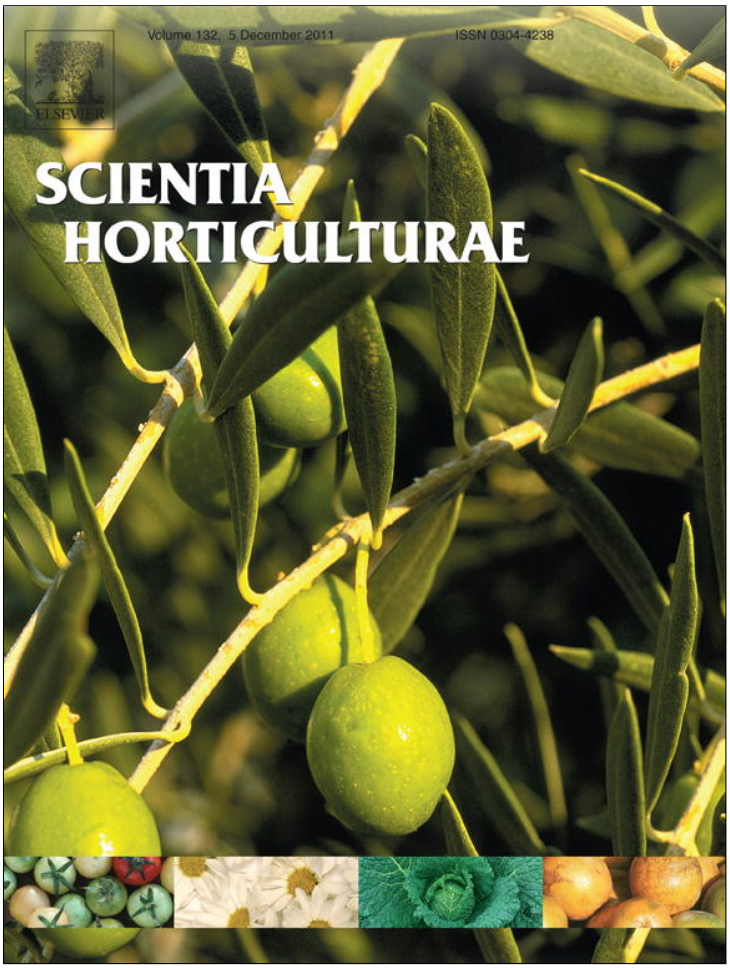

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Standardizing germination protocols for diverse raspberry and blackberry species

Sugae Wada [a,1], Barbara M. Reed [b,*]

[a] Department of Horticulture, Oregon State University, 4017 Ag and Life Sciences Bldg, Corvallis, OR 97331-7304, USA
[b] USDA-ARS National Clonal Germplasm Repository, 33447 Peoria Rd, Corvallis, OR 97333-2521, USA

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ABSTRACT

Most blackberry and raspberry (*Rubus*) seed exhibit delayed or poor germination because of a deep double dormancy. The objective of this study was to improve seed scarification and germination protocols for the diverse *Rubus* species in two subgenera often used for breeding improved cultivars. We also defined the germination potential of freshly collected seed and the seed characteristics of 17 *Rubus* species in three subgenera. Only four of 17 species had ≥50% germination of immediately-germinated, non-scarified seed, indicating primary seed dormancy. Seed-coat thickness was better correlated with seed size ($R = 0.82$) than was hardness ($R = 0.71$). Dry seed of three species each in the subg. *Idaeobatus* and *Rubus* were scarified with concentrated sulfuric acid (98% H_2SO_4) or sodium hypochlorite (14% NaOCl) followed by germination treatments of deionized water (DI), smoke gas solution, gibberellic acid (2.03 mg/L GA_3) with potassium nitrate (34 mg/L KNO_3) or GA_3 alone. Germination after H_2SO_4 scarification was significantly better than NaOCl for four of the six species despite equal reduction in the seed coat by the scarification treatments. H_2SO_4-scarified seed had maximum germination in 6–8 months compared to 12 months for NaOCl-scarified seed. Scarification treatments were not uniform for the subgenera. Increased H_2SO_4 scarification durations monitored by viability testing with 2,3,5 triphenyl tetrazolium chloride (TZ) were very effective in determining optimal scarification timing. *R. georgicus* and *R. occidentalis* H_2SO_4-scarified seed treated with $GA_3 + KNO_3$ or smoke germinated significantly better than the other treatments; $GA_3 + KNO_3$, smoke and DI water were equally effective for the other four species.

