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# Germination of *Pulsatilla* Seeds as Influenced by Seed Morphology, Moist 5 °C and Gibberellin ($GA_3$) Treatment, and Detection of Nickel in Seeds

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***Abstract.*** **Seeds of *Pulsatilla turczaninovii* were categorized to full and empty seeds based on observations under a light microscope and on X-ray images. A germination test for full or empty seeds was evaluated as affected by the duration of gibberellin $GA_3$ and the moist 5 °C cold stratification (CS) treatment. The morphological and elemental components of *P. turczaninovii* and *P. cernua* var. *koreana* seeds were analyzed using low-temperature scanning electron microscopy (LT-SEM) and energy dispersive X-ray diffraction analysis. The results showed that 64% of full and semifull *P. turczaninovii* seeds 10 weeks after harvesting germinated in 17 to 19 days; however, the germination rate, including empty and semiempty seeds, was lower (52.6%). Full seeds with damaged or dried vegetative organs (embryo or endosperm) and semifull seeds with severely damaged vegetative organs were observed, and this result could be related to low germination rates. Germination patterns of seeds stored dry at 5 °C for 44 weeks that showed a sigmoid pattern were increased by immersing seeds in a $GA_3$ solution for 8 hours and treating seeds with 16 or 32 days of CS. More seeds germinated between 12 and 17 days (as compared with 17 to 29 days), especially when they were treated with $GA_3$ and received 32 days of CS. Comparison of germination rates of visually full seeds upon harvest (52.6%) with those that had been stored dry for 44 weeks (26.3% to 29.7%) suggested that the viability of seeds may have decreased. Dormancy could be a factor that decreased germination and can be removed by low temperature and $GA_3$ treatment. LT-SEM revealed a valley-like, sunken streak in empty seeds of *P. cernua* var. *koreana*. The nickel content in the trichome and seedcoat of full and empty seeds of both taxa ranged from 2.98 to 4.62 (weight %), as determined on X-ray images. Our study suggested that the low germination rate was due to either the presence of dormancy, damage to either embryo or endosperm, a loss in viability, or the presence of nickel in the seeds.**

The genus *Pulsatilla* includes 26 species native to North America, Europe, and Asia (USDA, Agricultural Research Service, National Plant Germplasm System, 2018). They are early flowering perennials ideal for border planting in gardens and thrive under conditions of well-drained soil and full sun. *Pulsatilla turczaninovii* Krylov and Serg. grows widely in Nei Mongol, China, and West Siberia, Russia (Wang and Bartholomew, 2001), while *Pulsatilla cernua* var. *koreana* (Yabe ex Nakai) Y. N. Lee grows in northeastern China and Korea.

*Pulsatilla cernua* var. *koreana* seeds germinate in 14 d at 25 °C; however, germination rates decrease after 6 to 8 weeks, and seeds do not germinate at all after 14 weeks of storage under unspecified room conditions (Sang et al., 1993). When stored dry in silica gel at 0 or 10 °C, however, the seeds have longevity of up to 24 weeks, with germination rates >66.3% (Sang et al., 1996). If good seeds are stored in moist vermiculite at room temperature under conditions of natural humidity, germination rates can dropped to 5.3% (Sang et al., 1996). However, no descriptions on the criteria of good seeds were provided.

Low germination rates might reflect improper storage conditions and for long storage duration of seeds, resulting in low viability (Sang et al., 1993, 1996) or poor seed quality, which cannot be identified visually (Baskin et al., 2006). The viability of *Swertia chirayita* (Roxb. Ex. Fleming) H. Karst seeds declined as dry storage duration increased over 24 months, even at 4 °C (Pradhan and Badola, 2012). In *Pulsatilla*, low germination rates may also result from the inclusion of both nonviable seeds, lacking a developed embryo (EM) or endosperm (EN) (vegetative organs) (Esau, 1965). Due to trichomes developed on the seedcoat, soaking *Pulsatilla* seeds in water to separate full, viable seeds from empty, nonviable seeds, as demonstrated with *Corylopsis coreana* Uyeki seeds (Kim et al., 2017), could not be performed.

Germination of viable seeds may be delayed or failed to germinate even at favorable temperatures due to dormancy (Baskin and Baskin, 2004). Seed dormancy could be a factor in low germination rates if dormancy is not released by low temperature or plant growth regulator treatments, especially those including gibberellin (Cadman et al., 2006). Germination of *P. cernua* seeds can increase to >92% if they are treated with kinetin, gibberellin GA3, and 2, 4-D for 24 h compared with 18% in untreated controls (Gu et al., 2014). The germination of fresh *P. turczaninovii* seeds is significantly improved by treatment with 100 $mg \cdot L^{-1}$ gibberellin $GA_3$ (64.0% in 32 d vs. 15.0% by the control in 60 d) (Shi et al., 2005). Li and Piao (2010) also reported that soaking seeds in 100 $mg \cdot L^{-1}$ $GA_3$ for 12 h increased germination. Germination of *P. turczaninovii* also increased compared with the control when treated with 100 $mg \cdot L^{-1}$ GA at 25 to 30 °C (Wang et al., 2013). However, in those studies, neither the age of the seeds nor their storage conditions were indicated.

Visual observations along with SEM to examine the surface of seeds may be useful to distinguish full from empty seeds. Therefore, X-ray scanning technology can be applied to evaluate images of seeds on a large scale, to assess seed development, and to improve seed lot quality and germination (Carvalho et al., 2010). X-ray diffraction techniques could be used to detect various elements in vivo, such as magnesium, silicon, and nickel (Bolton et al., 2014; Roh et al., 2012). Another factor influencing low seed germination rates could be the occurrence of nickel, as this factor has been shown to inhibit the germination of radish (*Raphnus sativus* cv. Early Menu) seeds (Yadav et al., 2009).

The objectives of this research were to 1) categorize *Pulsatilla turczaninovii* seeds as full or empty using X-ray imaging and to study the germination of these seeds; 2) investigate the germination of dry stored seeds at 5 °C as influenced by gibberellin $GA_3$ and durations of CS; 3) compare the morphologies of full and empty seeds of *P. turczaninovii* and *P. cernua* var. *koreana* using low-temperature SEM (LT-SEM) and visual observation under a light microscope; and 4) analyze the elemental components of *P. turczaninovii* and *P. cernua* var. *koreana* seeds using energy dispersive X-ray diffraction analysis.

