nevertheless, cold stratification significantly shortened the MGT of the seeds. Although the germination rates of the *A. septentrionalis* seeds treated with 500 and 1,000 ㎎·L^{-1} GA_3 at 25/15℃ did not significantly differ, the GS of the seeds treated with 500 ㎎·L^{-1} GA_3 was higher than that of the seeds treated with 1,000 ㎎·L^{-1} GA_3. Therefore, the optimal conditions for breaking the dormancy of *A. septentrionalis* seeds are 25/15℃ modified temperature condition and 500 ㎎·L^{-1} GA_3 treatment.

However, the bioactive substances and anatomical observations involved in the maturation of *A. septentrionalis* embryos were not observed in this study, and it is difficult to explain the inability of *A. septentrionalis* to germinate in low-temperature environments. Thus, future studies should be conducted.

This study was conducted to determine the seed dormancy type and the appropriate conditions for the germination of *A. septentrionalis* seeds, in an effort to conserve the forest genetic resources in North Korea. The results of this study revealed that *A. septentrionalis* seeds are non-deep type MPD that requires 25/15℃ temperature and 500 ㎎·L^{-1} GA_3 treatment to germinate. We believe that our findings will be an important resource for technical support and field guidance for farmers and industries that have difficulties in the seed propagation and production of *A. septentrionalis*.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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