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Scarification and stratification protocols for breaking dormancy of *Rubus* (Rosaceae) species in Korea

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Abstract

Some *Rubus* species are slow to germinate due to their thick, impermeable endocarp and physiological dormancy. We studied the effect of scarification and stratification on the breaking of dormancy in five *Rubus* species (*R. parvifolius*, *R. phoenicolasius*, *R. buergeri*, *R. takesimensis* and *R. corchorifolius*). On anatomical observation, *Rubus* stones are generally shown to have a three-layered endocarp (outer-, middle-, and inner-endocarp) that conveys physical dormancy. For the scarification treatment, stones were treated with sulphuric acid. The most effective treatment for breaking dormancy resulted in 50-70% scarification of the middle-endocarp. The combined treatment of scarification and stratification considerably increased germination of *Rubus* seeds. In particular, sulphuric acid scarification followed by cold stratification (eight weeks at 4°C) was most effective for three species: *R. parvifolius*, *R. phoenicolasius* and *R. takesimensis*. However, *R. corchorifolius* showed very similar results in all stratification treatments and *R. buergeri* was affected by neither single nor combined scarification and stratification treatments. In summary, three of the studied species of *Rubus* were found to exhibit deep combinational dormancy caused by a hard endocarp and physiological inhibitory mechanisms. In contrast, *R. corchorifolius* seeds only showed physical dormancy.

Introduction

Rubus L., which is divided into 12 subgenera, is a large genus of the family Rosaceae including approximately 750 deciduous or evergreen, often prickly, erect or trailing shrub species (Thompson, 1995; Alice, 2001). The genus has a worldwide distribution, from the lowland tropics to the subarctic, and is economically and ecologically important as a fruit crop, ornamental, invasive weed and in early forest succession (Thompson, 1995; Peacock and Hummer, 1996; Howarth *et al*., 1997). Flowers bloom in the spring or early summer and the fruit, containing several seeds, ripen from summer to fall. In natural conditions seed dispersal generally occurs by birds or mammals (USDA, 1949). The seeds of *Rubus* species are surrounded by a thick, hard endocarp; together, the seed and endocarp make up the stone.

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In most cases, animal-dispersed seeds are fleshy and the endocarp prevents destruction of the seed by dehydration or predation. Endocarps generally comprise multiple layers with a protective mechanical layer that is formed from all or part of the middle- or inner-endocarp. In some species the mechanical layer consists of one or more rows of elongated, palisade-like cells that play an important role in the plant life cycle by supporting the development of the embryo and controlling dormancy and germination (Rudall, 1997; Moïse *et al.*, 2005; Wada *et al*., 2011). Stones of some *Rubus* species have a thick, hard endocarp that impedes water imbibition and restricts availability of oxygen required for germination (Rose, 1919). In addition, some *Rubus* species also contain proanthocyandins in their seed coat (in fact and hereafter referred to as the endocarp) that limit the exchange of gases and prolong the life of the dormant embryo (Wada *et al*., 2011).

Most studies of *Rubus* are restricted to the economically important blackberries (subgenus *Rubus*) and raspberries (subgenus *Idaeobatus*). Both have deep combinational dormancy caused by a hard endocarp and one or more additional mechanisms such as an impermeable endocarp, chemical inhibitors or embryonic dormancy (USDA, 1949; Nyborn, 1980). Physical dormancy is determined by the outer layer of the stones (endocarp) that restricts the uptake of water and oxygen and ultimately prevents imbibition. To break physical dormancy, mechanical or chemical scarification, resulting in weakening of the covering layer and hence allowing the radicle to penetrate, has been widely used (Baskin and Baskin, 2004). Typically, breaking dormancy in seeds with physical dormancy, in both natural and artificial conditions, has been assumed to involve making an opening in the endocarp or seed coat through which water can reach the embryo (Baskin *et al*., 2000). On the other hand, breaking of combinational dormancy (physical and physiological dormancy) might require a few weeks of warm or cold stratification after physical dormancy has broken and seeds have imbibed adequate water to germinate.

