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Acid Scarification Completes the Germination of *Hovenia dulcis* Seeds

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Abstract: Seeds have been reported to have a combination of physical and physiological dormancy. However, this study revealed that *H. dulcis* seeds only have physical dormancy. The water absorption of the seeds after different periods of sulfuric acid scarification was measured, and the water gap through which water was absorbed after scarification treatment was specified. Cold stratification treatment and gibberellic acid treatment were performed after sulfuric acid scarification to determine whether *H. dulcis* seeds had physiological dormancy. *H. dulcis* seeds could absorb water completely when scarified for more than 60 min, and water was absorbed only through the hilar fissure near the micropyle, indicating that *H. dulcis* seeds have physical dormancy. However, there was no synergistic effect on the final seed germination percentage after the cold stratification or gibberellic acid treatments, and germination was delayed under cold temperature conditions. Thus, it was concluded that *H. dulcis* seeds have no physiological dormancy but only physical dormancy. This study not only clarifies the kind of dormancy in *H. dulcis* seeds but also provides a method to expedite seed germination without a long cold stratification treatment period of 2 or 3 months.

Keywords: combinational dormancy; physical dormancy; scarification

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1. Introduction

Hovenia dulcis Thunb. is a deciduous plant that belongs to the family Rhamnaceae. It is mainly distributed in East Asia, including Korea, Japan, and East China, and has been introduced in the United States, Australia, and Central Africa, as an ornamental plant [1]. The extracts of *H. dulcis* are effective against liver diseases and can detoxify alcohol poisoning [2]. They are also used as ingredients in food supplements in Korea and Japan [3]. More than 50 phytochemical compounds have been isolated from the leaves, roots, bark, and seeds of *Hovenia* spp. in the last 30 years [4], and some novel compounds have been isolated from *H. dulcis* and structurally determined [5], indicating that *H. dulcis* is an important resource plant.

Using tissue culture methods, in vitro propagation of *H. dulcis* from its axillary buds and stems has been successful [6,7]. However, the percentage of callus induction is low in such methods, and the redifferentiation efficiency varies depending on the source of the plant. Although *H. dulcis* can be propagated using cuttings of a vegetative organ, this vegetative propagation method requires considerable labor, and the yields vary according to personal skills. Propagating this plant from seeds is more practical. However, *H. dulcis* seeds do not germinate well immediately after seeding [8].

In the first report on dormancy in seeds of *H. dulcis*, seeds required scarification for germination [9,10], and *H. dulcis* was classified as having a physical dormancy [11]. However, it was later revealed that *H. dulcis* seeds also needed stratification for more than 90 days along with scarification for germination [12]. This study stated that cold temperature treatment is required after sulfuric acid treatment to break physiological

dormancy of the embryo. Thus, the seeds of *H. dulcis* are defined as having combinational dormancy, i.e., they have both physical and physiological dormancy [8]. Because there are conflicting interpretations of the dormancy in seeds of *H. dulcis*, research was conducted to determine the kind of dormancy in seeds of this species. Moreover, in many commercial applications, cold temperature treatment for a long period is required for the propagation of this species.

However, further studies are necessary on whether physical dormancy and/or physiological dormancy are present in seeds of *H. dulcis*. The present study not only investigated the dormancy type of this plant species but also suggested a method to obtain seedlings in a shorter time for *H. dulcis* breeding by shortening the cold temperature treatment period. Therefore, this study first revealed the presence of physical dormancy in *H. dulcis* seeds and then attempted to verify whether it is necessary to break the physiological dormancy after breaking the physical dormancy.

2. Materials and Methods

2.1. Seed Materials

The seeds used in the experiments were collected on 26 October 2021, from plants growing in Gongju, Chungcheongnam-do, Korea (36°32′17″ N, 126°58′32″ E). The *H. dulcis* plants bloomed in June, and the seeds were harvested at the end of October. The average air temperatures in June, July, August, September, and October were 23.5, 27.8, 25.8, 22.4, and 15.7 °C, respectively. Immediately after harvesting, the pulp was removed by hand rubbing, and seeds were washed with running water. The seeds were placed on the table in the laboratory to dry at room temperature (20–25 °C) and humidity of 45–50% for 6 h. The seeds were stored in a refrigerator at 5 °C in a dry state until their use in the experiment, and subsequent experiments were carried out on 4 November 2021.