## Materials and Methods

*Plant materials and seed germination.* *Pulsatilla turczaninovii* seeds were collected

from their natural habitats at Balin You Right Banner, Chifeng City, Nei Mongol, PR China on 9 June 2017, and those of *P. cernua* var. *koreana* from Jecheon, Chungbuk Province, Korea, during the first week of May 2017. The seeds were stored dry at 5 °C until use in the evaluation, except for the periods during which the seeds were mailed and imaged, as described below. The stigma attached to the seed was removed before the germination tests. Seeds were immersed in distilled water at 20 °C for 4 h or as otherwise stated for visual observation of the external and internal anatomical analysis. Germination tests started on 4 Sept. 2017 and seeds were sown individually in 50-cell plug trays (Taizhou Longji Plastic Industry Co. Ltd., Zhejiang, China) filled with premixed peatmoss and vermiculite (1:1 by volume). The trays were then placed in a growth chamber maintained at 20 °C for 12 h and watered every 1 to 2 d; they were maintained under fluorescent tubes with a photosynthetic photon flux density of 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

*Germination of seeds after dry storage at 5 °C as influenced by $GA_3$ and a moist 5 °C treatment (CS).* *Pulsatilla turczaninowii* seeds that were considered full were stored dry at 5 °C for 44 weeks and were then stored at 20 °C for 32, 16, and 0 d, and treated with gibberellin acid ($GA_3$) 0, 50, and 100 ppm for 0 and 8 h. After $GA_3$ treatment, seeds were packed in moist vermiculite (50% by weight) in a zipper bag for 0, 16, and 32 d of CS. Seeds in moist vermiculite were spread over premixed peatmoss : vermiculite (1:1 by volume) medium, and covered with about 0.5 cm of the same medium in an open tray (54 × 27 × 6 cm) on 28 Dec. 2017. There were 100 seeds per replication and three replications per treatment. The number of seeds germinating at 20 °C was counted at 2- to 3-d intervals for the first 19 d, and then at 1- to 2-d intervals until germination data collection ended 29 d after sowing.

The data were subjected to ANOVA using SAS software (ver. 9.0; SAS Institute Inc., 2002). Differences in means were determined using Duncan's multiple range test. Based on the germination rate, germination data at 12, 17, and 29 d after sowing are presented and differences between days 12 and 17 and days 17 and 29.

*Observations of external features and internal anatomical analysis using a light stereoscope and LT-SEM.* The surfaces of *Pulsatilla turczaninovii* seeds were visually observed. Seeds were immersed in distilled water at 20 °C for 4 h, and they were longitudinally dissected for observation under a light microscope at ×25–100 magnification (N-117M; Ningbo Yongxin Optical Co., Ltd., Ningbo City, Zhejing Province, P.R. China). Up to five seeds of each type of seeds were observed; and based on the images, seeds were classified as full, semifull, semiempty, or empty seeds (Fig. 1). Vegetative organs (EM and EN) (Esau, 1965) were also examined (Fig. 2). Only representative images are shown here, depicting vegetative organs and turgid vegetative organs, full seeds with damaged or dried internal structures, semifull seeds with severely damaged vegetative organs, and empty seeds exhibiting valley-like sunken streaks at the surface of the seedcoat and hollow cavities.

Additional seeds were germinated and observed under a light microscope after storage at 5 °C. Seeds after removal of stigma were immersed in distilled water for 8 h and kept at 20 °C for 12 d; images were then obtained (Fig. 3). Photographs of the longitudinal and of cross-sections of the seeds were collected at early germination stages; well-developed roots, hypocotyls, and cotyledons were also collected.

The seedcoat was observed using LT-SEM according to the methods described by Bolton et al. (2014) and Roh et al. (2012). Microscopic images were obtained from two seeds each of *P. turczaninovii* and of *P. cernua* var. *koreana* at the Electron & Confocal Microscopy Unit (U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD).

*X-ray scanning images and seed germination test.* For X-ray scanning, 50 full and empty *P. turczaninovii* seeds, determined by visual observation, were fixed to index cards in triplicate and mailed to the Ornamental Plant Germplasm Center (Ohio State University, Columbus, OH) on 1 Aug. 2017. Seed germination of X-ray imaged seeds, following exposure to 20 kV for 15 s, was as described in Kim et al. (2017). Based on the X-ray images (Fig. 4), the seeds were classified as full if they had a barrel shape and white transparent organs were visible inside the seedcoat (full seeds). Semifull seeds were peanut-shaped and contained white organs in their centers. Semiempty seeds consisted of degenerated organs with a distinct line underlining the seedcoat, and empty seeds were those with only a seedcoat.

After scanning, the seeds were mailed back to Beijing, where they were used in germination tests starting on 4 Sept. 2017. Seeds were mailed by China's express mail system and the U.S. Postal Service, which generally took 3 to 4 d, followed by scanning that took about 7 d. During these periods, temperature and humidity were not available.

The number of germinated seeds was recorded on 27 Sept. 2017, and data collection ended on 16 Oct. 2017, when no more seeds germinated. The data were subjected to ANOVA, and means were compared using Duncan's multiple range test.

*Chemical composition of seed by energy using energy dispersive X-ray diffraction analysis.* A variable pressure scanning electron microscope (SEM) (Hitachi High Technologies America, Inc., Pleasanton, CA) was used, with an INCA® X-ray diffraction system (Oxford Instruments, Oxford, UK) attached to the SEM. INCA® software was used to determine chemical composition based on images at ×300 magnification and 246,000 to 328,000 counts per sample (Roh et al., 2012). Counts of each element were recorded from the surface of the seedcoat of *P. turczaninovii*. Energy dispersive X-ray diffraction analysis was performed from the surface of the seedcoat and the long, hair-like calyx tissue attached to the ovary. Representative data from the seedcoat and trichome of full seeds are presented. Quantitative analyses were performed only from the seedcoat surface of full and empty seeds of *P. turczaninovii* and *P. cernua* var. *koreana*.

## Results and Discussion

*Analysis of the external features and internal structure by light microscope and by low-temperature SEM (LT-SEM).* Full and empty seeds could be recognized by visual observation under a light microscope (Figs. 1 and 2), or more accurately by using X-ray images (Fig. 4). However, distinguishing between full and semifull seeds, and between semiempty and empty seeds was challenging. Due to an enlargement of the visual images of the small seeds (≈2 mm in length), the images may be insufficiently clear to show noticeable differences between full and semifull, or between empty and semiempty seeds. A further study is required to obtain good X-ray images by testing various exposure conditions (Carvalho et al., 2010). Full seeds showed turgid and expanded shapes (Fig. 1A) and well-developed internal structures (vegetative organs; EM/EN) in the cross-section perpendicular to the proximal and distal end line of the seed (Fig. 1B). Semifull seeds have a valley-like sunken area at the distal end of the seed (Fig. 1C). Semiempty seeds also have a valley-like area (Fig. 1D). Shriveled empty seeds are shown as well (Fig. 1E).

When seeds soaked in water were dissected longitudinally, the full seeds with well-developed vegetative organs (EM/EN) and other tissues (Fig. 2A) were also observed in the cross-section (Fig. 1B), and to the full seeds showing transparent or translucent on X-ray images (Fig. 4A). Only a seedcoat lacking vegetative organs (EM/EN) was observed in the empty seeds (Fig. 2F). The difficulty in differentiating between full