According to Wada and Reed (2011a, b), scarification with sulphuric acid, ranging from 30 minutes to more than three hours, was required to degrade the endocarp of some *Rubus* species before germination could occur. Before Wada and Reed, Peacock and Hummer (1996) used sulphuric acid for endocarp scarification in six *Rubus* species (*R. ursinus* Cham, *R. leucodermis* Douglas ex Torrey & A. Gray, *R. eustephanos* Focke ex Diels, *R. multibracteatus* A. Leveille & Vanoit, *R. chamaemorus* L. and *R. parviflorus* Nutt.) and suggested that 30 minutes of sulphuric acid scarification significantly improves the germination of *R. eustephanos*, *R. leucodermis* and *R. ursinus* compared with control and liquid nitrogen treatments. In the *Woody Plant Seed Manual*, 15-20 minutes scarification with concentrated sulphuric acid is recommended for raspberry (*R. idaeus* L., *R. occidentalis* L.) and up to three hours for blackberry (*Rubus* spp.) (USDA, 2003). However, the great variation in the endocarp thickness and differences in endocarp surface structure may require some variation in the period of treatment (Wada and Reed, 2006, 2010, 2011a). Therefore, protocols for breaking physical dormancy and stratification treatments for physiological dormancy perhaps differ among *Rubus* species.

The object of this study was to determine the germination patterns of seeds from five *Rubus* species in Korea in response to scarification and stratification treatments. We also investigated the structure of the endocarp of each species, especially the layers and tissues contributing to endocarp texture and hardness.

Materials and methods

Plant material

Mature fruits of five *Rubus* species, including *R. takesimensis* which is endemic to Korea, were collected from different plant populations in Korea (table 1). After collection, the fleshy tissue was removed manually and the stones (seeds + endocarp) poured through a strainer and rinsed in tap water. The cleaned stones were dried for a month at room temperature and then stored at 4°C for almost six months prior to the experiment.

Table 1. Investigated species of *Rubus* and their collection information.

Species	Subgenus	Distribution	Fruiting season	Collection site	Collection date
R. parvifolius	*Idaeobatus*	Korea, China, Japan	July-August	Jeonju si, Jeollabuk do, Korea	3 June 2013
R. phoenicolasius	*Idaeobatus*	Korea, China, Japan	July-August	Jinan gun, Jeollabuk do, Korea	26 June 2013
R. buergeri	*Malachobatus*	Korea, China, Japan, Taiwan	November-January	Seogwipo si, Jeju do, Korea	8 December 2012
R. takesimensis	*Idaeobatus*	Korea (endemic)	July-August	Ulleung gun Gyeongsangbuk do, Korea	13 June 2013
R. corchorifolius	*Idaeobatus*	Korea, China, Japan, Taiwan, Vietnam	April-June	Gunsan si Jeollabuk do, Korea	9 May 2013

Stone characteristics

Samples of dry stones of each species were weighed as three lots of 1000 using an electronic microbalance. Prior to scarification treatment, size (length, width and thickness) of 30 stones from each species was measured using a Leica MZ16FA stereomicroscopic zoom microscope (Leica, Germany) with infinity capture imaging software. Endocarp thickness was measured on four different areas of each stones including the micropylar region. A subjective endocarp hardness rating of 1-5 was assigned after stones samples were cut manually with a scalpel. Hardness ratings were: 1, soft; 2, slightly hard; 3, hard; 4, very hard; and 5, extremely hard (Wada and Reed, 2011b).

Scarification procedure

Before scarification and stratification, water uptake by intact and scratched stones were examined. Three replicate samples of 20 intact and scratched stones of each species were equally supplied with water and the weight of each replicate was measured at regular intervals. Following specific guidelines for Korean *Rubus* species, the scarification period was estimated for each accession based on subgenus and data on stones characteristics.

The five species were treated with concentrated sulphuric acid for 0, 10 and 20 minutes. A treatment of five minutes was applied only to *R. corchorifolius* because stones of this species are very small with a thin endocarp. After sulphuric acid treatment, stones were rinsed with running water for one hour.

Endocarp observation (surface and anatomy)
Scarified stones were dried and mounted directly on stubs using double-sided adhesive tape, and sputter-coated with gold (Kic-1A, Coxem, Korea). Stones were then observed by scanning electron microscope (CX-100s, Coxem) to examine the extent of endocarp surface rupture. To examine endocarp anatomy, dry stones were dehydrated with an ethanol series (50, 70, 80, 90, 95 and 100%). After complete dehydration the stones were passed through alcohol/Technovit combinations (3:1, 1:1, 1:3 and 100% Technovit) and then embedded in Technovit 7100 resin. Embedded materials were sectioned by microtome (4-6 μm) and then stained with 0.1% Toludine blue O for 60-90 seconds. The permanent slides were observed under a Hirox 3D microscope (Hirox, Japan). Photographs were taken with the attached camera system. The scarified layer of endocarp was measured for each specific scarification treatment.

