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Germination and abscisic acid analysis in the seeds of forest resource trees after storage at different temperatures

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ABSTRACT

Abscisic acid (ABA) is a plant hormone that plays a significant role in various developmental and physiological processes in higher plants. In this study, ABA was quantified in *Chamaecyparis obtusa*, *Pinus rigida* × *Pinus taeda*, and *Pinus densiflora* seeds stored for 20 years at −18 °C, 4 °C, or 25 °C using high-performance liquid chromatography. At all temperatures, *C. obtusa* had the highest ABA concentration, followed by *P. rigida* × *P. taeda* and then *P. densiflora*. The germination rates of the seeds were also investigated. For the two *Pinus* species, there was an inverse relationship between ABA content and germination rate. However, as all *C. obtusa* seeds failed to germinate, no such correlation was observed for this species. This study provides important information for advancing ABA research in gymnosperms, which may provide insight into their physiological and biochemical mechanisms.

ARTICLE HISTORY

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Seed germination; seed storage; ABA; content analysis; HPLC/DAD

1. Introduction

Abscisic acid (ABA) is a phytohormone that is intricately involved in seed development and maturation, as well as protein synthesis that helps plants overcome or endure stressors like desiccation, cold, and high salinity (Nambara et al. 2010; Wan et al. 2015; Abrams and Loewen 2019; Oh et al. 2022). It also functions as a general growth and metabolic activity inhibitor.

In higher plants, ABA is synthesized by the oxidative breakdown of C₄₀ carotenoid 9-cis neoxanthin into C₁₅ xanthorin and C₂₅ allenic-apo-aldehyde (Abrams and Loewen 2019; Cardoso et al. 2020; Chen et al. 2020). Ring modification and side chain oxidation are then applied to xanthoxin in a two-step process to create ABA (Abrams and Loewen 2019; Cardoso et al. 2020). These two steps are catalyzed by enzymes that are produced enzymatically. In contrast, the initial carotenoid cleavage process, which is the essential regulatory step in the synthesis of ABA, is catalyzed by 9-cis-epoxy carotenoid dioxygenase (Wan et al. 2015). Throughout a plant's life cycle, ABA is engaged in numerous physiological and developmental processes (Abrams and Loewen 2019; Ali et al. 2022). It controls the maintenance of embryonic dormancy and seed maturation during the early stages of a plant's development (Ali et al. 2022).

However, there is scant data on ABA extraction and quantification from plants in general, let alone from gymnosperms, including the species used in this study.

Studies characterizing other natural compounds found in these species have been conducted by Hong et al. (2004), Lee (2009), and Park and Woo (2022), but there have been few to none targeting ABA. The biomedical properties and applications of ABA may be further elucidated with more research. Hence, primary studies on ABA extraction will provide a backbone for further research advancements in this topic. Based on previous studies, there is an inverse relationship between ABA content and germination rate, this is because ABA plays a strong role in maintaining dormancy in plants (Rodríguez-Gacio et al. 2009; Kim et al. 2021; Wang et al. 2021).

Chamaecyparis obtusa (Cupressaceae, Gymnospermophyta), commonly known as Hinoki cypress, is a common gymnosperm tree in Japan famous for its timber and wood (Osone et al. 2020; Tanaka et al. 2021). It can grow to as high as 35 m with a trunk diameter of up to 1 m (Ogawa 2019). The wood from *C. obtusa* is preferred for light construction, boxes, veneers, and plywood because it is simple to saw and season (Osone et al. 2020; Tanaka et al. 2021). The species has been found to have several biological activities (Ikei et al. 2018; Park et al. 2019; Eltayeb et al. 2023). In fact, Ikei et al. (2018) showed that just touching the Hinoki cypress can induce physiological relaxation. Using a different approach, Park et al. (2019) extracted essential oils from *C. obtusa* and tested their modulating effects on

autonomic nervous system activity. The results were promising, implying that essential oils from this species can be used in drug development research. Another study employing *C. obtusa* essential oils examined their overall antioxidant activity using the ABTS assay and discovered that *C. obtusa* contains natural products with a range of food, cosmetic, and medicinal applications (Eltayeb et al. 2023).

Pinus (Pinaceae)-often found as tall, strong trees and infrequently as shrubs-is a genus characterized by needle-shaped evergreen foliage and tissues that store resin (Trueba et al. 2022). Like *C. obtusa*, species from this genus are also used as timber and wood with various applications in housing and construction (Ramage et al. 2017). The biological activities of the seeds and cones of *Pinus* species have been tested by various studies (Jeong et al. 2014; Jo et al. 2012; Choi et al. 2020; Kim et al. 2013a; Kim et al. 2013b). Jeong et al. (2014) investigated the antibacterial effects of the *P. densiflora* cone extracts on different microorganisms. Needle extracts of the same species also exhibit anticancer properties against YD-8 human oral cancer cells (Jo et al. 2012). *Pinus rigida* extracts, on the other hand, showed antimicrobial properties against *Bacillus subtilis* (Kim et al. 2013a) and *Staphylococcus aureus* and *Escherichia coli* (Kim et al. 2013b). Extracts from this species also exhibited antioxidant activity when evaluated using the DPPH assay, ABTS radical scavenging assay, reducing power assay, and Fe^{2+} chelating assay (Choi et al. 2020).