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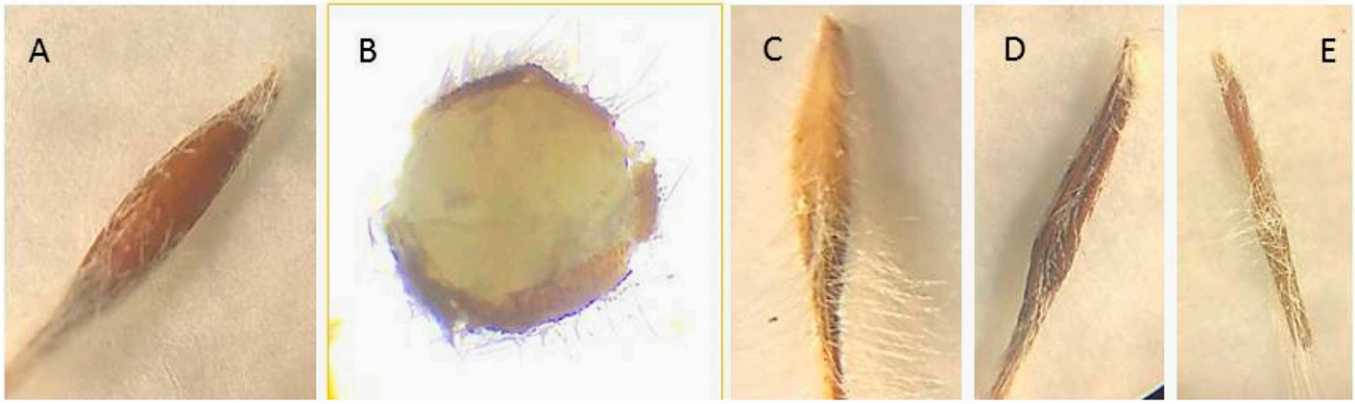


Fig. 1. Classification of *Pulsatilla turczaninovii* seeds based on visual observation under a light microscope. Seeds were immersed in water at 20 °C for 4 h. A full seed with a turgid and expanded shape (**A**) and cross-section of tissues/organs of a full seed (**B**). Semifull seeds have a valley-like sunken area at the distal end (DE) of the seed (**C**). A semiempty seed with a valley-like area (**D**). A shriveled, empty seed (**E**).

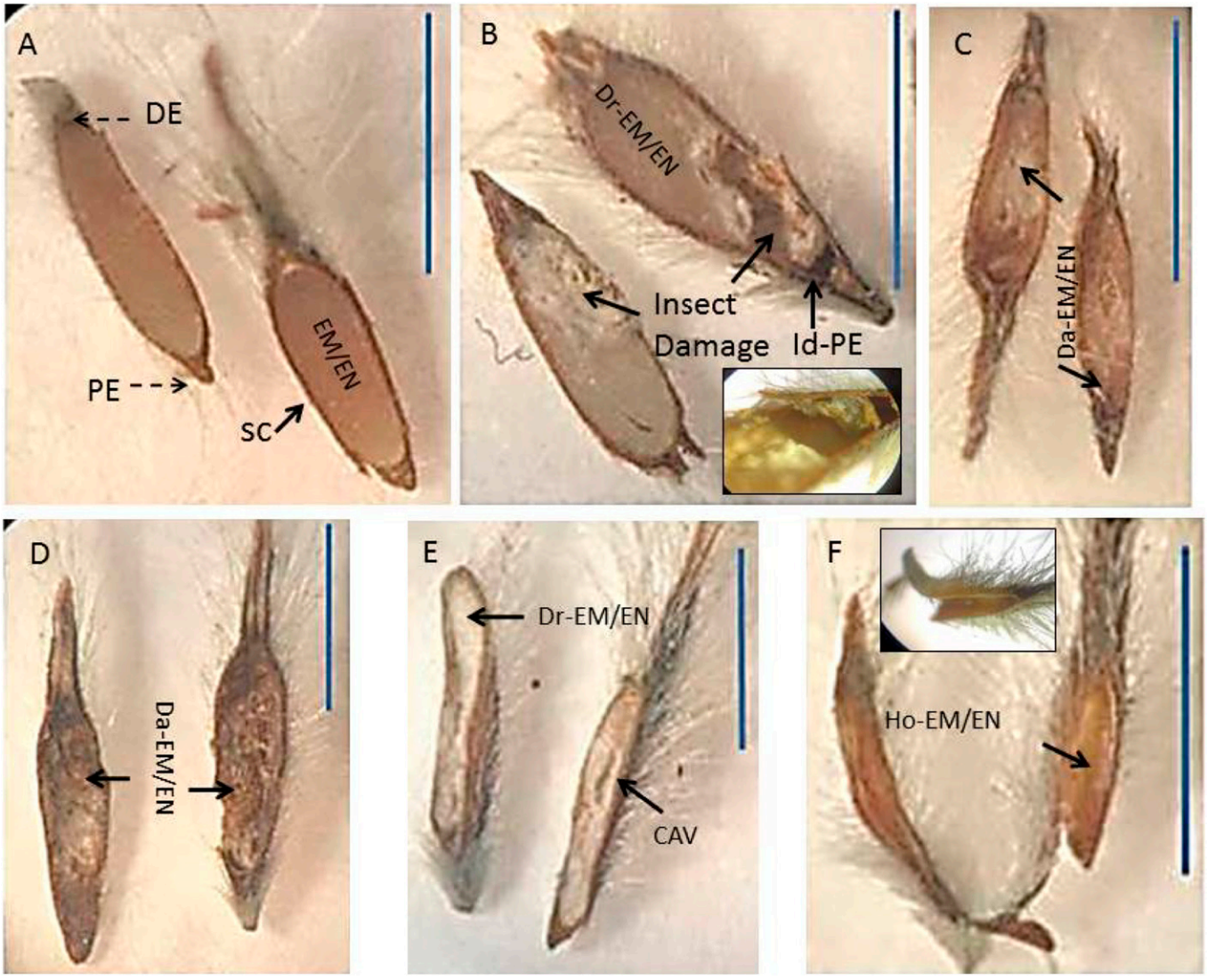


Fig. 2. Visual observations of the vegetative organs (embryos (EM)/endosperms (EN)) in various seeds (longitudinal sections). Seeds were immersed in water, except for those in (**E**) (dry seeds), before observation under a light microscope. A full seed showing turgid vegetative organs from the proximal end (PE) to the distal end (DE) (**A**). A full seed with dried and damaged vegetative organs at the PE due to insect damage (Id-PE; insert) (**B**). Full seeds with damaged or dried vegetative organs in their centers (Da-EM /EN) (**C**). Semifull seeds with severely damaged embryos (Da-EM/EN) (**D**). A dry seed with dried vegetative organs (Dr-EM /EN) showing a cavity (CAV) (**E**). An empty seed with a hollow cavity (Ho-EM/EN) (**F**; insert). Bar = 2.25 mm. Magnification at ×25, except at ×100 (insert in **E**) and 10× (insert in **F**). All seeds except those in (**E**) were immersed in water at 20 °C for 4 h.