2.2. Imbibition Test

This experiment was conducted to determine whether the seeds of *H. dulcis* have physical dormancy. The seeds were immersed in 98% sulfuric acid for 0, 5, 10, 30, 60, 90, 120, and 180 min at room temperature (20–25 °C). To completely remove the sulfuric acid from the seeds, the seeds were placed in a plastic mesh and washed with running water for 5 min. According to treatments, three replicates of 25 seeds were placed on a filter paper moistened with distilled water. Seeds were placed at 25/15 °C (12/12 h of day/night temperature) in a light- and temperature-controlled incubator (Multi-Room Incubator; WiseCube, Wonju, Korea) using a 12 h daily photoperiod of 25 $mol \cdot m^{-2} \cdot s^{-1}$ PPF (Photosynthetic Photon Flux), with light provided by cool white fluorescent lamps. The change in the weight of the seeds absorbing water was measured for up to 4 days. Water absorption was calculated using the formula $\%W_s = [(W_i - W_d)/W_d] \times 100$, where W_s = increased seed weight, W_i = seed weight after water absorption, and W_d = initial weight.

2.3. Seed Water Absorption Tracking

To determine whether the seeds absorb water after sulfuric acid scarification, the seeds of *H. dulcis* treated with sulfuric acid for 1 h were immersed in a 0.1% safranin (Kisan Bio, Seoul, Korea) solution at a constant temperature of 25 °C. Then, the seeds were sampled at 0, 1, 6, 12, and 24 h, cut in half with a razor, and staining observed. The stained seeds were observed under a digital microscope (AM 4113 Dino-Lite Premier; AnMo Electronics Co., New Taipei City, Taiwan).

2.4. Optimal Germination Temperature Experiment

This experiment was conducted to determine the optimum temperature for germination after scarification. The germination percentage was determined in a growth chamber controlled at 25/15, 20/10, 15/6 °C for 12/12 h of day/night temperature, and 5 °C after scarification treatment with sulfuric acid for 60 min. Seeds were placed on river sand moistened with distilled water in 9-cm Petri dishes, which were sealed with parafilm

(Pechiney Plastic Packaging, Menasha, WI, USA) to prevent water loss during incubation. The 25 seeds were placed in a Petri dish, with four replicates per treatment. At the start of the experiment, seeds were sterilized with 1000 mg·L^{-1} of benomyl (FarmHannong, Seoul, Korea) for 1 h, and seeds that were continuously contaminated after disinfection were removed. Seed germination was checked once a week, and germination was established when the root grew more than 2 mm. The experiment was terminated if there was no change in the germination percentage for more than three weeks after the last seed germinated. At the end of the experiment, all the seeds that did not germinate were cut and tested for viability with tetrazolium, and empty seeds or inactive seeds were excluded when calculating the total germination percentage. The seed vigor test was performed after immersion in a tetrazolium solution of 1.0% at 40 °C for 2 h, and the staining was confirmed with a digital microscope (AM 4113 Dino-Lite Premier, Taiwan).

2.5. Cold Stratification Treatment

This experiment was conducted to determine whether the seeds have physiological dormancy. They were divided into non-scarified seeds and seeds scarified with sulfuric acid for 60 min. Then, cold stratification treatment was performed for each group. Four replicates containing 25 seeds each were incubated at 5 °C for 0, 4, 8, and 12 weeks. Then, all seeds were moved to 25/15 °C (day/night temperature) and checked for germination.

2.6. Gibberellic Acid Treatment

The ability of gibberellic acid (GA) to break dormancy is used to check for physiological dormancy in seeds [8]. Similar to the cold stratification treatment, the GA treatment was conducted by dividing the seeds into non-scarified and scarified seeds. The scarified seeds were soaked for 1 h in sulfuric acid, dried at room temperature (20–25 °C) for 6 h and then soaked in GA solution. The seeds were soaked in distilled water (control) or in solutions of 10, 100, and 1000 mg·L^{-1} GA_3 for 24 h at room temperature before incubation. All the seeds were incubated at 25/15 °C.