In this study, the seeds of three different gymnosperm species - *C. obtusa*, *P. rigida* $\times$ *P. taeda*, and *P. densiflora* - were stored at three temperatures, and then their ABA content was quantitated via high-performance liquid chromatography (HPLC). To determine whether there is a correlation between ABA content and germination, the germination rate of the seeds was also tested.

2. Materials and methods

2.1. Plant materials

Experimental plants - *C. obtusa*, *P. rigida* $\times$ *P. taeda*, and *P. densiflora* - were provided by the National Institute of Forest Science (NIFoS), Korea. To test seed

vitality after long-term storage, seeds of each species were collected from the NIFoS orchard in 2003 and stored at three storage temperatures: $-18^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$, and $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

2.2. Instruments and reagents

HPLC was performed using an Agilent 1260 Infinity II Quat Pump (CA, USA) and DAD WR detector (CA, USA). HPLC-grade water and acetonitrile (ACN) was purchased from J. T. Baker (PA, USA), and ABA (Figure 1) was provided by Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea.

2.3. Seed culture conditions

After storage under each condition for 20 years, 100 seeds were sown on a 1% agar plate. The agar plate was then incubated for 30 days in a BOD incubator set at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with 16 h of light, and 8 h of darkness. Germination was recorded when radicle root lengths exceeded 2 mm, and the germination rate was measured for 30 days to evaluate the effects of long-term storage by species and storage condition.

2.4. Preparation of standard and sample solutions

To create a series of standard solutions, ABA was dissolved at 1 mg/mL. Then, by diluting this standard stock solution to the necessary concentrations, the calibration curve was generated. The seeds of *C. obtusa*, *P. rigida* $\times$ *P. taeda*, and *P. densiflora* were extracted three times in ethanol (EtOH) using reflux cooling at 75°C for three hours. From the resultant extract, a concentrated EtOH extract was produced by filtration and evaporation. Each extract was diluted in 1 mL of methanol (MeOH), filtered through a $0.45\ \mu\text{m}$ filter, and then analyzed by HPLC.

2.5. HPLC conditions and calibration curve

A quantitative HPLC analysis was performed on the samples (Tables 1 and 2). The standard compounds were prepared in five concentrations, and then by first plotting the peak area of each solution against its matching concentration, a calibration curve was created. Following that, linearity was assessed using the correlation coefficient (r^2). The analyte's content was then determined by matching it to the calibration curve. Finally, the peak area (Y) and concentration (X,

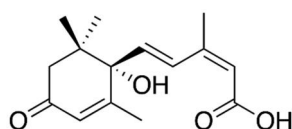


Figure 1. Chemical structure of ABA.

Table 1. Operating conditions of HPLC.

Item	Condition
HPLC	Agilent 1260 Infinity II Quat Pump, CA, USA DAD WR detector, CA, USA
Column	INNO C ₁₈ column (25 cm $\times$ 4.6 mm, 5 μm)
Injection	10 μL
Mobile	Gradient [A Water : B 50% MeOH in ACN : C 1% TFA in Water]
Flow rate	1.0 mL/min
Wavelength	UV 250 nm

mg/mL) were used to generate the standard correction function. Table 3 displays the mean and standard deviation (SD) for this calculation ($n = 5$).

Table 2. Mobile condition of HPLC.

Time (min)	Solvent		
	A (%)	B (%)	C (%)
0	98.2	1	0.8
20	54.2	45	0.8
22	54.2	45	0.8
28	0	99.2	0.8
33	98.2	1	0.8
40	98.2	1	0.8

A is Water, B is 50% MeOH in ACN, and C 1% TFA in Water.

Table 3. Calibration curve of ABA.

Compound	t_R	Calibration equation ^z	Correlation factor, r^2 ^y
ABA	21	$Y = 27.571X - 0.6042$	0.9995

^z Y = peak area, X = concentration of standard ($\mu\text{g/mL}$).

^y r^2 = correlation coefficient based on three data points in the calibration curve.

2.6. Statistical analysis

Results were subjected to a two-way analysis of variance (ANOVA) using SAS analytical software. The values were expressed as the mean $\pm$ SD.