Stratification procedure
After scarification was complete, each stone lot underwent one of four stratification treatments: (1) control (no stratification, moving directly to germination); (2) four weeks at 4°C (cold stratification); (3) eight weeks at 4°C; or (4) four weeks at 25/15°C (14 hours / 10 hours) followed by four weeks at 4°C (warm + cold stratification). The stratification of stones were carried out on wet filter paper with 14-/10-hour light/dark cycle. After each treatment the experimental lots were transferred to a germinator set at 25/15°C with a 14-hour photoperiod.

Germination test
Stones were sown in 90 mm-diameter Petri dishes lined with two layers of wet Whatman No. 1 filter paper. A total of 200 stones were counted randomly from each treatment and sown as four lots of 50 stones for the trial. The Petri dishes were placed at alternating 15°C for 10 hours (dark) and 25°C for 14 hours (with 14 hours of fluorescent white light). The counting of germinations was done on a daily basis until no more germinations occurred. Mean germination time (MGT) was recorded according to Alvarado *et al.* (1987) using the following formula:

$$MGT = \frac{\Sigma T_i N_i}{\Sigma N_i}$$

where N_i is the number of newly germination seeds at time T_i.
Germination index (GI) was calculated according to McKenzie *et al.* (1980):

$$GI = \sum_{n=0}^{t-1} X_n \, (t-n) / t$$

where, X_n = germination percentage on n^{th} count, n = the n^{th} day of counting germination -1 and t = total days of counting germination.

Data analysis

A completely randomised design with four replications was used. Data were analysed by two-way ANOVA using the SPSS statistical package, version 17.0. Treatment means were compared using Tukey's HSD mean separation at the 1% probability level.

Results

Stone characteristics

Variation was apparent in stone weight, size, endocarp thickness and endocarp hardness among the five species. Weight of 1000 stones ranged from 0.04 to 2.76 g and endocarp thickness varied from 0.196 to 0.264 mm (table 2). *R. parvifolius* had the heaviest and largest stones, with the thickest and hardest endocarp (length 2.860 mm; width 2.125 mm; thickness 1.498 mm). On the other hand, *R. corchorifolius* had the lightest stones, with a thin endocarp and the smallest stone size (length 1.580 mm; width 1.089 mm; thickness 0.715 mm). *R. phoenicolasius*, *R. buergeri* and *R. takesimensis* had medium stone thickness and moderate endocarp hardness and stone size. Consequently, endocarp thickness and hardness were found to be directly proportional to stone size and weight.

Table 2. Stone morphology of five *Rubus* species. Values are the mean of 30 stones each species.

Rubus species	Thousand stones weight (g)	Stone size			Endocarp		
		Thickness (mm)	Length (mm)	Width (mm)	Thickness[1] (mm)	Thickness[2] (mm)	Hardness (1-5)
R. parvifolius	2.76a	1.498a	2.863a	2.125a	0.598a	0.264a	4
R. phoenicolasius	1.11bc	0.996b	2.105c	1.365b	0.482b	0.212bc	3
R. buergeri	1.36ab	1.135c	2.250b	1.500c	0.485ab	0.245ab	4
R. takesimensis	0.91bc	0.898d	2.097c	1.234d	0.400ab	0.224abc	3
R. corchorifolius	0.40c	0.715e	1.580d	1.089e	0.380c	0.196c	2

[1] micropylar region; [2] excluding micropylar region. Values followed by the same letter within a column are not significantly different ($P > 0.05$).

Endocarp scarification

The weight of scratched stones increased by 40-50% more than intact stones after 48 hours in water (figure 1). Under scanning electron microscope, the endocarp surface structure was of the scalariform or reticulate-foveate type with little variation among the five *Rubus* species (figure 2). The amount of endocarp rupture was directly related to the period of sulphuric acid pretreatment. The longer the pretreatment period, the more highly ruptured the endocarp. Endocarp anatomy revealed that all studied species had a comparatively thin outer-endocarp and thick middle- and inner-endocarp; each of the three distinct layers exhibited lignified sclerenchymatous cells. On the whole, the outer-endocarp comprised 1-3 layers of cells and the middle-endocarp comprised 5-20 layers of