2.7. Statistical Analysis

The significance of the seed germination percentage in the last week according to each imbibition, optimal germination temperature, cold stratification, and GA treatment was analyzed using ANOVA, followed by Duncan's multiple range test at the 5% significance level. A significance test and graph were generated using SigmaPlot 11.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Imbibition

The increase in water absorption in the seeds not treated with sulfuric acid was less than 10%, even after 96 h (Figure 1). However, when the sulfuric acid treatment was increased from 5 min to 10, 30, 60, 90, 120, and 180 min, the water absorption increased by 19, 21, 39, 79, 80, and 82%, respectively. When the seeds were treated with sulfuric acid for 60 min or more, there was no significant increase in water absorption (Figure 1). The hilar fissure near the micropyle of the seeds was opened during sulfuric acid treatment (Figure 2), and water gradually diffused through the hilar fissure (Figure 3). In non-scarified seeds, no part of the macrosclereid was stained (Figure 3A). The reddish area indicates that water was absorbed into the seed. After 1 h of sulfuric acid treatment, the micropyle part started to exhibit red coloration (Figure 3B); after 12 h, half of the seed coat was stained red (Figure 3C), and after 24 h, the entire macrosclereid was stained red (Figure 3D).

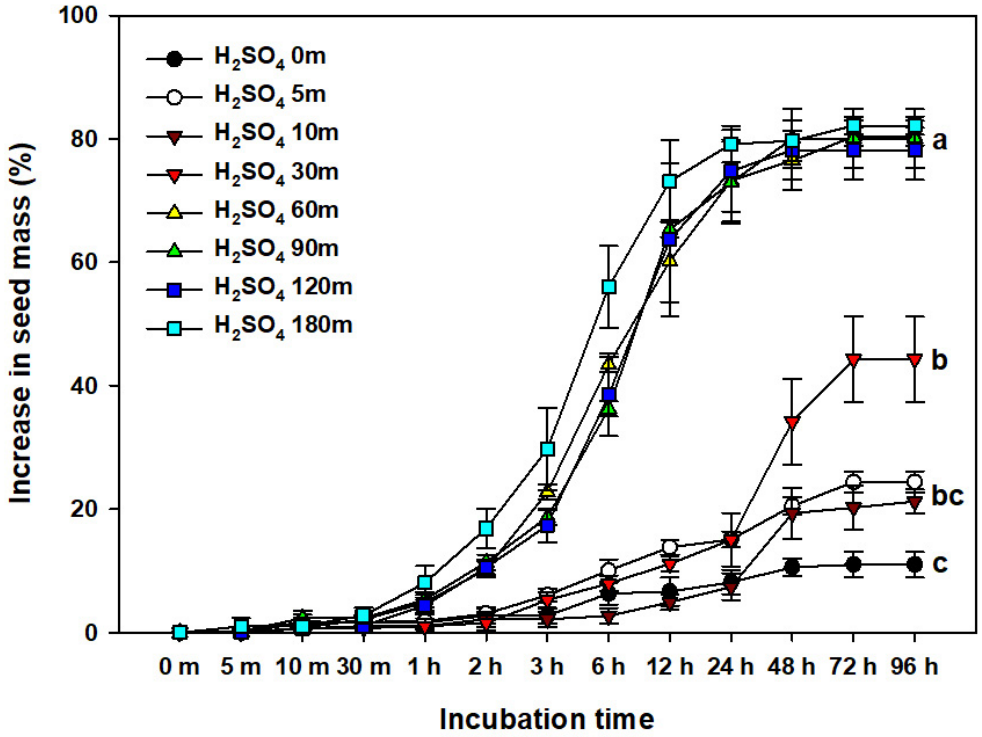

Figure 1. Effects of sulfuric acid scarification times on the increase in mass of *Hovenia dulcis* seeds. Seeds were scarified with 98% sulfuric acid at room temperature (20–25 °C). Vertical bars show ± SE of three replicates. The different letters represent statistically significant differences for germination percentages according to treatments in 96 h as determined by Duncan's multiple range test ($p < 0.05$).