3. Results and discussion

The ABA content of the seeds from the three species was analyzed. The seeds were stored for 20 years under three storage temperatures (-18 , 4 , and 25°C), and the germination rate was tested yearly. A correlation

Table 4. Content of ABA in EtOH extracts of the seeds of three forest resource trees after storage at different temperatures.

Storage temperature	ABA content (mg/g DW)		
	<i>C. obtusa</i>	<i>P. rigida</i> $\times$ <i>P. taeda</i>	<i>P. densiflora</i>
-18°C	3.50 ± 0.81	2.86 ± 0.69	2.17 ± 0.04
4°C	3.97 ± 0.54	4.27 ± 1.60	3.33 ± 0.62
25°C	6.26 ± 0.61	5.93 ± 1.15	5.09 ± 0.71

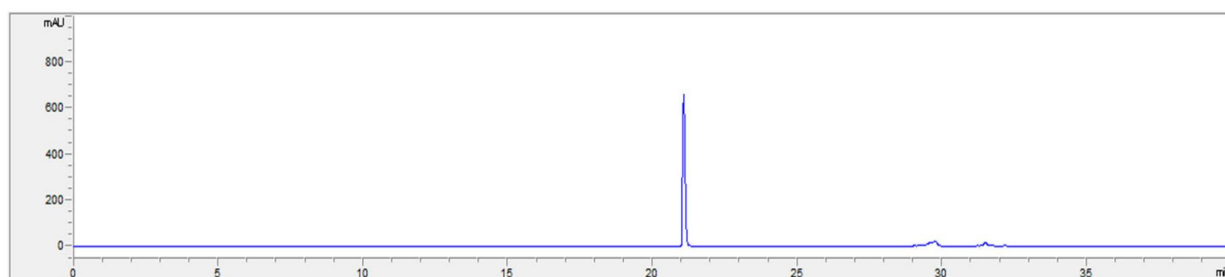
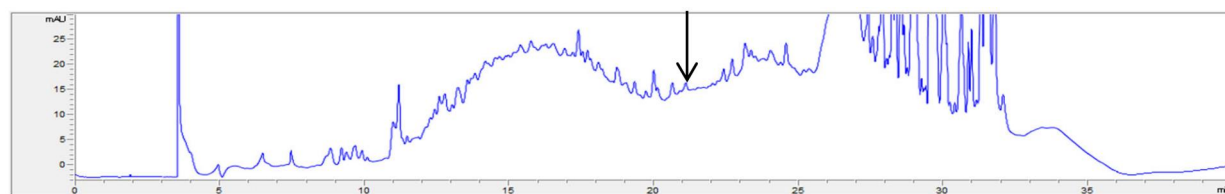
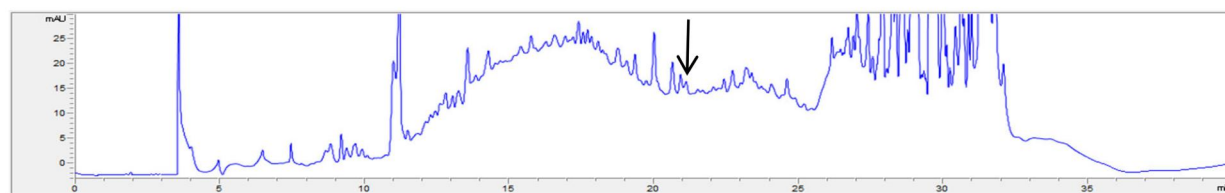


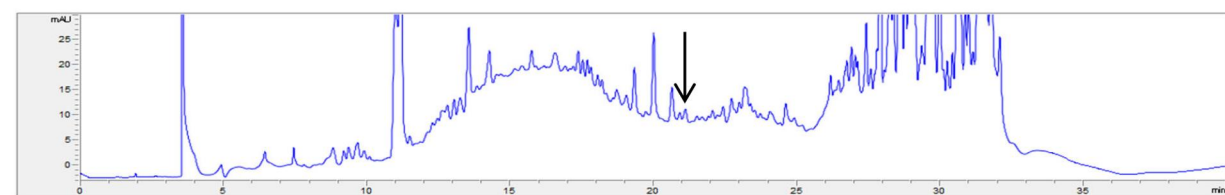
Figure 2. HPLC Chromatogram of ABA.