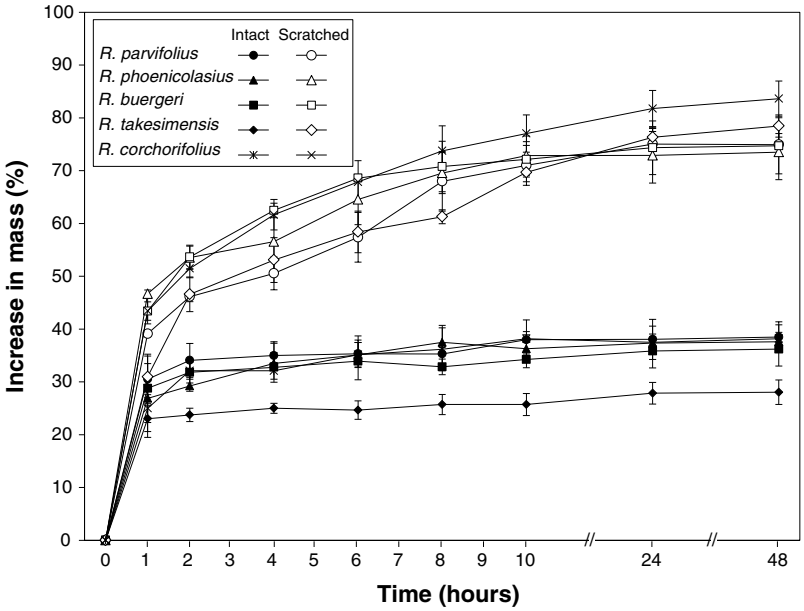

Figure 1. Time course for increase in mass of mechanically scratched and of intact seeds of five *Rubus* species.

30-70 µm-long slender and highly lignified cells (figure 3). The inner-endocarp consisted of 5-6 horizontally arranged layers of cells. Therefore in *Rubus* species breaking of dormancy mostly requires breaking of the middle-endocarp.

The endocarp anatomy of scarified stones showed the effectiveness of treatment with concentrated sulphuric acid (H_2SO_4). The largest stones those of *R. parvifolius* with a very thick and hard endocarp, only showed 10-20% scarification of the middle-endocarp after 10 minutes of treatment and 50-70% after 20 minutes treatment. On the other hand, in *R. phoenicolasius*, *R. buergeri* and *R. takesimensis*, with medium thickness and moderately hard endocarps, the middle-endocarp was scarified by 50-70% within 10 minutes. After 20 minutes of treatment, scarification had usually reached the inner-endocarp; sometimes even the endosperm was damaged by the sulphuric acid. In *R. corchorifolius*, both the longer 10- and 20-minute treatments were highly destructive and thus we applied a 5-minute treatment. The middle-endocarp in this species was scarified by 50-70% after this time.

Effect of scarification on germination

Untreated seeds of all five species were dormant. Sulphuric acid scarification was somewhat effective in breaking physical dormancy and each species had a different optimal treatment time (table 3). The results showed that sulphuric acid significantly improved the germination for *R. parvifolius* (24% after 20 minutes), *R. phoenicolasius* (12% after 10 minutes) and *R. takesimensis* (31% after 10 minutes). Meanwhile, was no germination observed in control seeds of all five species. On the other hand, *R. corchorifolius* seeds were very successfully germinated after five minutes of scarification (71%). No sulphuric acid treatment was successful for the germination of *R. buergeri* seeds.