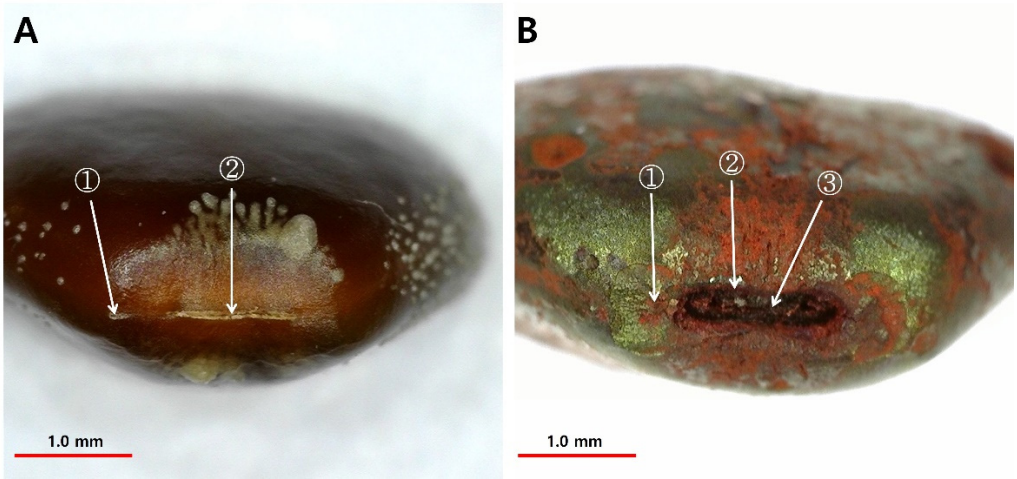

Figure 2. Water gap opening of *Hovenia dulcis* seeds after 24 h in water after (**A**) no treatment and (**B**) sulfuric acid treatment for 1 h. Arrows ①, ②, and ③ indicate micropyle, hilum, and hilar fissure, respectively.

3.2. Optimal Germination Temperature Experiment

In non-scarified seeds, the final germination was less than 25%, regardless of the incubation temperature (Figure 4A). However, when the seeds were scarified with sulfuric acid for 1 h, germination was 100% for all temperature treatments (Figure 4B). The higher the incubation temperature, the faster the seeds germinated. At 25/15 °C, germination was close to 100% in 4 weeks, but the seeds required approximately 15 weeks to germinate 100% at a temperature of 5 °C.

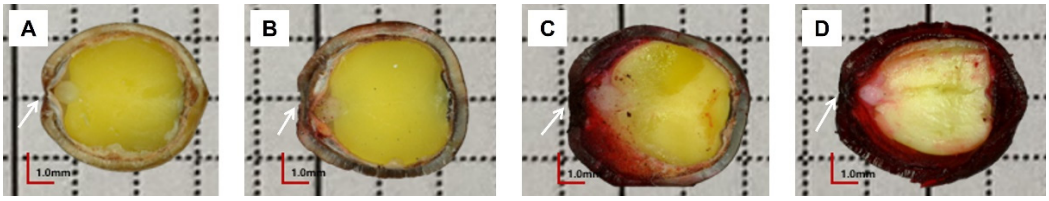

Figure 3. Time periods of water absorption by *Hovenia dulcis* seeds after sulfuric acid scarification for 1 h at (**A**) 0, (**B**) 1, (**C**) 12, and (**D**) 24 h. Dyeing was performed using safranin stain. Arrows indicate hilar fissure.