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1. Introduction

The genus *Rubus* is a highly diverse taxon that includes more than 750 species (Thompson, 1995) in 12 subgenera and occurs on all continents except Antarctica (Focke, 1914; Hummer, 1996). The most studied are the economically important blackberries (subg. *Rubus*) and raspberries (subg. *Idaeobatus*); both have a deep double dormancy caused by a hard seed coat and one or more additional mechanisms such as an impermeable seed coat, chemical inhibitors, or presence of a dormant embryo (Nybom, 1980; Taylor, 2005; Zasada and Tappeiner III, 2003). Growth inhibiting substances are concentrated in the endosperm and testa of dormant seeds of 'Lawton' blackberry (Lasheen and Blackhurst, 1956). External dormancy is determined by the outer layer of the seed (endocarp); it restricts the uptake of water and oxygen and mechanically prevents the embryo from swelling. This dormancy is broken if the seed coat is removed or scarified, while internal dormancy is regulated by biochemical or biophysical processes that occur during an after-ripening at 0–5 °C (Taylor, 2005).

Although cultivars of raspberries and blackberries are clonally propagated, important traits for improved performance, flavor, disease or pest resistance are available in wild germplasm that is accessed from stored seed of wild collected plants. Protocols for germination of wild species are not available, often resulting in little or no germination of the stored seed, and therefore lack of access to possibly important genetic resources. Dormancy of *Rubus* seeds can also cause substantial problems for blackberry and raspberry breeding programs, resulting in little or no germination from desired hybrid seed (Clark et al., 2007). Due to the great diversity of *Rubus* species, a single standard germination protocol is unlikely to be useful for germinating seed of diverse germplasm. This genus is especially difficult due to the disparity in seed-coat thickness and structure (Daubeny, 1996) and specific germination procedures are not available for most *Rubus* species. Standardization of seed scarification and germination protocols using freshly collected seed would provide the optimum conditions for germination with seeds of known origin and storage conditions.

Acid scarification followed by warm and cold stratification is considered the best procedure for breaking dormancy in seeds with an intact stony endocarp (Heit, 1967). A widely used two-step scarification procedure for *Rubus* seed includes treating thoroughly

* Corresponding author. Tel.: +1 541 738 4216; fax: +1 541 738 4205.
E-mail addresses: wadas@hort.oregonstate.edu (S. Wada), Barbara.Reed@ars.usda.gov, reedbm@hort.oregonstate.edu (B.M. Reed).
[1] Tel.: +1 541 738 4218.

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dried seed with concentrated H_2SO_4 for up to 3 h and washing in running water followed by several days soaking in a saturated sodium bicarbonate solution and rinsing in water (Clark et al., 2007). Calcium hypochlorite [$Ca(ClO)_2$] in calcium hydroxide [$Ca(OH)_2$] solution is used for neutralizing H_2SO_4 and removing carbonized endocarp before stratification. The Woody Plant Seed Manual suggested H_2SO_4 scarification for 15–20 min for raspberry (subg. *Idaeobatus*; *R. idaeus* L., *R. occidentalis* L.) and up to 3 h for blackberry (*Rubus* subg. *Rubus* spp. Watson) (Zasada and Tappeiner III, 2003). NaOCl (14%) scarification was effective for sand blackberry (*R. cuneifolius* Pursh) seeds and reduced endocarp thickness, resulting in structural weakening (Campbell et al., 1988).

Drying interferes with the immediate germination of blackberry seeds. Freshly collected, moist, halved *R. laciniatus* Willd and 'Navaho' blackberry seeds germinated well *in vitro* but dried halved seeds did not (Mian et al., 1995). This indicates that the lack of germination of dried blackberry seed is due to other factors than the hard seed coat. Dale and Jarvis (1983) reported that freshly harvested, scarified raspberry (*R. idaeus*) seed germinated readily, but when dried for 7 wk the germination rate was greatly reduced. Naruhashi et al. (1999) examined 47 Japanese *Rubus* species to clarify their germination pattern. A great deal of interspecific variation in germination rates was evident when the fresh seed was germinated *in vitro* for 100 days and a low rate or lack of germination of many species was explained by seed dormancy. Little is known about scarification requirements for most *Rubus* species, but 30 min H_2SO_4 scarification significantly improved germination of four of six species, compared to unscarified controls (Peacock and Hummer, 1996). Our earlier study of cold-stored seed found wide variation in scarification requirements for eight *Rubus* species in six subgenera (Wada and Reed, 2011).