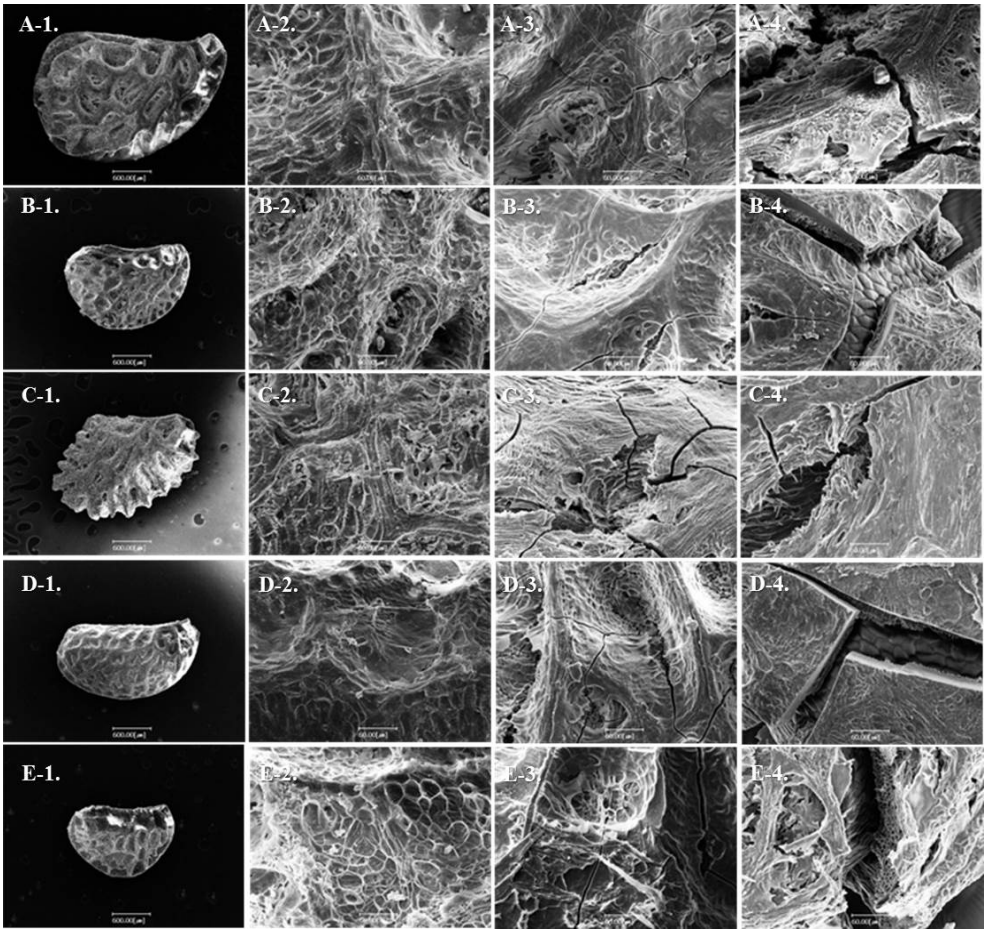

Figure 2. SEM image of stones surface with and without H_2SO_4 scarification: (**A**) *R. parvifolius*; (**B**) *R. phoenicolasius*; (**C**) *R. buergeri*; (**D**) *R. takesimensis*; (**E**) *R. corchorifolius*; (1) non-scarified (× 50); (2) non-scarified (× 500); (3) 10-minutes scarified (× 500); (4) 20-minutes scarified (× 500).

Effect of scarification combined with stratification

There were significant differences in the effects of scarification treatments depending on the species. A combination of scarification and stratification significantly improved germination (table 4). In general, sulphuric acid scarification followed by cold stratification was very effective in three species (*R. parvifolius*, *R. phoenicolasius* and *R. takesimensis*). Percentage germination also increased with the duration of cold stratification, although not significantly ($P > 0.05$). In *R. parvifolius*, over 90% germination was observed with 20 minutes of sulphuric acid scarification followed by either (4 or 8 weeks of cold stratification and 83% seeds germinated after 20 minutes of sulphuric acid scarification followed by warm/cold stratificationt. *R. phoenicolasius* and *R. takesimensis* seeds germinated in the range of 59-74% after 10 minutes sulphuric acid scarification followed

by the warm/cold stratification or four weeks cold stratification, while 10 minutes of sulphuric acid scarification followed by cold stratification for eight weeks resulted in > 90% germination (table 4). Stratification had no effect on *R. corchorifolius* because seeds germinated well after scarification (71% seeds germinated after five minutes of sulphuric acid); cold stratification for four weeks only increased germination by 4% in this species.