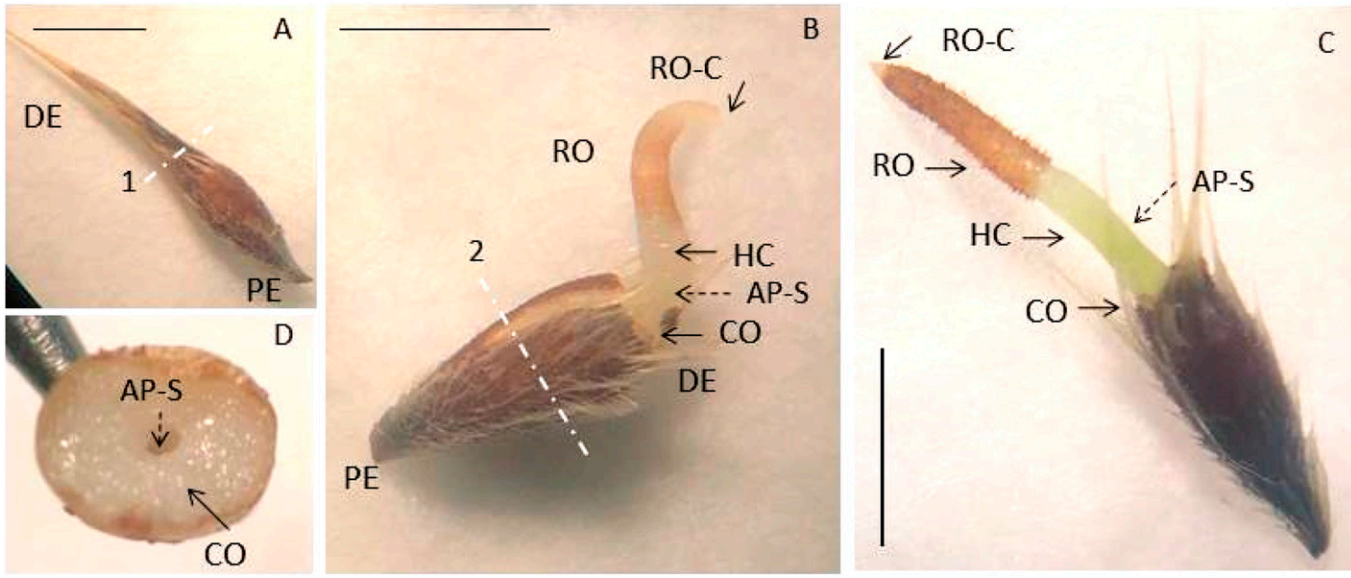


Fig. 3. Development of young seedlings following immersion in distilled water for 8 h and storage at 20 °C for 12 d. Seeds germinated after removal of the stigma at dotted line 1 and showing the distal end (DE) and proximal end (PE) (**A**). Emergence of the root (RO) and root cap (RO-C), the hypocotyl (HC), and the base of the cotyledon (CO) (**C**); and the apical shoot apex (AP-S) developing from the DE of the seed (**B**). Advanced seed germination showing the RO, RO-C, the green HC, and the CO emerging from the DE of seeds (**C**). The AP-S of the seed in a longitudinal cross-sectional view (dotted line 2, **B**). The exposed CO and hole that the AP-S will grow through (**D**). Bar = 0.5 mm.

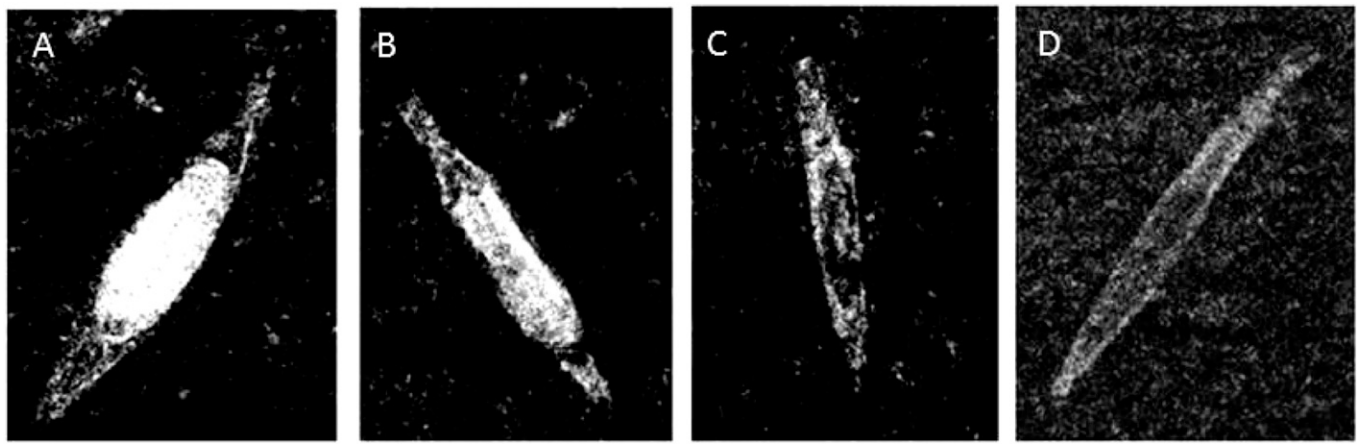


Fig. 4. Classification of *Pulsatilla turczaninovii* seeds based on X-ray images. Full seeds have well-developed vegetative organs (EM and EN) (**A**); semifull seeds have degenerated vegetative organs (**B**). Semiempty seeds with lines surrounding damaged vegetative organs (**C**). Empty seeds showing only a seedcoat (**D**).

and semifull seeds by X-ray images can be attributed to the condition of the internal structures. Seeds having damaged vegetative organs (Da-EM/EN) (Fig. 2C and D) when dried may be considered semifull seeds, and these seeds may present with narrower diameters than the full seeds. Even if seeds are partially damaged by an unidentified insect (Id-PE, insect damage) (Fig. 2B), they may still be considered full seeds. If the vegetative organs are severely damaged and have a cavity (CAV) in the center of the seed but still include dried vegetative organs (Dr-EM/EN) (Fig. 2E), seeds may be classified as either semifull or semiempty seeds, depending on the degree of desiccation of the vegetative organs. By examining images under light microscope, full seeds have well-developed vegetative organs composed of EM, cotyledons (CO), and EN were observed, although these organs cannot be distinguished clearly at the seed stage. Full seeds showed insect damage and deposits at their proximal end of seeds, while the distal end showed undamaged vegetative organs. Damaged areas in some full seeds may have been the cause of failed seed germination. Such damage at the proximal end can contribute to failed seed germination due to damage of vegetative organs and radicle, which will be discussed in the next section.

Full seeds that were immersed in distilled water for 8 h and stored at 20 °C for 12 d germinated (Fig. 3). Development of normal seedlings after removal of the stigma at dotted line 1 (Fig. 3A) showed emerging root (RO) and root cap (RO-C), the hypocotyl (HC), and the base of the cotyledon (CO) (Fig. 3B). Although comparison between stigma removed and attached seeds were not made, seeds with stigma attached tended to take a few more days to germinate (Yoo and Yuan, personal observation, 2017), and this result should further be investigated on the possible presence of inhibitor in the stigma and morphological restriction of the seedcoat and stigma for radicle development.

Some seeds that appear full may also exhibit damaged internal structures that do not show valley-like streaks visible at the surface of the seedcoat (Fig. 1). Further anatomical examination should be carried out to conform this finding. Therefore, X-ray scanning should focus at the DE, which may not be too practical when many small seeds have to be scanned (Fig. 4). Seed germination progressed showing the RO, RO-C, the green HC, and the CO emerged from the DE of seeds. The apical shoot apex (AP-S) of the seed in a cross-sectional view

(dotted line 2, Fig. 3B) will grow out through the AP-S (Fig. 3D).