After scarification, additional treatments during stratification significantly enhance seed germination for many plant species. These treatments include gibberellic acid (GA_3) (Koornneef et al., 2002; Warr et al., 1979); smoke (Flematti et al., 2004; Keeley and Fotheringham, 2005; Nuzzo et al., 1996); and potassium nitrate (KNO_3) (Bethke et al., 2007a; Centibas and Koyuncu, 2006). In most studies *Rubus* seed is germinated in sand, soil, peat moss or *in vitro* without seed treatments. In stored *Rubus* seed we found that some species have improved germination following a combined GA_3 and KNO_3 seed treatment (Wada and Reed, 2011).

The objective of this study was to improve seed scarification and germination protocols for diverse species in two commercially important *Rubus* subgenera. Fresh seed was studied so that the collection, drying and storage conditions were standardized and initial viability was high. Specifically, we defined scarification and germination requirements for fresh dry seed of three species in each subgenus of *Idaeobatus* and *Rubus*, including time course studies. In addition we evaluated seed-coat characteristics, primary dormancy and immediate germination potential of fresh moist seed of 17 *Rubus* species in three subgenera.

2. Materials and methods

2.1. Plant materials

Fully mature fruit of 17 *Rubus* species (Table 1) were collected from plants in the USDA-ARS small fruit breeding program research field at the Oregon State University, Lewis Brown Farm (Corvallis, OR) in the summer of 2006. Fruit was soaked in pectinase (Novozymes, Fresno, CA) (15 mL/500 g fruit) for 24 h at room temperature, then mashed and blended for 1 min in a blender with plastic-covered blades. The mixture was poured through a strainer and rinsed in tap water as the seeds were rubbed against the mesh of the strainer. Thoroughly cleaned seed were scraped off the strainer and spread onto paper towels to dry (22 °C for 5 days) or immediately set up for fresh seed germination trials.

2.2. Fresh seed

2.2.1. Seed viability

Fresh seeds ($n = 25$) were soaked for 12 h in water, cut in half longitudinally, treated with a 1% solution of 2,3,5 triphenyl tetrazolium chloride (TZ) for 24 h at room temperature, and drained. A few drops of 85% lactic acid solution were added to the seed for 0.5 h. The embryos were visually evaluated under a low magnification dissecting microscope (AOSA, 2000). The entire or most of the embryo on viable seeds stained red while white embryos with red seed coats were not viable.

2.2.2. Stratification and germination

Two sets of 100 fresh seeds of each of the 17 species, were placed in germination boxes ($n = 200$) on blotters fully soaked with deionized (DI) water. All seeds were treated for 1 month under a warm stratification regime at 18 ± 2 °C with a 16 h photoperiod (Hoffman Manufacturing Inc. Jefferson, OR) followed by a 3 month cold stratification in the dark at 4 ± 1 °C, and finally they were placed in the germinator with 8 h dark at 15 ± 2 °C and 16 h light at 30 ± 2 °C for 8 months.

2.3. Dry seed

The remaining seeds were dried 5 days at room temperature (22 °C), desiccated for 24 h (Drierite, W.A. Hammond Drierite Company, Xenia, OH) and held for 3 months at 22 °C in plastic dishes (15 × 2.5 cm) before germination testing.

2.3.1. Seed weight

Dry seed for each species was weighed in 3 lots of 100 seeds each.

2.3.2. Seed-coat thickness and hardness

Seed-coat thickness was measured for 10 dry seeds of each species using a Nikon SMZ 1000 stereomicroscopic zoom microscope (Nikon Instruments, Tokyo, Japan) with Infinity Capture Imaging software (Lumenera Corporation, Ottawa, Canada). For six species the seed-coat thickness was measured before and after scarification treatments. Measurements were taken in the center of the seed equidistant from the micropylar region and the hilar end. Hardness ratings of 1–5 were assigned after dry seeds were soaked in DI water for 2 days and hand sectioned with a scalpel. A subjective hardness grading of 1 soft, 2 slightly hard, 3 hard, 4 very hard and 5 extremely hard was used.