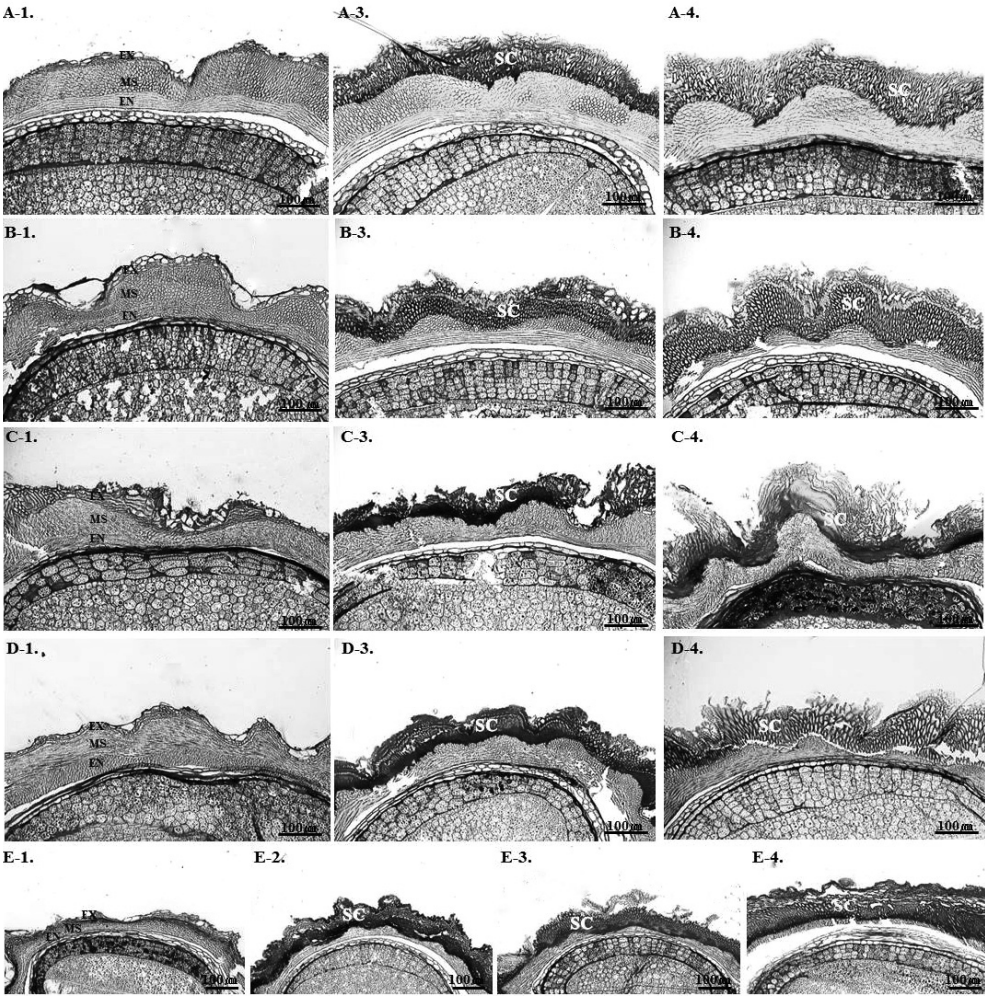

Figure 3. Cross-section of stones showing the scarification of endocarp of five *Rubus* species: (**A**) *R. parvifolius*; (**B**) *R. phoenicolasius*; (**C**) *R. buergeri*; (**D**) *R. takesimensis*; (**E**) *R. corchorifolius*; (1) non-scarified (control); (2) 5-minutes scarified (*R. corchorifolius* only); (3) 10-minutes scarified; (4) 20-minutes scarified endocarp. OE = outer-endocarp; ME = middle-endocarp; IE = inner-endocarp; SC = scarification area.
[Colour version of this figure available in the online publication.]

Table 3. Germination characteristics of *Rubus* species as affected by H_2SO_4 treatment.

Species	H_2SO_4 treatment (minutes)	Final germination (%)[1]	Mean germination time (days)	Germination index
R. parvifolius	0	0f	-	0
	10	0f	-	0
	20	23.8e	57.75	2.26
R. phoenicolasius	0	0f	-	0
	10	10.8e	20.83	2.82
	20	10.0e	15.00	2.83
R. buergeri	0	0f	-	0
	10	1.0ef	72	0.06
	20	2.0e	22	0.10
R. takesimensis	0	0f	-	0
	10	31.3ed	16.32	6.82
	20	27.0e	15.63	7.53
R. corchorifolius	0	18.0ed	50.00	1.51
	5	71.0a	18.37	18.76
	10	14.2cde	14.07	4.19
	20	3.0f	11.00	1.11

[1] Values followed by the same letter are not significantly different ($P > 0.05$).

Discussion

In this study we found that non-scarified, fresh seeds of five *Rubus* species did not germinate even though they were highly viable (tetrazolium test; data not shown). Four species (*R. parvifolius*, *R. phoenicolasius*, *R. takesimensis* and *R. corchorifolius*) showed physical seed dormancy and efficient use of concentrated sulphuric acid broke the dormancy and promoted germination (table 3). Generally a short scarification period (maximum 20 minutes) is effective for these five *Rubus* species compared with 30 minutes to more than three hours required to damage the endocarp before germination in many other *Rubus* species (USDA, 1949; Thompson, 1995; Wada and Reed, 2011a, b). This implies that the endocarps of the species studied here are relatively thin and soft.