(A)

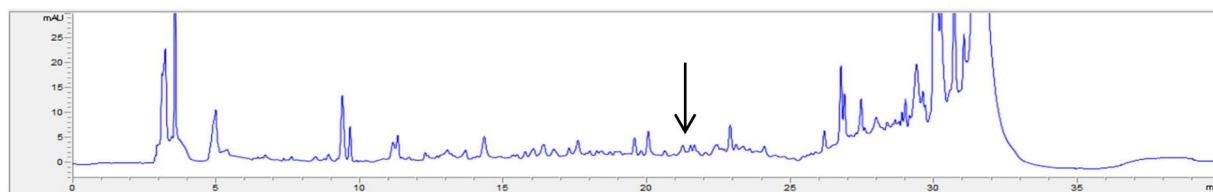


(B)

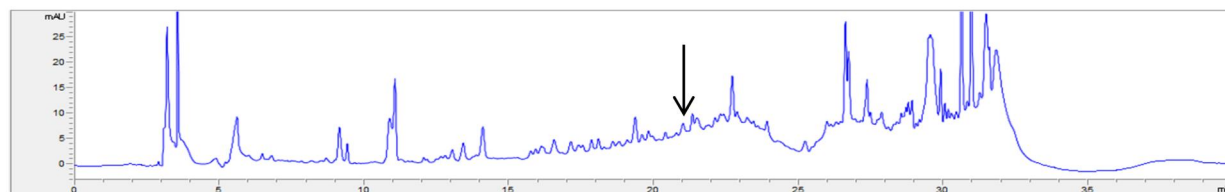


(C)

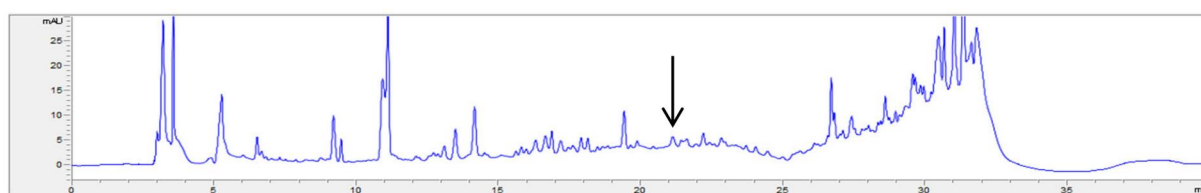
Figure 3. HPLC chromatograms of *C. obtusa* seed extracts from seeds stored for 20 years at -18°C (A), 4°C (B), and 25°C (C). The arrows point to the peaks representing ABA.



(A)

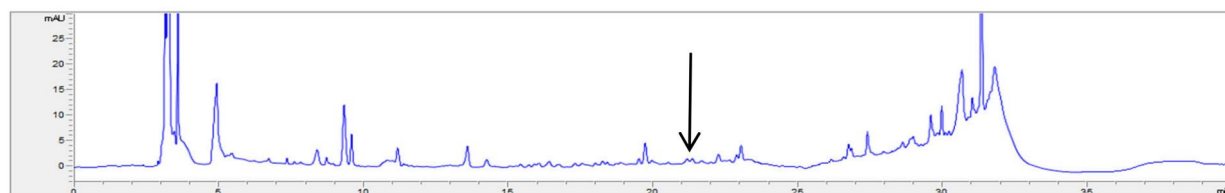


(B)

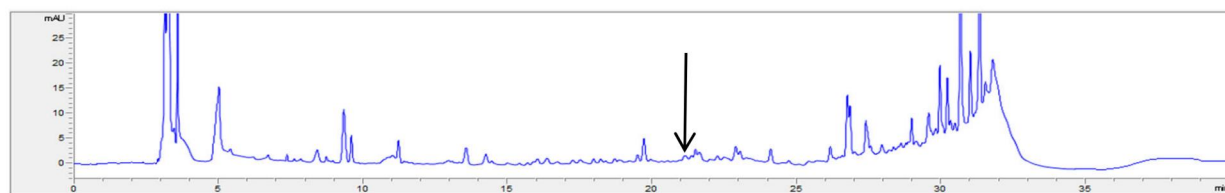


(C)

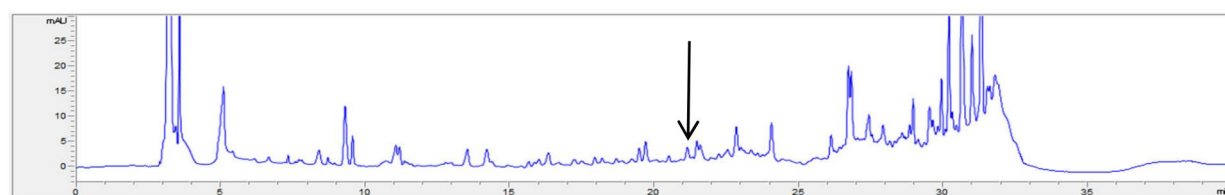
Figure 4. HPLC chromatograms of *P. rigida* $\times$ *P. taeda* seed extracts from seeds stored for 20 years at -18°C (A), 4°C (B), and 25°C (C). The arrows point to the peaks representing ABA.



(A)



(B)



(C)

Figure 5. HPLC chromatograms of *P. densiflora* seed extracts from seeds stored for 20 years at -18°C (A), 4°C (B), and 25°C (C). The arrows point to the peaks representing ABA.

analysis between concentrations of the seed germination-related hormone-ABA, a germination inhibitor and germination rate for each condition was performed.