On the LT-SEM micrographs (Fig. 5), sunken areas resembling a stretched valley were observed in the empty seeds of *P. cernua* var. *koreana*, but not in the full seeds. Full seeds of *P. cernua* var. *koreana* and *P. turczaninowii* were rounder and more swollen in appearance compared with the empty seeds as observed under the light microscope (Fig. 1D and E)—as was also observed on the X-ray images (Fig. 4). A crack in the seedcoat was observed in the empty seeds of *P. turczaninowii* (dotted arrow), which could result from harvesting and handling of seeds. In both species, trichomes were cylindrical, not branched, and were also densely distributed and more abundant at the distal end of the seed. Although seed morphology can be used to differentiate *Cyanea* and other genera in Lobeliodae (Buss et al., 2001), the SEM micrographs obtained in this study did not show any clear differences between *P. turczaninowii* and *P. cornea* var. *coreana*, which are grown in close geographic proximity in northeastern China and Korea (Wang and Bartholomew, 2001).

*X-ray scanning, seed germination and a possible cause for the low germination rates of full seeds.* From a total of 50 visually categorized full seeds, 36 seeds were full examined by X-ray (Fig. 4A), and 26 of the full seeds (72.3%) germinated in 17 d (Table 1). However, only 4.7% semifull seeds germinated in 19 d. Among the visually categorized empty seeds, 5.4 semiempty and 25.6 empty seeds failed to germinate; and from the 12 seeds identified as full based on the X-ray images, 7.3 (60.5%) germinated in 17 d.

The final germination rate of the full and semifull seeds was 64% based on the total number of the two types of seeds; however, this represented only 52.6% of the 50 seeds examined (Table 1). One reason why the germination rate did not exceed 72.3% in full seeds could be because X-ray scanning may not be able to differentiate between seeds with healthy vegetative organs (Fig. 2A) and those damaged by insects or desiccated dry seeds (Fig. 2B–D). If there was insect damage at the proximal end of the seed (Fig. 2B), or if the vegetative organs were dried or damaged (Fig. 2C and D), they were often not detected by X-ray scanning. As a result, these seeds may have been categorized as full seeds, but did not germinate. We also observed dried seeds that had dried vegetative organs (Dr-EM/EN) with notable CAV (Fig. 2E), and empty seeds with valley-like sunken streaks at the surface of the seedcoat and hollow cavities (Ho-EM/EN) (Fig. 2F).

Overall, the germination rate of the seeds that germinated in 17 to 19 d was comparable to that reported by Sang et al. (1996).

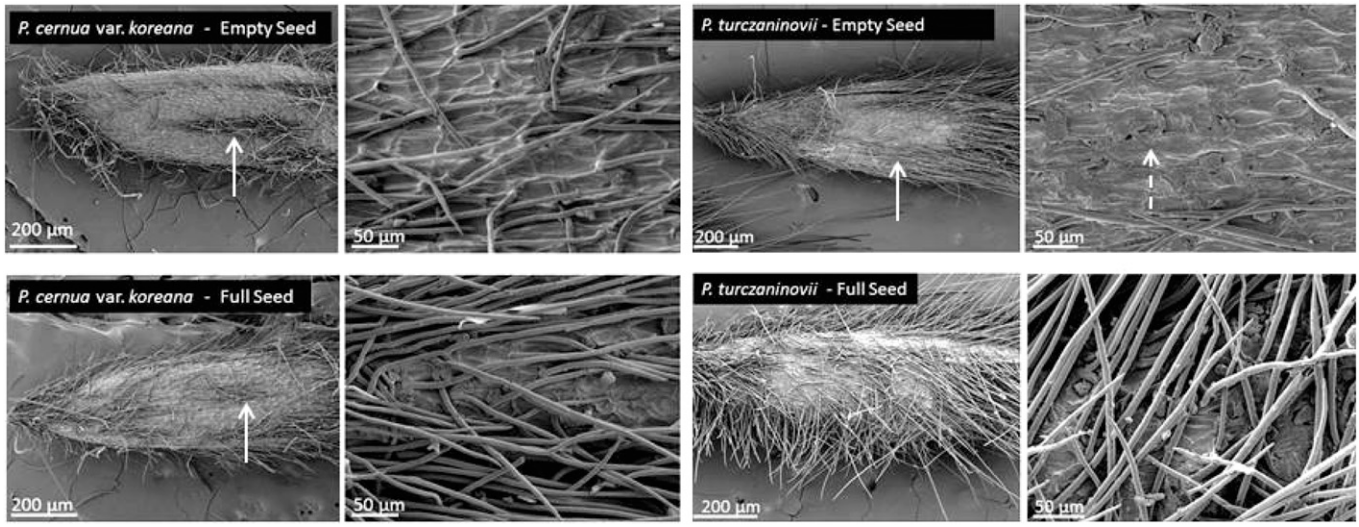


Fig. 5. Low-temperature SEM analyses of full and empty seeds of *Pulsatilla cernua* var. *koreana* collected from Korea and of *P. turczaninovii* collected from China. The valley-like sunken streaks in the empty seeds are indicated with a solid arrow, and the cavities in the empty seeds of *P. turczaninovii* are indicated with a dotted arrow.

Table 1. Germination of *Pulsatilla turczaninovii* seeds based on their classification by visual observation and X-ray imaging.

| Classification of seeds | | No. of seeds | | | |
|---|---|---|---|---|---|
| Visual[z] | X-ray images[y] | Each X-ray image | Germinated | Germination rate | No. of days to germinate |
| Full seed | Full seed | 35.7 a | 26 a | 72.3 a | 17 a |
| | Semifull seed | 5.3 d | 0.3 b | 4.7 b | 1.9 b |
| | Semiempty seed | 4.0 d | 0 b | 0 b | 0 b |
| | Empty seeds | 5.0 d | 0 b | 0 b | 0 b |
| | All full and semifull seeds[x] | | | 64 (52.6) | |
| Empty seed | Full seed | 12 c | 7.3 b | 60.5 a | 17 a |
| | Semifull seed | 7 d | 0.3 b | 4.9 b | 16 a |
| | Semiempty seed | 5.4 d | 0 b | 0 b | 0 b |
| | Empty seeds | 25.6 b | 0 b | 0 b | 0 b |
| | All full and semifull seeds[x] | | | 4.0 (0.15) | |
| Level of significance[w] | | | | | |
| Visual: Full and empty (Visual F to E) | | NS | ** | NS | NS |
| X-ray images (X-ray) | | ** | ** | ** | ** |
| Visual F to E × X-ray | | ** | ** | NS | NS |

[z]Refer to Figs. 1 and 2 for the visual classification of full and empty seeds.
[y]Refer to Fig. 4 for an explanation of the four different seed categories: full, semifull, semiempty, and empty seeds.
[x]The germination rate was calculated as the number of full and semifull seeds that germinated divided by the number of full and semifull seeds or by the total number of seeds (in parentheses). These data were excluded from the ANOVA.
[w]Nonsignificant (NS), significant at $P \leq 0.05$ (**), *F*-test.

However, nine full seeds and five semiempty seeds, as identified on the X-ray images of visually full seeds, did not germinate. This result explained the low germination rate, especially when determined without specifically considering the number of full vs. empty seeds (Sang et al., 1993, 1996). The inability to separate empty from full seeds will lower the germination rate. Thus, the low germination rate in this study may not have been due to seeds that were no longer viable. That low rate may be because the germination tests were performed about 10 weeks after the seeds were harvested, which is shorter than both the 14 weeks reported for seeds that did not germinate after storage at unspecified room conditions (Sang et al., 1993) and the 24 weeks for seeds stored dry at low temperatures (0 or 10 °C), of which 66.3% germinated (Sang et al., 1996).