It has already been suggested that endocarp thickness and hardness is the key factor in the strength of physical dormancy. However, just because the stone feels hard when touched or has a tough endocarp, it does not necessarily mean that it is impermeable to water. Water absorption by stones with scratched endocarp increased by more than two times that of intact seeds after 48 hours indicating that the endocarp of *Rubus* species

Table 4. Germination characteristics of five *Rubus* species after sulphuric acid scarification and then either no, warm (W; 25/15°C) and/or cold (C; 4°C) stratification for four or eight weeks, as indicated.

Species	H_2SO_4 treatment (minutes)	Stratification treatment (weeks)	Final germination (%)[1]	Mean germination time (days)	Germination Index
R. parvifolius	0	0	0e	-	0
		W 4- C 4	0e	-	0
		C 4	0e	-	0
		C 8	0e	-	0
	10	0	0e	-	0
		W 4- C 4	4.3e	83.00	0.19
		C 4	73.0c	34.88	11.58
		C 8	70.0c	23.99	19.14
	20	0	23.7d	57.75	2.23
		W 4- C 4	82.7b	66.66	6.32
		C 4	90.0a	28.73	19.85
		C 8	92.0a	16.26	26.67
R. phoenicolasius	0	0	0g	-	0
		W 4- C 4	0g	-	0
		C 4	0g	-	0
		C 8	0g	-	0
	10	0	10.7f	20.83	2.82
		W 4- C 4	25.0e	55.81	5.50
		C 4	73.0b	25.45	14.09
		C 8	92.0a	19.61	36.30
	20	0	10.0f	15.00	2.83
		W 4- C 4	49.0b	24.04	13.85
		C 4	55.7cd	15.39	14.43
		C 8	64.0bc	7.89	53.21
R. buergeri	0	0	0b	-	0
		W 4- C 4	0b	-	0
		C 4	0b	-	0
		C 8	0b	-	0
	10	0	1.0ab	72.00	0.06
		W 4- C 4	0b	-	0
		C 4	0b	-	0
		C 8	0b	-	0

Table 4. *con't*

Table 4. *Continued*

Species	H_2SO_4 treatment (minutes)	Stratification treatment (weeks)	Final germination (%)[1]	Mean germination time (days)	Germination Index
R. buergeri	20	0	2.0a	22.00	0.09
		W 4- C 4	0b	-	0
		C 4	0b	-	0
		C 8	0b	-	0
R. takesimensis	0	0	0d	-	0
		W 4- C 4	0d	-	0
		C 4	0d	-	0
		C 8	0d	-	0
	10	0	31.2cd	16.32	6.82
		W 4- C 4	78.0a	21.15	12.77
		C 4	74.0a	27.55	15.10
		C 8	90.0a	25.27	19.74
	20	0	27.0c	15.63	7.53
		W 4- C 4	47.2b	27.17	7.73
		C 4	37.1cd	14.22	11.62
		C 8	45.0b	14.78	13.30
R. corchorifolius	0	0	18.0cd	50.00	1.51
		W 4- C 4	0f	-	0
		C 4	15.1cde	36.00	1.74
		C 8	18.0cd	25.56	3.21
	5	0	71.0a	18.37	18.76
		W 4- C 4	53.0b	19.30	13.96
		C 4	75.0a	15.27	22.66
		C 8	71.1a	22.62	12.98
	10	0	14.2cde	14.07	4.19
		W 4- C 4	8.0def	13.88	2.38
		C 4	20.5c	10.70	10.15
		C 8	7.6ef	9.00	3.11
	20	0	3.0f	11.00	1.11
		W 4- C 4	0f	-	0
		C 4	1.0f	6.00	0.67
		C 8	8.0def	10.00	3.20

[1] Values followed by the same letter are not significantly different ($P > 0.05$).

included here could restrict water uptake and might cause physical dormancy. It was also found that the degree of endocarp scarification necessary for germination was related to stone size and endocarp thickness (tables 2 and 3). It was observed that at least half of the thickness of middle-endocarp should be scarified for germination to occur (table 3; figure 3). The optimum period of scarification for *R. phoenicolasius* and *R. takesimensis* was 10 minutes (70-90% germination) and that for *R. parvifolius* was 20 minutes. *R. corchorifolius* seeds were the smallest with the thinnest endocarp of the studied species and thus was scarified within five minutes (≥ 70% germination). Although more than half of the middle-endocarp of *R. buergeri* was scarified within 10 minutes, seed germination for this species was similar following all the scarification treatments, with very little germination. These results show that standard scarification treatments of 30 minutes or three hours based on subgenus and stone size are not applicable to Korean *Rubus* species.