ABA showed good linearity at a retention time of 21 min (Figure 2). As shown in Table 4, *C. obtusa* seeds had the highest overall ABA content of the three species, and *P. rigida* × *P. taeda* had more ABA content than *P. densiflora*. At the storage temperatures, −18, 4, and 25 °C, *C. obtusa* contained 3.50, 3.97, and 6.26 mg/g DW, respectively, of ABA (Figure 3); *P. rigida* × *P. taeda* contained 2.86, 4.27, and 5.93 mg/g DW, respectively (Figure 4); and *P. densiflora* contained 2.17, 3.33, and 5.09 mg/g DW, respectively

Table 5. Two-way ANOVA treatment of ABA content.

Source	DF	Anova SS	Mean square	F value	Pr > F
C	2	39.60462301	19.80231151	24.30	<0.0001
T	2	3.14154118	1.57077059	1.93	0.1744
C*T	4	1.48993966	0.3724891	0.46	0.7662

C is species, T is temperature, and C*T is species average compared with average time.

(Figure 5). Therefore, we observed a linear relationship wherein the ABA content increased as the storage temperature increased, and the highest content was observed at 25 °C.

According to the statistics treatment (Table 5), the ABA content from the three species is not significantly affected by temperature ($p > 0.1744$). However, the species source was observed to affect ABA content significantly ($p < 0.0001$). These results are visualized in Figure 6. Environmental stressors, the age of the parent tree, the time of year the seeds were gathered, light and temperature levels, and the overall health of the parent tree may all have an impact on ABA concentrations (Kraus and Ziegler 1993; Zhang and Zhu 2022; Oh et al. 2022). One possible reason behind the observed phenomenon is that environmental stressors like heat and desiccation induce ABA production (Toh et al. 2008; Tuan et al. 2018). However, due to a scarcity of related data, we cannot parse how the different responses to heat and desiccation may have contributed to the observed differences in ABA content (Maia 2014). Toh et al. (2008) investigated the role of

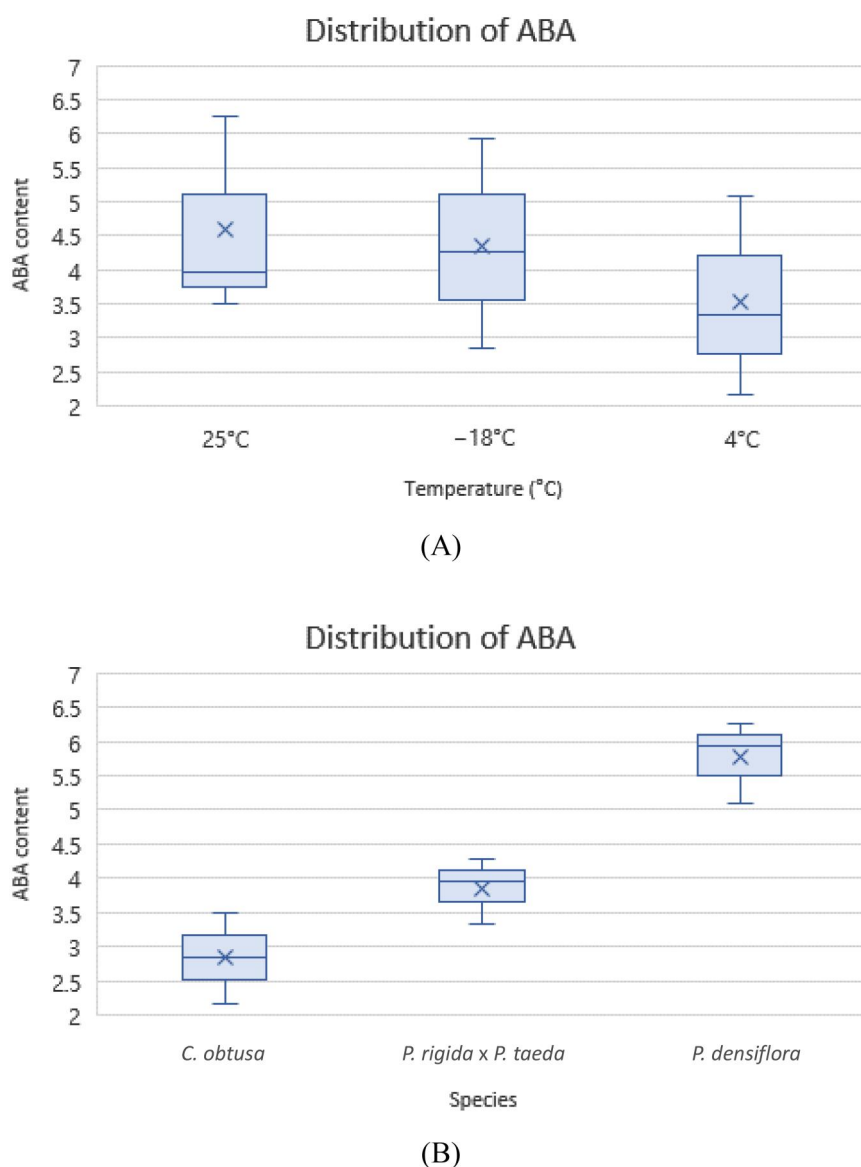


Figure 6. Two-way ANOVA treatment of ABA content according to temperature (A) and species (B).