2.3.3. Plant materials

Scarification and germination tests were done with seeds from six species in two subgenera selected by seed weight, and represented a range of seed-coat thickness and hardness. Four replications of 50 seeds each were used per accession for each germination test ($n = 200$). Seeds were categorized as small, medium or large for the subgenus. For subg. *Idaeobatus*, small – *R. hoffmeisterianus* Kunth & C. D. Bouché (0.04 g), medium – *R. coreanus* Miq. (0.10 g), and large – *R. occidentalis* (0.19 g). In subg. *Rubus*, small – *R. ursinus* Cham. & Schltdl. (0.12 g/100 seed), medium – *R. georgicus* Focke (0.26 g), and large – *R. caesius* L. (0.37 g).

2.3.4. Scarification procedures

Scarification procedures were modified from the methods of Campbell et al. (1988), Jennings and Tulloch (1965), and Moore et al. (1974). Preliminary tests employed scarification times commonly used for each subgenus and visually determined that no embryo

Table 1
Fresh seed germination of 17 *Rubus* species at 12 months and dry seed characteristics.

Rubus species	Subgenus	Dry seed characteristics			
		% fresh seed germination[z]	Hardness rating[y]	Seed-coat thickness (mm)[x]	Weight of 100 seeds (g)[w]
R. caesius L.	*Rubus*	89.5	3	0.185 abcd	0.366 a
R. canadensis L.	*Rubus*	0.0	5	0.210 abc	0.154 e
R. caucasicus Focke	*Rubus*	57.5	2	0.167 cde	0.183 d
R. coreanus Miq.	*Idaeobatus*	10.0	2	0.136 defg	0.100 h
R. corchorifolius L.	*Idaeobatus*	19.0	2	0.129 defg	0.050 i
R. crataegifolius Bunge	*Idaeobatus*	2.5	2	0.121 defg	0.107 g
R. cyri Juz.	*Rubus*	1.0	5	0.231 ab	0.362 a
R. georgicus Focke	*Rubus*	0.0	5	0.175 bcde	0.255 c
R. hoffmeisterianus Kunth & C. D. Bouché	*Idaeobatus*	1.0	2	0.087 g	0.040 j
R. insularis F. Aresch.	*Rubus*	0.0	4	0.238 a	0.280 b
R. leucodermis Douglas ex Torr. & A. Gray	*Idaeobatus*	0.0	3	0.118 efg	0.109 g
R. occidentalis L.	*Idaeobatus*	0.0	5	0.176 bcde	0.188 d
R. parvifolius L.	*Idaeobatus*	0.5	3	0.157 cdef	0.153 e
R. sachalinensis H. Lév.	*Idaeobatus*	1.0	4	0.171 bcde	0.187 d
R. setchuenensis Bureau & Franch.	*Malachobatus*	99.0	1	0.102 fg	0.048 i
R. swinhoeii Hance	*Malachobatus*	60.0	2	0.166 cde	0.110 g
R. ursinus Cham. & Schltdl.	*Rubus*	0.5	4	0.144 defg	0.120 f

[z] Freshly collected seeds germinated with DI water only ($n = 200$).
[y] A subjective hardness grading of 1 soft, 2 slightly hard, 3 hard, 4 very hard and 5 extremely hard was used.
[x] Means in a column followed by the same letter are not significantly different calculated by Duncan's multiple range test ($\alpha = 0.05$) ($n = 10$).
[w] 3 replicates of 100 seeds ($n = 300$).

injury occurred. Treatments were: (1) Control – no treatment. (2) H_2SO_4 (98%) in an ice bath for 0.5 h for *Idaeobatus* or 3 h for subg. *Rubus*, rinsed in running water for 1 h; then 5 min in $Ca(ClO)_2$ (3 g/L) completely dissolved in water with an excess of $Ca(OH)_2$ (3 g/L) in each treatment beaker, and finally rinsed for 5 min in running water. Seeds were rubbed against a strainer before stratification to remove the carbonized portions of testa. (3) NaOCl (14%) in an ice bath for 6 h for *Idaeobatus* and 20 h for *Rubus* and rinsed for 1 h in running water.