In our study, most empty and semiempty seeds identified by X-ray scanning, regardless of whether they were visually categorized as full or empty, did not germinate. Because we divided the seeds into "full" and "empty," we were able to attribute the failed germination in full seeds to dormancy rather than a loss of viability only 10 w after harvesting, and this finding was most likely due to desiccation shortly after the seed was harvested, or to insect damage occurring at the proximal end of the seeds where the radicle emerges. Because full seeds were evaluated visually and X-ray images showed germination rates of 72.3% in 17 d, we believe that mailing of the seeds from China to the United States for X-ray imaging did not greatly affect germination, despite not controlling for temperature or humidity.

*Germination of dry stored seeds at 5 °C: Influence of dry storage/concentration and duration of $GA_3$/moist 5 °C treatment (CS).* Germination rates were unaffected by the concentration of $GA_3$; however, immersing seeds in a $GA_3$ solution for 8 h significantly increased the germination rates of seeds to the rates that were not treated $GA_3$. Germination rates showed similar curves whether seeds received 0, 16, or 32 d of CS, as they germinated in 12, 10, and 10 d, respectively (data not presented). Seeds that did not receive CS did not germinate for the first 12 d after sowing (Table 2). Temperatures between 0 °C and 10 °C are considered suitable for most species (Baskin et al., 2006). Germination patterns showed a sigmoid curve regardless of the duration of CS, and data for storage of 16 d duration are presented (Fig. 6).

Seeds dry stored for 44 weeks at 5 °C and not treated with CS did not show higher germination rates when treated with $GA_3$ over the course of 29 d (Table 2). However, when seeds were treated with CS for 16 or 32 d, germination started earlier than for seeds that did not receive CS. At 17 d after sowing, germination of seeds treated with CS for 16 d was accelerated under 50 and 100 ppm $GA_3$ treatments, but no such accelerated germination was observed when seeds were treated with CS for 32 d. Although there was a great variation among replications in a given treatment, there were significant differences among treatments: 24.7% (16 or 32 d of CS, no $GA_3$ treatment) vs. 38.7% (32 d of CS, 100 ppm $GA_3$).

Table. 2. Effects of $GA_3$ concentration, treatment duration, and cold treatment on the number of *Pulsatilla turczaninovii* seeds germinated at 20 °C. Seeds were dry stored at 5 °C for 44 weeks and then treated with $GA_3$ followed by a 5 °C cold stratification (CS) treatment.

| $GA_3$ treatment[z] | | | No. of seeds germinated (germination rate) | | | | |
|---|---|---|---|---|---|---|---|
| | | | No. of days after sowing[x] | | | No. of days (as ranges) after sowing[w] | |
| Concn (ppm) | Treatment duration (h) | No. of days at 5 °C (CS)[y] | 12 | 17 | 29 | 12–17 | 17–29 |
| 0 | 0 | 0 | 0.0 b[v] | 16.7 e–g | 28.0 d–g | 16.7 c–f | 11.3 a |
| 0 | 8 | 0 | 0.0 b | 22.0 d–g | 29.7 c–f | 22.0 b–f | 7.7 a–d |
| 50 | 0 | 0 | 0.0 b | 13.7 f–g | 24.3 e–g | 13.7 ef | 10.7 ab |
| 50 | 8 | 0 | 0.0 b | 15.3 e–g | 21.7 fg | 15.3 ef | 6.3 a–e |
| 100 | 0 | 0 | 0.0 b | 8.3 g | 14.0 g | 8.3 f | 5.7 b–e |
| 100 | 8 | 0 | 0.0 b | 12.3 fg | 14.7 g | 12.3 ef | 3.3 de |
| 0 | 0 | 16 | 5.0 ab | 28.0 b–e | 29.7 c–f | 21.3 b–f | 1.7 e |
| 0 | 8 | 16 | 7.0 a | 42.0 ab | 52.0 a | 29.3 a–d | 9.3 a–c |
| 50 | 0 | 16 | 7.0 a | 24.7 c–f | 32.0 c–f | 15.7 d–f | 7.3 a–d |
| 50 | 8 | 16 | 7.3 a | 36.3 a–c | 48.0 ab | 25.3 b–e | 8.3 a–d |
| 100 | 0 | 16 | 5.0 ab | 28.0 b–e | 38.3 a–e | 23.0 b–e | 10.3 ab |
| 100 | 8 | 16 | 10.0 a | 43.0 a | 52.0 a | 33.0 ab | 8.3 a–d |
| 0 | 0 | 32 | 1.0 b | 24.7 c–f | 26.3 d–g | 23.7 b–e | 2.7 de |
| 0 | 8 | 32 | 7.0 a | 47.7 a | 52.3 a | 40.0 a | 3.7 de |
| 50 | 0 | 32 | 4.7 ab | 34.7 a–d | 37.3 b–e | 30.0 a–c | 2.7 de |
| 50 | 8 | 32 | 5.7 ab | 38.7 a–c | 44.0 a–c | 33.0 ab | 5.3 b–e |
| 100 | 0 | 32 | 4.7 ab | 38.0 a–c | 39.3 a–d | 33.0 ab | 1.3 e |
| 100 | 8 | 32 | 7.7 a | 38.7 a–c | 40.0 a–d | 31.0 ab | 4.7 c–e |
| Level of significance[u] | | | | | | | |
| $GA_3$ concentration (ppm) (A) | | | NS | NS | NS | NS | NS |
| $GA_3$ duration (h) (B) | | | * | *** | *** | ** | NS |
| Days at 5 °C (C) | | | *** | *** | *** | *** | *** |
| A × B | | | NS | NS | * | NS | NS |
| B × C | | | NS | NS | ** | NS | ** |
| A × C | | | NS | NS | NS | NS | * |
| A × B × C | | | NS | NS | NS | NS | NS |

[z]Seeds were treated with 0, 50, or 100 ppm $GA_3$ solution for 8 h.
[y]Seeds were stored at 20 °C for 32, 16, or 0 d followed by respective $GA_3$ treatments, and then received 0, 16, and 32 d of CS, respectively.
[x]Only data of selected dates are presented.
[w]Data regarding the number of seeds germinated between days 12 and 19 (19 to 12 d), and between days 19 and 29 (29 to 19 d), are presented.
[v]Means with the same letter within a column are not significantly different at $P \leq 0.05$ by Duncan's multiple range test.
[u]Nonsignificant (NS), significant at $P \leq 0.05$ (*), 0.01 (**), and 0.001 (***), *F*-test.