temperature in the synthesis of ABA in *Arabidopsis* seeds. When the seeds were subjected to high temperatures, the ABA production increased, the production of gibberellins was suppressed, and even the expression of ABA synthesis genes was upregulated. A similar mechanism may have been behind the results seen in this study.

The age of the plant can also affect the amount of ABA in the plant body (Kraus and Ziegler 1993; Zhang and Zhu 2022). Older and more developed plants may yield samples with more ABA than younger ones (Kraus and Ziegler 1993). The ABA content of the seeds might also be influenced by any injuries to the plant's body parts at the time of sampling (Kraus and Ziegler 1993). A study conducted by Pashkovskiy et al. (2019) showed that there is a tendency for ABA to accumulate in the needles of a tree when it is subjected to stress or injury, which might explain why no ABA was detected in the seeds used in that study. Similar results were observed in De Diego et al. (2013) when they subjected *Pinus* sp. to drought. *via* its influence on light and temperature, season, in effect, also influences the ABA levels in plants (Zhang and Zhu

2022). Studies using gymnosperms as samples have found that ABA content was higher during spring and early summer (Kraus and Ziegler 1993). Therefore, the time or season of sampling might have also played a role in the ABA content of the seeds used in this study.

The germination rates of the seed samples were also measured. As seen in Table 6, *C. obtusa* seeds failed to germinate after storage at all temperatures. Based on Table 6, we would expect *C. obtusa* to germinate in at least the lower storage temperatures, but even at -18°C , where ABA content was lowest, it did not. The average germination percentage of *C. obtusa* seeds for the first five years of storage (2003–2008) was 52.0, 5.2, and 0.8% at -18 , 4, and 25°C , respectively, while for the last five years (2015–2019), it was 6.4% at -18°C and 0% at both 4 and 25°C . In addition, the germination percentage in 2019, the last year of seed viability, was 4.0% at -18°C . To gain insights into the non-germination of *C. obtusa*, the internal structures of seeds collected in 2003 and 2020 were compared using an optical microscope. This confirmed that the seeds collected in 2003 were abnormal, with deteriorated internal tissues, compared to seeds collected in 2020 (Figure 7). The examination of the internal structures of the seed revealed that the internal tissue had deteriorated when compared to the normal seed in 2020. These results are consistent with previous research showing a shelf life of seven years for cypress seeds. The non-germination could also be explained by either the physical or physiological dormancy

Table 6. The germination rates of the seeds of three forest resource trees after storage at different temperatures.

Storage temperature	Germination rate (%)		
	<i>C. obtusa</i>	<i>P. rigida</i> × <i>P. taeda</i>	<i>P. densiflora</i>
-18°C	0	95	84
4°C	0	80	48
25°C	0	0	0