2.3.5. Germination treatments

Germination blotters were fully soaked with DI water. Scarified seed were placed on the blotters and 5 mL of one of four solutions was added (without adjusting the pH). Control seeds were not scarified and were germinated with DI water. Treatments were: (1) DI water (pH 6.8–7.0), (2) GA_3 (2.03 mg/L, pH 4.8), (3) $GA_3 + KNO_3$ (34 mg/L, pH 4.9) and (4) smoke gas solution (Super Smoke Plus, Claremont, South Africa, pH 2.8) made by soaking five pieces of smoke-infused paper in 250 mL DI water for 24 h at 4 °C. KNO_3 (pH 5.5) alone was initially tested, but not scored due to extensive fungal contamination during germination. All treatments were replicated with 50 seeds per 9 cm plastic Petri dish and 4 dishes per species. All seeds were stratified as for moist seed (above). Plates were sprayed with thiophanate methyl fungicide (3336 WP, Cleary Chemical Corporation, Dayton, NJ; 0.13 mL/L) as needed to control fungi.

2.3.6. Additional scarification tests

Initially *R. occidentalis* did not germinate with 0.5 h H_2SO_4 scarification, perhaps because of the thick, extremely-hard seedcoat. Additional scarification tests were run with H_2SO_4 in an ice bath for 0.5, 1.0, 1.5, 2.0, and 3.0 h (4 reps; $n = 200$). All were stratified and germinated on blotters with DI water. From this test the 3 h scarification was chosen and a second set of the four germination treatments were run. Later prolonged H_2SO_4 scarification tests were performed with three species (*R. occidentalis*, *R. ursinus*, and *R. georgicus*) to determine seed viability after long-duration scarification. Seeds were scarified for 3.0 h, 3.5 h and 4.0 h. After each scarification treatment, embryo damage was evaluated with TZ as described above.

2.4. Data set and analysis

All plates were placed in a randomized complete block design with each incubator shelf considered a block. Germination counts were taken at 1 month after warm stratification and then monthly from 4 to 12 months in the germinator. Raw data of germination counts were used for the statistical analysis, and presented in the figures and tables as percentages. Data were analyzed for ANOVA using SAS 9.2 (2008). Means were separated with Duncan's multiple range test significant at *alpha* = 0.05. Dunnett's test of pair-wise multiple comparisons significant at 95% confidence limits level were used to analyze for interactions between genotypes and treatment effects.

3. Results

3.1. Fresh seed

3.1.1. Seed viability

All fresh seed samples were confirmed as >95% viable by the TZ test (data not shown).

3.1.2. Fresh seed germination

Unscarified fresh seed of the 17 species had variable germination at 12 months (Table 1). At 6 months only 2 species had germinated; *R. setchuenensis* (subg. *Malachobatus*) germination reached 99% during the warm stratification, so no cold stratification, scarifications, or pretreatment was required for this species (data not shown). By 12 months only 4 species had germination >50%. All of the species with very-hard (rated 4) or extremely-hard (rated 5) seed coats had very little or no germination.

3.2. Dry seed

3.2.1. Seed characteristics

Seed size ranged from 0.040 g to 0.366 g per 100 seed (Table 1). Seed-coat thickness ranged from 0.087 to 0.238 mm. Seed weight and seed-coat thickness were well correlated ($R = 0.82$) ($P < 0.05$) (Fig. 1a). The smallest seed, *R. hoffmeisterianus*, had the thinnest seed coat and the three largest seeds had some of the thickest seed coats. Ten species had hard to extremely-hard seed coats and seven