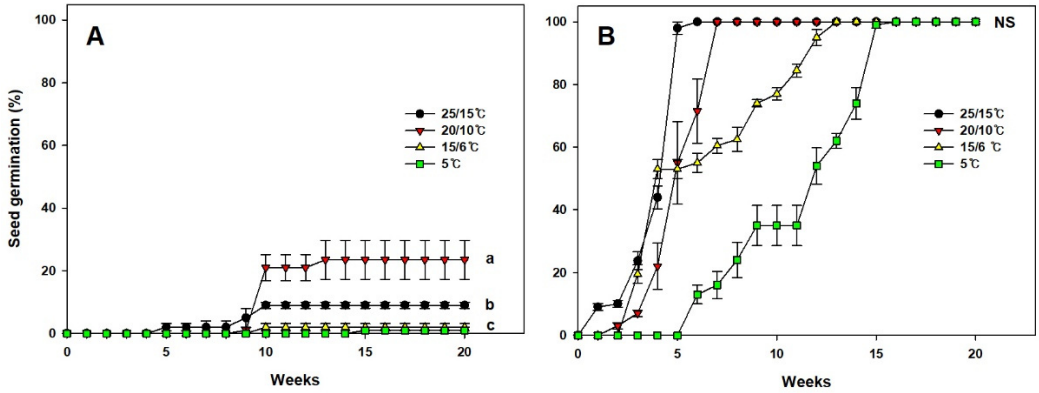

Figure 4. Germination of *Hovenia dulcis* seeds at various temperature regimes after (**A**) no treatment and (**B**) 98% sulfuric acid scarification for 1 h. Vertical bars show ± SE of four replicates. The different letters represent statistically significant differences for germination percentages according to treatments in the 20th week as determined by Duncan's multiple range test ($p < 0.05$).

3.3. Cold Stratification Treatment

When cold stratification treatment was performed without scarification, only approximately 20% of the seeds germinated, regardless of the period of cold stratification treatment (Figure 5A). However, all seeds germinated after scarification, regardless of the cold stratification period (Figure 5B). The scarified seeds that were not subjected to the cold stratification treatment achieved the fastest germination, and the increase in the cold stratification treatment period increased the time required to reach final germination.

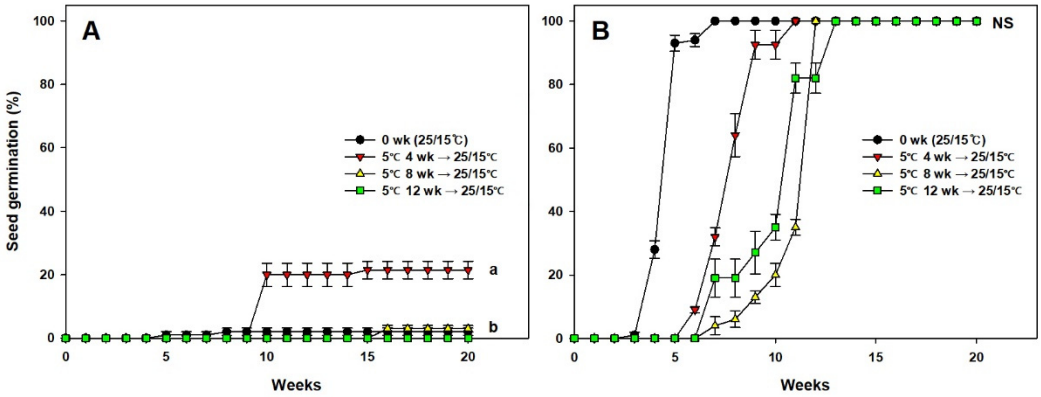

Figure 5. Germination of *Hovenia dulcis* seeds by cold stratification treatments after (**A**) no scarification and (**B**) 98% sulfuric acid scarification for 1 h. Vertical bars show ± SE of four replicates. The different letters represent statistically significant differences for germination percentages according to treatments in the 20th week as determined by Duncan's multiple range test ($p < 0.05$).

3.4. Gibberellic Acid Treatment

In the non-scarified seeds, the germination was the highest (50%) at 1000 ppm of GA3 and less than 20% at the other GA concentrations (Figure 6A). When treated with sulfuric acid followed by GA treatment, all seeds germinated, regardless of GA concentration, and there was no difference in germination speed (Figure 6B).