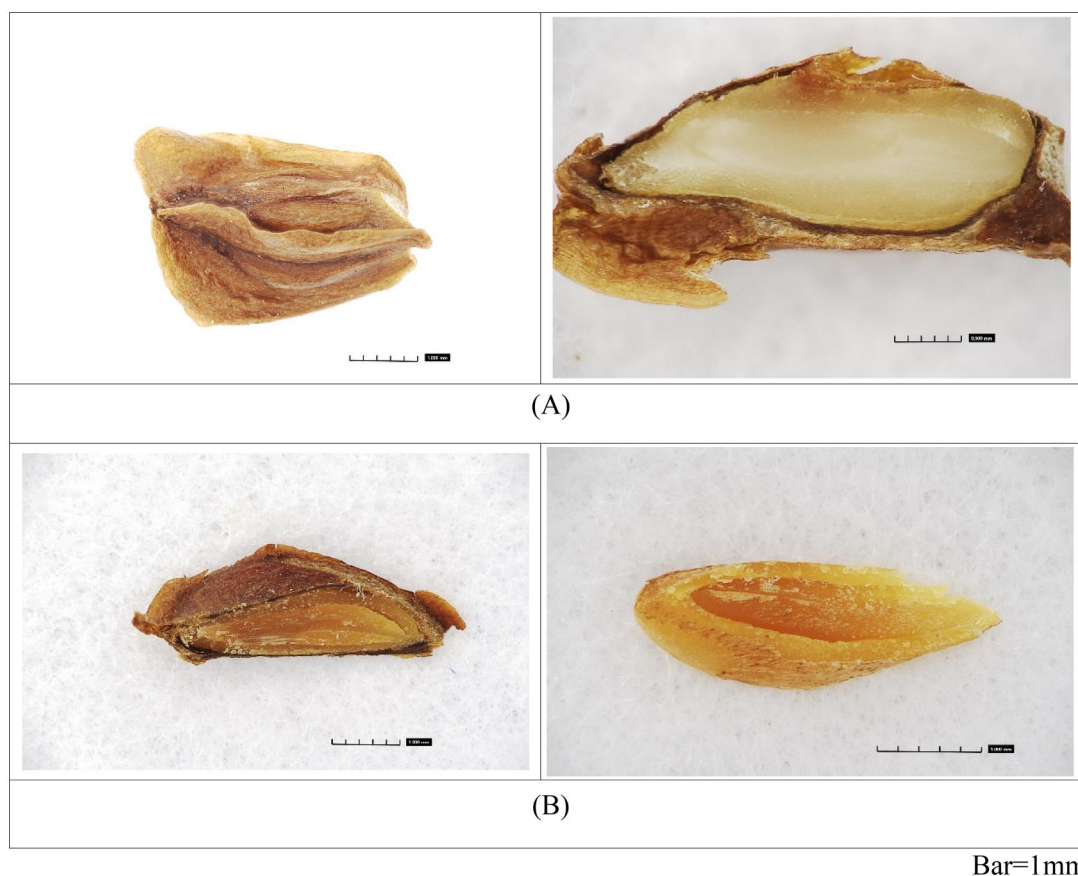
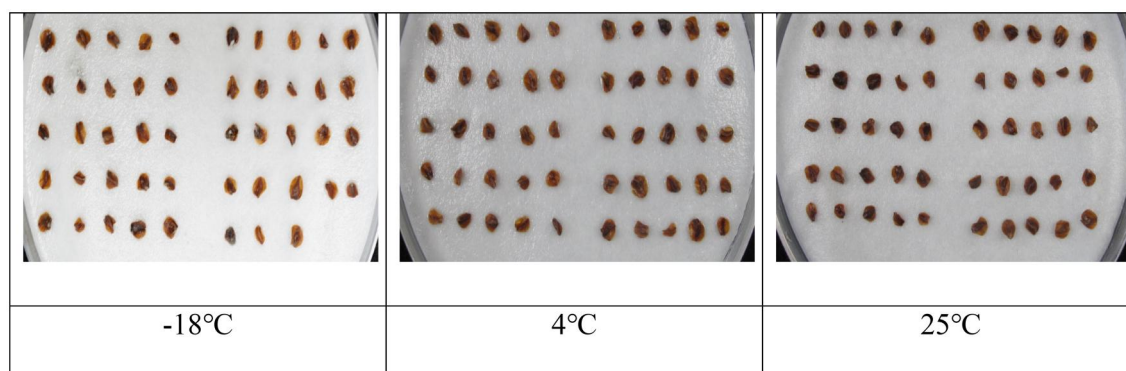
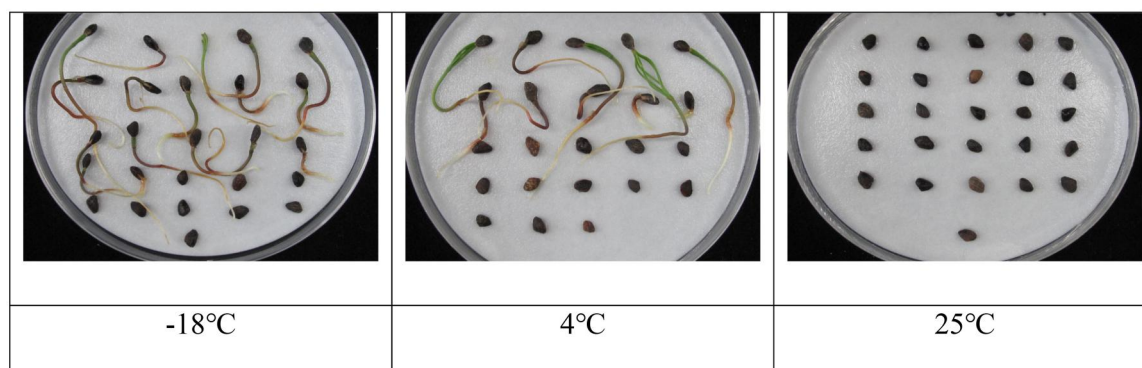