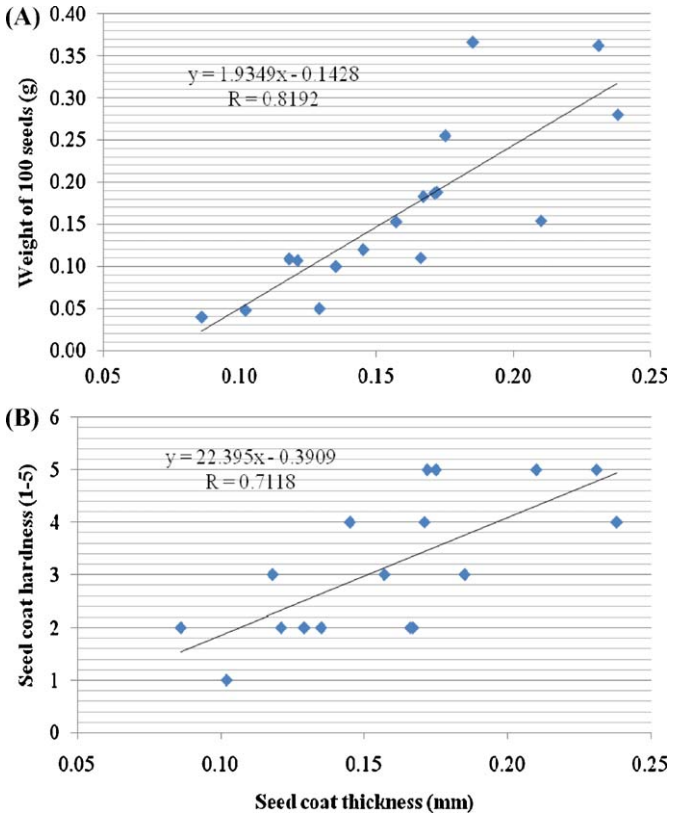

Fig. 1. Correlation between (A) seed weight and seed-coat thickness, (B) seed-coat hardness and thickness of seed of 17 *Rubus* species.

were soft or only slightly hard. Seed-coat hardness and thickness were correlated ($R = 0.71$) ($P < 0.05$) (Fig. 1b).

3.2.2. Seed-coat thickness following scarification

Seed-coat thickness was significantly reduced with either H_2SO_4 or NaOCl scarification for five of the six species and there were no significant differences between the scarification treatments (Table 2). Only *Rubus occidentalis* seed treated with 0.5 h H_2SO_4 had less seed coat removed than the NaOCl treatment.

3.2.3. Scarification treatments

There were significant differences among the treatments and among the species; there was a significant species by treatment interaction ($P < 0.05$). At 12 months, germination in the H_2SO_4 treatments (except *R. occidentalis* with 0.5 h treatment) was significantly better than the NaOCl or control treatments for four of six the species (except *R. coreanus* and *R. caesius*). The NaOCl + DI treatment was better than the control for all species except for *R. occidentalis*. Unscarified control seeds of *R. caesius* and *R. coreanus* began to germinate at 6 months (data not shown) and had low germination at 12 months; no other control seed germinated (Table 3). Significant scarification treatment effects were noted ($P < 0.05$) for five of the six *Rubus* species.

3.2.4. Extended scarification treatments

Rubus occidentalis seed had essentially no germination when scarified with H_2SO_4 for 0.5 h; but a more complete set of scarification tests indicated that a much longer scarification was needed. There was no germination for the control or on DI with 0.5 h scarification (0%), and little with 1 h (12%) or 2 h (20%), but with 3 h the germination reached 74%. TZ testing after longer H_2SO_4 scarification of *R. occidentalis*, *R. ursinus*, and *R. georgicus* indicated that >95% of unscarified and 3 h scarified seeds were viable, and this declined to about 80% of 3.5 h scarified seeds. All viability was lost with 4 h scarification resulting in white embryos (data not shown).

3.2.5. Germination treatments

R. georgicus and *R. occidentalis* (3-h scarified) seed treated with $GA_3 + KNO_3$ or smoke germinated better than the other H_2SO_4 treatments. Germination of *R. ursinus* with the GA_3 treatment was significantly less ($P < 0.05$) than the other treatments at 12 months. For three species there were no significant differences among the H_2SO_4 treatments (Table 3). Germination treatments following NaOCl scarification were either less effective or not significantly different from the DI water treatment, except for GA_3 or smoke for *R. ursinus* where they were significantly more effective ($P < 0.05$). None of the germination treatments were effective after NaOCl scarification for *R. occidentalis*, with all germination <10%.

3.3. Time course of dry-seed germination

The species varied in how quickly they germinated depending on the scarification and germination treatments (Fig. 2). A few germinated during warm stratification but minimal germination was observed during the cold stratification period. Germination for most species accelerated after the cold stratification period. Germination started for most species at 4 months and by 5 months all of the species responded vigorously to the treatments. Most H_2SO_4 treated seeds reached their maximal germination at 6–8 months and while most of the NaOCl treated seed had very low germination at 6 months and a lower total germination at 12 months compared to the H_2SO_4 treated seed.

4. Discussion

This study of freshly collected *Rubus* seed provides information on a range of species in the economically important raspberry (*Idaeobatus*) and blackberry (*Rubus*) subgenera. This information is useful for developing germination protocols so that seeds of diverse species can be used for breeding improved cultivars. Stored seed of wild-collected *Rubus* species are distributed to plant breeders from germplasm collections; however many of these species do not germinate following commonly used procedures. Determining the optimum scarification protocols and germination treatments for diverse species will make these collections more useful and more easily utilized by plant breeders.