In spite of breaking the physical dormancy by a single treatment of sulphuric acid scarification, *Rubus* species still exhibited low germination. Therefore, the studied *Rubus* species perhaps have deep combinational dormancy caused by not only a hard endocarp but also by physiological inhibitors. Previous studies reported that *Rubus* seeds can be stimulated to germinate by soaking them in concentrated sulphuric acid followed by stratification (USDA, 1949; Peacock and Hummer, 1996) and the results of this study concurs with these earlier findings. In one report, Reman *et al*. (2011) described both physical and physiological dormancy in black raspberry (*R. coreanus*) and the best germination was achieved by stratifying seeds for 30-45 days before scarifying with sulphuric acid for 15 minutes. Similar results were presented by Peacock and Hummer (1996) for six *Rubus* species; seeds were effectively germinated at low temperature following warm stratification after soaking in sulphuric acid for 30 minutes.

The results of this study suggest that the longer the stratification period, the higher the percentage germination in three species: *R. parvifolius*, *R. phoenicolasius* and *R. takesimensis*. This clearly indicates that the endocarp alone is not the factor restricting germination and *Rubus* seeds may also have physiological dormancy. In the case of *R. parvifolius*, the best germination treatment was eight weeks stratification after 20 minutes sulphuric acid scarification. *R. phoenicolasius* and *R. takesimensis* were similar in that the optimum pretreatment was eight weeks stratification after 10 minutes sulphuric acid scarification (table 4). Interestingly, *R. corchorifolius* did not require stratification and thus germinated only after sulphuric acid scarification, suggesting that the seeds of this species have physical dormancy. Wada *et al*. (2011) reported a similar result for *R. hoffmeisterianus* Kunth & Bouch as the seeds germinated well after 30 minutes of sulphuric acid scarification without cold/warm stratification treatment. Similarly, *R. idaeus* seeds treated with concentrated sulphuric acid or sodium hydroxide germinated well without any stratification (Rose, 1919).

R. buergeri showed low or no germination following all treatments, even though seeds were determined to be highly viable by tetrazolium test (data not shown). Furthermore, *R. buergeri* has a medium thickness and moderately hard endocarp, and the middle-endocarp was effectively scarified by 50-70% within 10 minutes. Cho *et al*. (2013) reported that *R. buergeri* seeds have an undeveloped embryo that remains dormant and thus it may require cold stratification for embryo growth and thence germination to occur. However,

this is not the case for our samples because we found fully developed embryos with two cotyledons and differentiated radicles in mature seeds. It has been suggested that some *Rubus* species contain phenolic compounds on the interior of the endocarp and micropylar region which contribute to endocarp hardness and result in seed dormancy (Wada *et al*., 2011). Given the wide range of concentration of phenolic compounds in *Rubus* seeds, with two to five times as much in the harder, thicker endocarps compared with the softer, thinner ones, sulphuric acid is likely to be effective in degrading those phenolics in the endocarp, resulting in successful scarification (Wada *et al*., 2011). Therefore, we cannot be sure that the lack of germination in *R. buergeri* was due to these phenolic compounds, because the endocarp was found to be well-scarified after 20 minutes. There may be some additional chemical inhibitors or some physiological disorder of the embryo that contribute to seed dormancy in *R. buergeri*.

R. parvifolius required strong scarification treatment because it had the largest stones with a thick and hard endocarp. In contrast, *R. corchorifolius*, with the smallest stones and a thin and comparatively soft endocarp, germinated after five minutes of scarification even without stratification. In summary, the diversity of *Rubus* stones morphological characteristics was demonstrated by investigating various aspects of germination. Hummer and Peacock (1994) noted that size and weight of stones differed significantly among 43 *Rubus* species, whereas Wada and Reed (2011b) pointed out that endocarp thickness is correlated with stone weight and endocarp hardness. In light of this study we suggest that an evaluation of *Rubus* stones characteristics is required to provide precise guidance for the realisation of effective scarification and germination protocols for different species. In addition, endocarp anatomical observation is useful as a basic indicator of what scarification treatment would be effective. However, for optimum germination, suitable stratification treatments are also required because some *Rubus* species have deep combinational dormancy.

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