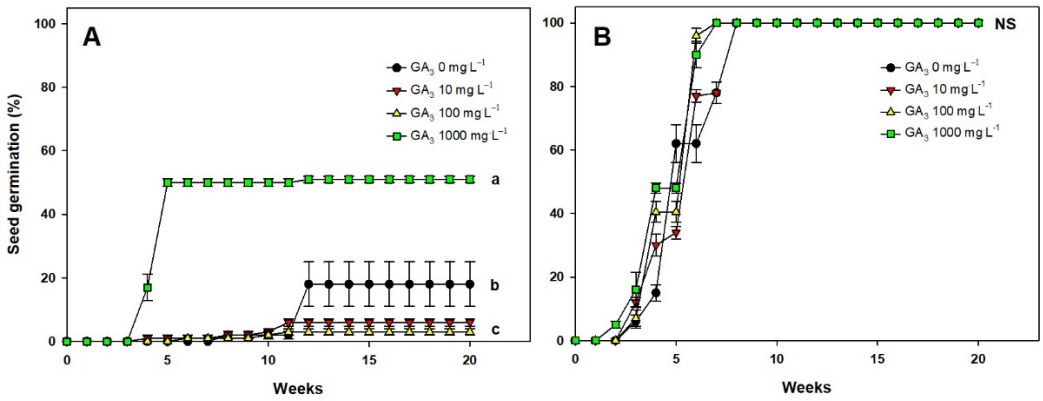

Figure 6. Germination of *Hovenia dulcis* seeds with gibberellic acid treatment after (**A**) no scarification and (**B**) 98% sulfuric acid scarification for 1 h. Vertical bars show ± SE of four replicates. The different letters represent statistically significant differences for germination percentages according to treatments in the 20th week as determined by Duncan's multiple range test ($p < 0.05$).

4. Discussion

For seeds that are physically dormant and cannot absorb water, either mechanical or acid scarification, enzymes, organic solvents, percussion, high atmospheric pressures, and heat treatment, are used to break the seed dormancy [8]. One of the most widely used methods is chemical scarification with sulfuric acid. The seed mass of *H. dulcis* increased up to 80% in seeds scarified with sulfuric acid for 1 h or more, but non-scarified seeds did not absorb water well (Figure 1), indicating that the seeds had physical dormancy. Seeds with physical dormancy have the potential to exhibit combinational dormancy with physiological dormancy. Combinational dormancy requires after-ripening at high temperatures or cold stratification to break physiological dormancy in addition to breaking physical dormancy [8]. A Frett's study reported *H. dulcis* seeds to exhibit combinational dormancy [12]. Although germination was only 36% when scarification was performed with 96% sulfuric acid for 45 min, germination increased by 25, 70, and 94% when cold stratification treatment at 5 °C after scarification was applied for 30, 60, and 90 days, respectively. Thus, it was established that 45 min of sulfuric acid treatment followed by 90 days of cold stratification treatment is required for the germination of *H. dulcis* seeds. This is even mentioned on the website https://www.treeshrubseeds.com/specieslist?id=971&ID2=8 (accessed on 8 November 2022). In the seed dormancy classification, *H. dulcis* seeds are categorized to have combinational dormancy [8].

However, in our experiment, we found that *H. dulcis* seeds have only physical dormancy and not physiological dormancy. The final germination did not increase with the cold stratification treatment period after scarification treatment, and 100% germination was achieved only with sulfuric acid treatment (Figure 5). Germination was increased close to 100% in 5 weeks at 25 °C but was delayed to 12 weeks in the cold stratification treatment at 5 °C. Even in the optimal germination temperature experiment, after scarification, the higher the incubation temperature was, the faster the seeds germinated, and the final germination percentage was the same among the various temperature regimes (Figure 4). This indicates that there was no effect of cold stratification treatment on breaking the dormancy. Instead, cold stratification treatment delayed the germination. Additionally, physiological dormancy can be determined by germination enhancement using GA. GA promoted germination in seeds of *Lysimachia coreana* Nakai [13] and *Nandina domestica* Thunb. [14]