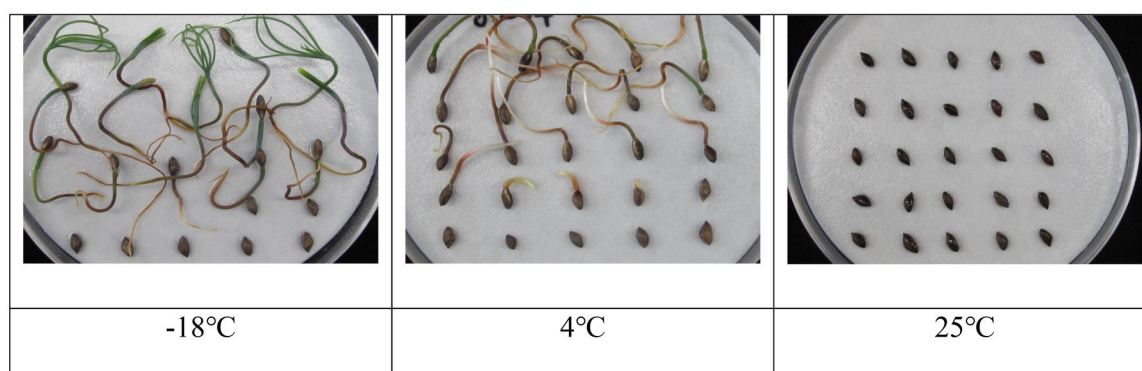
Figure 7. Anatomical features of normal *C. obtusa* seeds from 2020 (A) and abnormal *C. obtusa* seeds from 2003 (B).



(A)



(B)



(C)

Figure 8. Germination of *C. obtusa* (a), *P. rigida* × *P. taeda* (B), and *P. densiflora* (C) seeds after storage at different temperatures for 20 years.

tendencies of the seeds, which vary from species to species in higher plants (Dalling et al. 2011). It is crucial to remember that different species have different dormancy-breaking requirements. This might also explain the germination rates of the two *Pinus* species (Figure 8).

For the two remaining species, *P. rigida* × *P. taeda* and *P. densiflora*, the germination rates at −18, 4, and 25 °C were 95, 80, and 0% and 84, 48, and 0%, respectively. The seeds stored at room temperature had the highest ABA content but the lowest germination rate. The ABA extracted from the seeds of the two *Pinus* species was relatively low, hence it might be one of the reasons why the seeds germinated. While the ABA content in the seeds of all species similarly varied according to the storage conditions, none of the stored *C. obtusa* seeds germinated, whereas the germination rates of *P. rigida* × *P. taeda* and *P. densiflora* varied

with storage temperature. A negative correlation between ABA content and germination rates, with regard to storage conditions, was observed: the lower the ABA content of the seed, the higher the germination rate and vice-versa. Seeds stored at −18 °C, which were not exposed to temperature stress, had the highest germination rate followed by seeds stored at 4 °C. On the other hand, seeds stored at 25 °C, room temperature, lost viability. The temperature change when exposing seeds to room temperature directly from low temperatures also induces stress that can consequently kill the seeds. Ultimately, seeds of all species (*C. obtusa*, *P. rigida* × *P. taeda*, and *P. densiflora*) had the highest ABA content after storage at 25 °C, and the long-term storage condition for best maintaining high seed viability was shown to be −18 °C. However, these results do not mean that only ABA content can influence germination and non-

germination of seeds. In fact, there are a lot of factors at play when seeds break dormancy. Nonetheless, the findings of this study, which suggest that ABA along with the aforementioned factors affect seed germination, are in line with those of existing literature.

4. Conclusion

This study investigated the ABA content and germination rates of seeds from three gymnosperm species stored at varying temperatures: -18°C , 4°C , and 25°C . The results showed that seeds of *C. obtusa* generally had the highest ABA content, followed by *P. rigida* $\times$ *P. taeda* and then *P. densiflora* at all three storage temperatures. The differences and absence of ABA in the samples can be attributed to factors like the sampling time of year, the environmental stressors acting on the parent tree, the age of the parent tree, the light and temperature levels, and the overall health of the parent tree. In higher plants, ABA is engaged in numerous physiological and developmental processes. Previous studies have demonstrated a relationship between ABA content and the germination rate of seeds. A negative correlation was observed between the ABA content and germination rate of the two pine tree species. No such relationship was observed in the cypress species, *C. obtusa*, because all seeds lost their viability during the experimental storage treatment. It is also worth noting that there have been few studies on ABA in gymnosperms, indicating a need for more research into the topic. Ultimately, studies like this one can help to further understand the role that ABA plays in the physiological processes of gymnosperms.

Author contributions

N P Uy: writing and data curation, J Choi: ABA analysis, J J Ku: germination and data curation, S Lee: writing and editing.

Disclosure statement

No potential conflicts of interest were reported by the authors.

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