Unscarified fresh seed of most *Rubus* species does not germinate even when determined to be highly viable by TZ testing. Only four of 17 species germinated >50% at 12 months (Table 1). Similar results were seen by Naruhashi et al. (1999) in a screen of 47 species of fresh unscarified *Rubus* seed *in vitro*. After 100 days, 10 species had high germination including *R. setchuenensis* (96%), *R. hakonensis* (83%) and *R. trifidus* (98%), seven species were moderate, including *R. palmatus* (49%) and *R. sieboldii* (43%), seven species had very small amounts of germination; 23 species did not germinate at all, including *R. coreanus* and *R. occidentalis*. In our study, the fresh seeds of *R. coreanus* germinated at only 10% after 12 months, while *R. occidentalis* did not germinate. Germination of the unscarified fresh seed did not correlate with seed weight (size) or seed-coat thickness or hardness (data not shown). None of the species with very-hard or extremely-hard seed coats germinated, but many others that did not germinate were only slightly hard and thin (Table 1). This indicates that the primary dormancy of fresh seed cannot be predicted by general seed-coat characteristics. Peacock and Hummer (1996) noted that *R. chamaemorus* had a difficult-to-cut (very hard) seed coat and low germination (<40%) while *R. multibracteatus* was easily cut (soft), germinated without treatment (85%) and likely had no endogenous dormancy. We also found that fresh collected *R.*

Table 2
Seed-coat thickness (mm) of six *Rubus* species in two subgenera before and after scarification.

Seed size[z]	Subg. *Idaeobatus*			Subg. *Rubus*		
	R. hoffmeisterianus (S)	*R. coreanus* (M)	*R. occidentalis* (L)	*R. ursinus* (S)	*R. georgicus* (M)	*R. caesius* (L)
Seed-coat thickness (Control)[y]	0.087 a	0.136 a	0.176 a	0.144 a	0.175 a	0.185 a
Thickness after H_2SO_4[x]	0.068 b	0.112 b	0.147 b (0.111 c)	0.129 b	0.145 b	0.148 b
H_2SO_4 reduction	22%	18%	37% (16%)	20%	17%	20%
Thickness after NaOCl[w]	0.060 b	0.105 b	0.106 c	0.118 b	0.130 b	0.141 b
NaOCl reduction	31%	23%	40%	20%	26%	24%

[z] Relative seed sizes in each subgenus: small (S), medium (M) and large (L).
[y] Mean of 10 seeds ± standard error (mm). Means in a column followed by the same letter are not significantly different calculated by Duncan's multiple range test ($\alpha = 0.05$).
[x] H_2SO_4 treatment 3 h for subg. *Rubus*, 0.5 h treatment for subg. *Idaeobatus* (3 h treatment for *R. occidentalis* in parenthesis).
[w] NaOCl treatment 6 h for subg. *Idaeobatus* and 20 h for subg. *Rubus*.

multibracteatus and *R. setchuenensis* seeds (both subg. *Malachobatus*) immediately germinated >90% after only 1 month of warm stratification (data not shown).

The diversity of *Rubus* species seed morphology was evident in our study through the seed size, hardness and thickness of seed coats (Table 1). Hummer and Peacock (1994) found that *Rubus* seed size differences were more than 80-fold as determined by seed weight of 43 species. The seed in this study, from fewer subgenera, varied by only 9-fold. We found that seed-coat thickness in this sample was correlated with seed weight and seed-coat hardness (Fig. 1). Moore et al. (1974) found a significant positive correlation between seed weight and seed-coat thickness in very-thick blackberry seed coats and also found a slight negative correlation between seed-coat thickness after scarification and percent germination, probably because many did not germinate at all. Initial evaluation of *Rubus* seed characteristics is required to provide guidance for determining effective scarification and germination protocols for these unique species.

Commonly used scarification techniques generally include treating raspberry seed in H_2SO_4 for 0.5 h and blackberry seed for 3 h (Clark et al., 2007). Other options include treating with 5% commercial bleach for 48 or 96 h (Galletta et al., 1989) or with 15% NaOCl for 18 h (Campbell et al., 1988). Scarification treatments in this study were originally based on these techniques published for a few species in the two commercially grown subgenera. They rely on subgenus designation and seed size for determining the duration of treatment. There are no studies of most species in these groups or most of the 10 less common subgenera. In a few cases the length of scarification treatment is based on the amount of seed coat removed/remaining (Moore et al., 1974). We found that effective scarification was related to the seed-coat thickness and hardness regardless of the subgenus and that each subgenus contained one or more species with seedcoat characteristics more common in the other subgenus (Table 1).