that have non-deep physiological dormancy, and they required cold stratification treatment. Scarification followed by GA treatment improved the germination of *Rhus javanica* seeds, which have combinational dormancy [15]. However, there were no differences in germination speed or final germination percentage when scarified *H. dulcis* seeds were treated with GA (Figure 6B), indicating that seeds did not have physiological dormancy. Physiological dormancy could be broken during dry storage called after-ripening if the experiment was not started immediately after harvesting the seeds. After-ripening could break seed dormancy at either high or low-temperature regimes, but many plant species required a dry storage period of 2 months or more at a temperature of 20 °C or higher rather than a low temperature [16]. The seeds used in our experiment were stored at 5 °C for 9 days from harvesting to starting the experiment. Physiological dormancy could be broken partially during this period, but it was a relatively short storage period to affect dormancy-break through after-ripening. The following reasons may explain why our results contradict those of previous studies. First, the 45-min sulfuric acid treatment of *H. dulcis* seeds described by a previous study is insufficient. In our experiments, more than 1 h sulfuric acid treatment was required for the seeds to absorb water sufficiently (Figure 1). Moreover, the optimal time for sulfuric acid treatment varies among different species. For example, 10 min of sulfuric acid treatment is sufficient for *Lespedeza tomentosa* seeds [17]; however, a longer sulfuric acid treatment time (180 min) is required for *Ulex europaeus* seeds [18]. Second, germination is believed to be related to seed viability and vigor. Woody plants show very different seed viabilities according to the region and climate. In a previous study, the average initial seed viability used in the experiment was mentioned, but there was no mention of seed vigor for all the seeds used in the experiment. However, in our experiment, all seeds were cut and their viability was determined using the tetrazolium test, which was reflected in the final germination percentage.

Although the seed coat of *H. dulcis* was completely immersed in sulfuric acid, water was absorbed only through a small opening in the water-gap area of the seed coat (Figure 2). After scarification, water absorption occurred through the hilar fissure near the micropyle (Figure 3). When seeds are treated with sulfuric acid, the seed coat and/or plugged natural openings are destroyed, but the location of water absorption differs depending on the plant species [8]. When *Rhus aromatica* seeds were treated with sulfuric acid for 1 h, macrosclereids of the seed coat were not removed but brachysclereids and osteosclereids in the micropyle were removed, and water absorption occurred [19]. When *Lupinus angustifolius* seeds were treated with sulfuric acid for 3 h, the counter palisade layer in the hilum region was partially destroyed, resulting in water absorption [20]. Similarly, in our study, after sulfuric acid treatment, water absorption gradually increased from the hilar fissure near the micropyle and the entire seed absorbed water (Figures 2 and 3).

The family Rhamnaceae includes 900 plant species in 58 genera, including *H. dulcis* [21]. *Hovenia dulcis* is the only species in the genus *Hovenia*, and the plant species that are taxonomically close to *Hovenia* include plants of the genera *Paliurus* and *Ziziphus* from the tribe Paliureae [22]. The germination percentage of *Paliurus spina-christi* seeds was low after no treatment or cold stratification treatment, but germination increased when the seeds were treated with sulfuric acid for more than 40 min [23]. However, we could not determine whether the seeds of this species have a combinational dormancy because sulfuric acid scarification followed by cold stratification was not applied in this study. The germination of *Ziziphus spina-christi* seeds was less than 40% without sulfuric acid treatment, but the germination percentages were 70, 73, and 91% after 30, 60, and 120 min of sulfuric acid scarification, respectively [24]. The dormancy of *Z. spina-christi* seeds was broken only by sulfuric acid treatment, with a treatment period longer than 2 h. Since the seeds of the plant species closely related to *H. dulcis* show the characteristics of physical dormancy, it would not be surprising if *H. dulcis* seeds also have a similar physical dormancy.

The present study concluded that *H. dulcis* seeds have only physical dormancy, and only sulfuric acid treatment is sufficient to break this dormancy, in contrast to a previous study that reported cold stratification after sulfuric acid treatment was necessary for seed

germination. Our results also have practical implications by shortening the long low-temperature period of 60–90 days required for breaking seed dormancy.

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