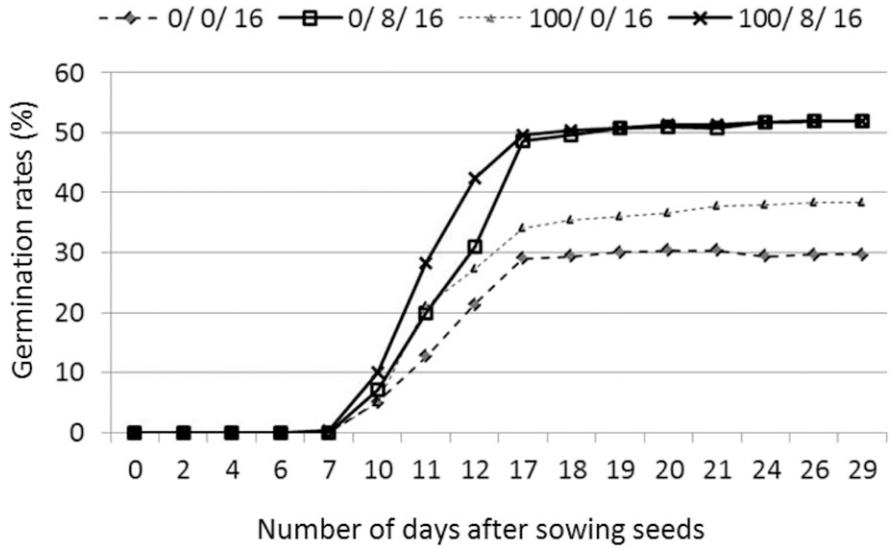


Fig. 6. Germination patterns of seeds that were stored dry at 5 °C for 44 weeks. Seeds were treated with 0 ppm $GA_3$ for 0 h followed by 16 d of cold stratification (CS) treatment (0/0/16) or 100 ppm $GA_3$ for 8 h, then followed by 16 d of CS treatment (100/8/16).

In general, more seeds germinated between 12 and 17 d compared with 17 and 29 d, especially when seeds were treated with $GA_3$ and CS. For example, seeds that

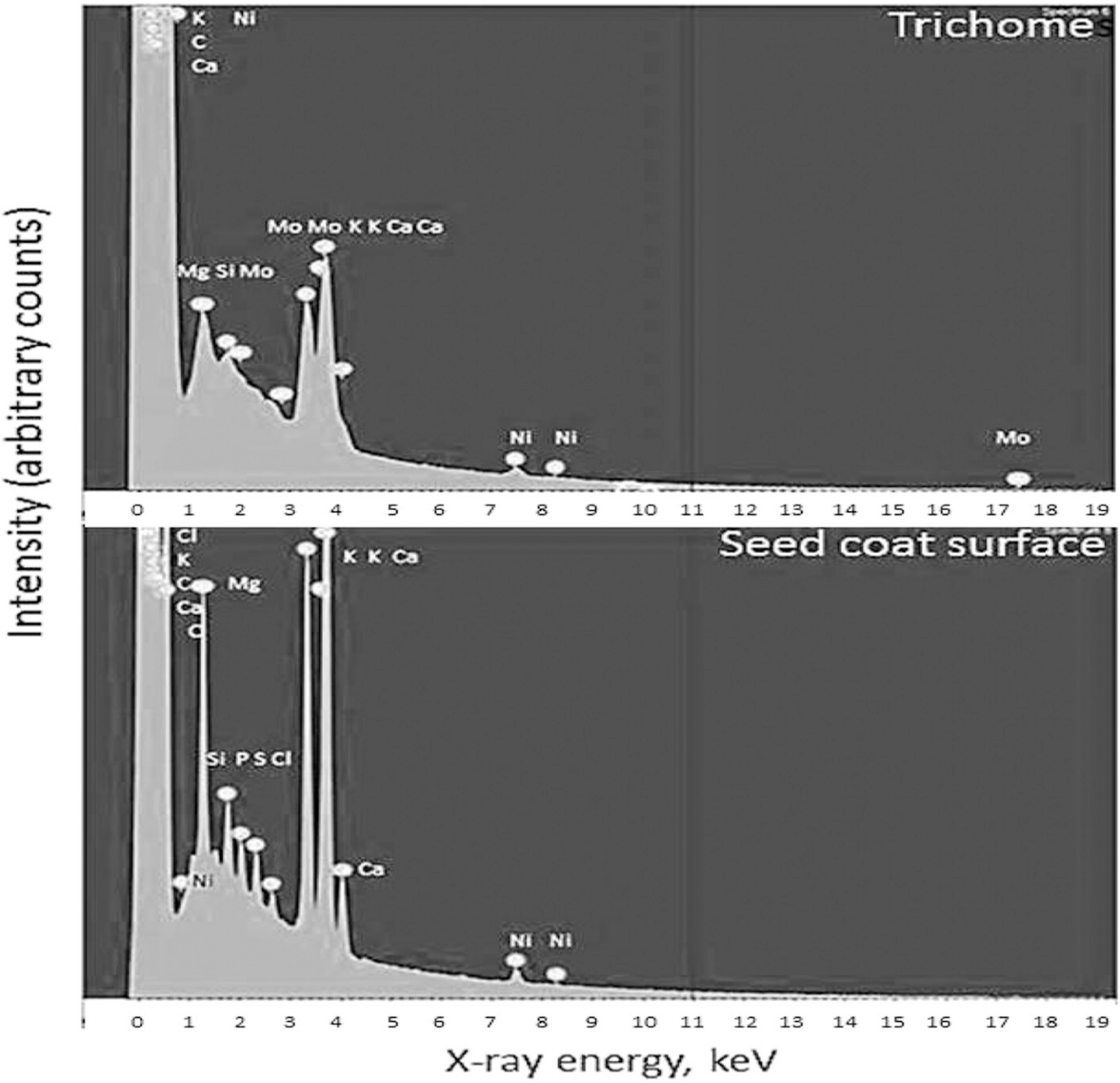


Fig. 7. Comparison of the energy-dispersive X-ray diffraction profiles of the trichome and seedcoat surface of full *Pulsatilla turczaninovii* seeds. The *y* axis shows the intensity of the peaks, and the *x* axis shows the applied X-ray energy (keV).

received 32 d of CS germinated at a rate significantly higher (30.0% to 33.0%) than those that received no CS (13.7% to 22.0%) (Table 2). The germination rate was 44% to 48% when the seeds were treated with 50 ppm solution for 8 h and received 32 d or 16 d of CS, which was significantly higher than the rate of 26.3% to 29.7% when seeds were not treated with $GA_3$, regardless of the duration of CS.

The germination rate of visually full seeds upon harvest (52.6%) (Table 1) was higher than that of seeds that were dry stored for 44 weeks (26.3% to 29.7%) and were not treated with $GA_{3n}$ or received any CS (Table 2); this result suggests that the viability of seeds may have decreased, reducing the germination rate by about 50%. The ideal temperature for germination of *Swertia chirayita* is considered 4 °C for 24 m, which produced a rate of germination exceeding 50% (Pradhan and Badola, 2012). It is assumed that other factors, such as dormancy, insect damage, and so forth may affect the development of vegetative organs, but these were not investigated or documented in *Pulsatilla* seeds.

Dormancy could be a factor for low germination rates and can be avoided by low-temperature treatments or plant growth-regulating treatments, especially true with gibberellin treatment (Cadman et al., 2006; Elhindi et al., 2016) or abscisic acid that affects the sensitivity of GA and the ratios between abscisic and GA (Finch-Savage and Leubner-Metzger, 2006). The overall germination rate of *P. turczaninovii* was 48% to 52.6%; these rates were significantly lower than that of *P. cernua*, which was >92% with a $GA_3$ treatment for 24 h, compared with 18% in an untreated control (Gu et al., 2014). Fresh *P. turczaninovii* seeds were also treated with 100 ppm $GA_3$ (64.0% in 32 d vs. 15.0% for the control in 60 d) (Shi et al., 2005). Similarly, Li and Piao (2010) reported that soaking seeds in $GA_3$ for 12 h increased germination. The germination rate of *P. turczaninovii* was enhanced as the temperature increased, and the optimum temperature is 25 to 30 °C following a 100 ppm $GA_3$ treatment (Wang et al., 2013), it is likely that the low germination rates in this study may be

Table 3. Normalized elemental analysis based on X-ray diffraction studies of the seedcoat surface of full and empty seeds distinguished on the X-ray images collected from China and Korea. The results are expressed on a % weight basis.

| | | Elemental analysis in wt bases (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Country of collection site | X-ray images[z] | Magnesium (Mg) | Silicon (Si) | Phosphorus (P) | Sulfur (S) | Potassium (K) | Calcium (Ca) | Nickel (Ni) | Total |
| China | Full | 11.13 a[y] | 7.03 a | 6.53 a | 3.28 | 52.01 a | 14.61 b | 4.62 a | 100 |
| | Empty | 5.68 b | 6.19 a | 5.62 a | 2.98 | 57.95 a | 17.75 b | 3.50 a | 100 |
| Korea | Full | 8.77 ab | 7.42 a | 2.73 b | 2.78 | 36.50 b | 42.69 a | 2.98 a | 100 |
| | Empty | 5.81 b | 3.73 b | 4.45 ab | 3.43 | 57.98 a | 20.89 b | 3.77 a | 100 |
| Level of significance[x] | | | | | | | | | |
| China vs. Korea (CK) | | NS | NS | NS | NS | * | ** | NS | |
| Full vs. empty (FE) | | * | * | NS | NS | * | * | NS | |
| CK × FE | | NS | * | * | NS | * | * | NS | |

[z]Refer to Fig. 4 for a classification of the seeds based on X-ray imaging.
[y]The means were compared using Duncan's multiple range test. Those with the same letter in a column did not differ significantly.
[x]Nonsignificant (NS), significant at $P \leq 0.05$ (*) and 0.01 (**), *F*-test.

attributable to differences in germination temperature.

Further research is required to understand why seed germination in this study was lower than in other reports. It is possible that seed maturity played a role, as seeds that are immature at the time of harvest may fail to germinate, which is not the same as dormancy. In *Styrax japonicus* Sieb. et Zucc, mature seeds were harvested at 16 to 19 weeks after anthesis, and this yielded germination rates exceeding 65% (Roh et al., 2004).

*Chemical composition of the seeds on energy-dispersive X-ray diffraction analysis.* Energy-dispersive X-ray diffraction analyses were performed in addition to SEM to analyze the elemental composition of the trichome and seedcoat surface of full seeds of *P. turczaninovii* (Fig. 7). This method has been widely used to determine the precise chemical composition based on correlations with high-resolution SEM images for element mapping and quantification (Wyroba et al., 2015). Nickel was detected in both the trichome and the seed surface, although on a weight basis (weight %) the quantities, as judged by arbitrary counts, were much lower than those of other elements, including potassium, calcium, and magnesium, as also reported for *Alyssum* (Broadhurst et al., 2009). Nickel was concentrated in the base of the trichome (8.0 weight %) and in cells adjacent to the site of trichome development (9.0 weight %) (Broadhurst et al., 2009). Therefore, both full and empty seeds of *P. turczaninovii* and *P. cernua* var. *koreana* were analyzed further. No significant differences in nickel accumulation with respect to the size or the position of the leaves, or the position of the trichome were found (Broadhurst et al., 2009).

Potassium was the most abundant element in the seedcoats of full and empty seeds, accounting for 36.5 weight % in empty *P. cernua* var. *koreana* seeds and >52.01 weight % in the full and empty seeds of *P. turczaninovii* (Table 3). Calcium levels (42.69 weight %) were the highest in full *P. cernua* var. *koreana* seeds; while in full and empty *P. turczaninovii* seeds, the levels were 14.61 and 17.75 weight %, respectively. Potassium levels were significantly lower in the full than in the empty seeds of *P. cernua* var. *koreana* and in the full and empty seeds of *P. turczaninovii*. In the common bean (*Phaseolus vulgaris* L.), the calcium concentration was higher in the seedcoat and the potassium concentration was higher in the EM (Ribeiro et al., 2012). Magnesium, silicon, phosphorus, and sulfur were also detected, in amounts of 5.68 to 11.13, 3.73 to 7.42, 2.73 to 6.53, and 2.78 to 3.43 weight %, respectively, while nickel made up 2.98 to 4.62 weight %. Because the analysis was replicated only twice for quantification and the roles of potassium and calcium in the seedcoat are not known, conclusions can only be drawn once a larger number of samples has been analyzed.

The role of nickel (even in low amounts) in *Pulsatilla* seeds is unclear, so it is unclear whether nickel in these seeds results in a low germination rate. In *Raphanus sativus* cv. Early Menu, 100 µM nickel (the lowest concentration tested; ranges of 100 to 1000 µM) inhibited seed germination and caused poor seedling growth (Yadav et al., 2009). When seedlings were transplanted, the growth of *Pulsatilla* was poor, showing necrotic regions at the tips of the young leaves (unpublished data). The role of nickel in the growth of seedlings should be further examined. Recovery from poor germination caused by Ni was achieved using copper and boron. *Alyssum murale* 'Kotodsch' and *A. corsicum* are nickel hyperaccumulators, reaching nickel contents of >2.5 weight % (Broadhurst et al., 2009).

## Conclusions

*Pulsatilla turczaninovii* seeds stored dry at 5 °C for 10 weeks after harvest germinated in 17 to 19 d at 20 °C, where the germination rate was 64%, based only on the number of full seeds, vs. 52.6% when full and empty seeds were counted. The majority of the seeds stored dry at 5 °C for 44 weeks and then treated with $GA_3$ and receiving 32 d of CS, also germinated in 17 d as compared with between 17 and 29 d. X-ray images were used to categorize the seeds as full, semifull, semiempty, or empty. Full seeds with well-developed vegetative organs observed under a light microscope corresponded to seeds that were transparent or translucent on X-ray images. Seeds showing damaged vegetative organs when dried can be considered semifull seeds. Seeds that were severely damaged and exhibited a cavity were considered as either semifull or semiempty seeds.

Comparison of the germination rates of visually full seeds at 10 weeks after harvest (52.6%) vs. those that were dry stored for 44 weeks and not treated with $GA_3$, or that received 0 d of CS (13.7% to 22.0%) and had germination rates of 44.0% to 48.0% when seeds were treated with 50 $GA_3$ ppm solution for 8 h, plus 16 d or 32 d of CS, suggests that viability of seeds may have decreased. Improvement of seed germination to >64% for full seeds may be possible by overcoming seed dormancy if dormancy is present upon harvest.

Nickel was detected in the full and empty seeds of both taxa by energy-dispersive X-ray images and accounted for 2.98 to 4.62 weight %. Our study suggests that the low germination rates can be attributed to dormancy, damage to the EM or EN (vegetative organs), and loss of viability after dry storage at 5 °C, or the presence of nickel in the seeds. Further research is required to elucidate the physiological role of nickel in seed germination, and in the growth and development of *Pulsatilla turczaninovii* seedlings.

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