Scarification with NaOCl reduced the seed coat thickness a similar amount to H_2SO_4 scarification, but usually did not result in equivalent germination (Tables 2 and 3). This indicates that the seed-coat thickness alone is not the only factor restricting germination and that H_2SO_4 may have additional effects on the seed-coat chemistry (Wada et al., 2011). Low germination following NaOCl may also be due to the high pH of the solution and its effects on the germination treatments that follow. Keeley and Fotheringham (1998) note that pH control is very important in germination studies. A pH < 6 germination solution was much more effective for germination of *Emmenanthe penduliflora* than solutions of neutral or higher pH; acidic solutions of water, nitrate and GA_3 were significantly more effective than neutral solutions (Keeley and Fotheringham, 1998). This indicates that it may be possible to acidify the pH of seeds after NaOCl scarification and increase the germination response.

Clark and Moore (1993) found that most of the blackberry group had >40% germination with 3 h H_2SO_4 scarification; however two sets of black raspberry (*R. occidentalis*) seeds did not germinate after the 0.5 h H_2SO_4 scarification that was effective for five red raspberry (*R. idaeus*) seed lots. *R. occidentalis* did not respond to either of the original scarification treatments in our experiments, possibly because it had a moderately thick (0.176 mm) and extremely-hard seed coat (Table 1). We surmised that, because the seed-coat thickness and hardness resembled those in the subg. *Rubus*, a longer scarification was needed. *R. occidentalis* had poor germination with the seed coat reduced by 16% (0.5 h scarification) but germinated well with a 37% reduction (3 h H_2SO_4 scarification) (Table 3). Moore et al. (1974) measured seed-coat thickness of open pollinated seed of 20 blackberry clones with extremely-thick seed coats (average 0.47 mm) and found that H_2SO_4 scarification reduced the seed coats an average of 6% at 0.5 h, 21% at 1 h and 43% at 3 h, but germination remained low. We found that measuring the amount of seed coat removed was not an effective technique, and TZ testing was

Table 3
Percent germination at 12 months of dry seeds of six species in two *Rubus* subgenera either untreated (control) or following scarification with H_2SO_4 or NaOCl and four post-scarification germination treatments.

	Subg. *Idaeobatus*[z]			Subg. *Rubus*[y]		
	R. hoffmeisterianus	*R. coreanus*	*R. occidentalis*	*R. ursinus*	*R. georgicus*	*R. caesius*
H_2SO_4 + DI	99.0 a[x]	65.6 ab	7.5 (61.6 b) b	95.0 a	69.6 b	74.0 abc
H_2SO_4 + GA_3	98.6 a	75.6 a	3.5 (63.0 b) c	64.6 b	68.6 b	91.0 a
H_2SO_4 + GA_3/KNO_3	98.6 a	66.0 ab	7.5 (73.6 a) b	94.0 a	95.6 a	86.6 abc
H_2SO_4 + Smoke	98.6 a	73.0 a	13.0 (74.0 a) a	98.0 a	86.0 a	88.0 ab
NaOCl + DI	31.0 bc	53.0 bc	6.6 b	15.6 d	19.0 cd	65.0 cd
NaOCl + GA_3	7.6 de	53.0 bc	0.6 c	35.6 c	29.0 c	66.6 bcd
NaOCl + GA_3/KNO_3	38.0 bc	45.0 c	0.6 c	9.6 de	11.0 def	51.0 de
NaOCl + Smoke	7.6 de	60.6 abc	1.6 c	26.6 c	18.6 cd	41.6 e
Control[w]	0.0 e	11.6 d	0.0 c	0.0 e	0.0 f	30.6 ef

[z] Subg. *Idaeobatus* seeds were scarified for 0.5 h with H_2SO_4 (3 h in parenthesis for *R. occidentalis*) or for 6 h in NaOCl.
[y] Subg. *Rubus* treated for 3 h with H_2SO_4 or for 20 h with NaOCl.
[x] Means in a column followed by the same letter are not significantly different calculated by Duncan's multiple range test ($\alpha = 0.05$) (4 replicates of 50 seeds, $n = 200$).
[w] Control seeds were not scarified and were germinated with DI water.

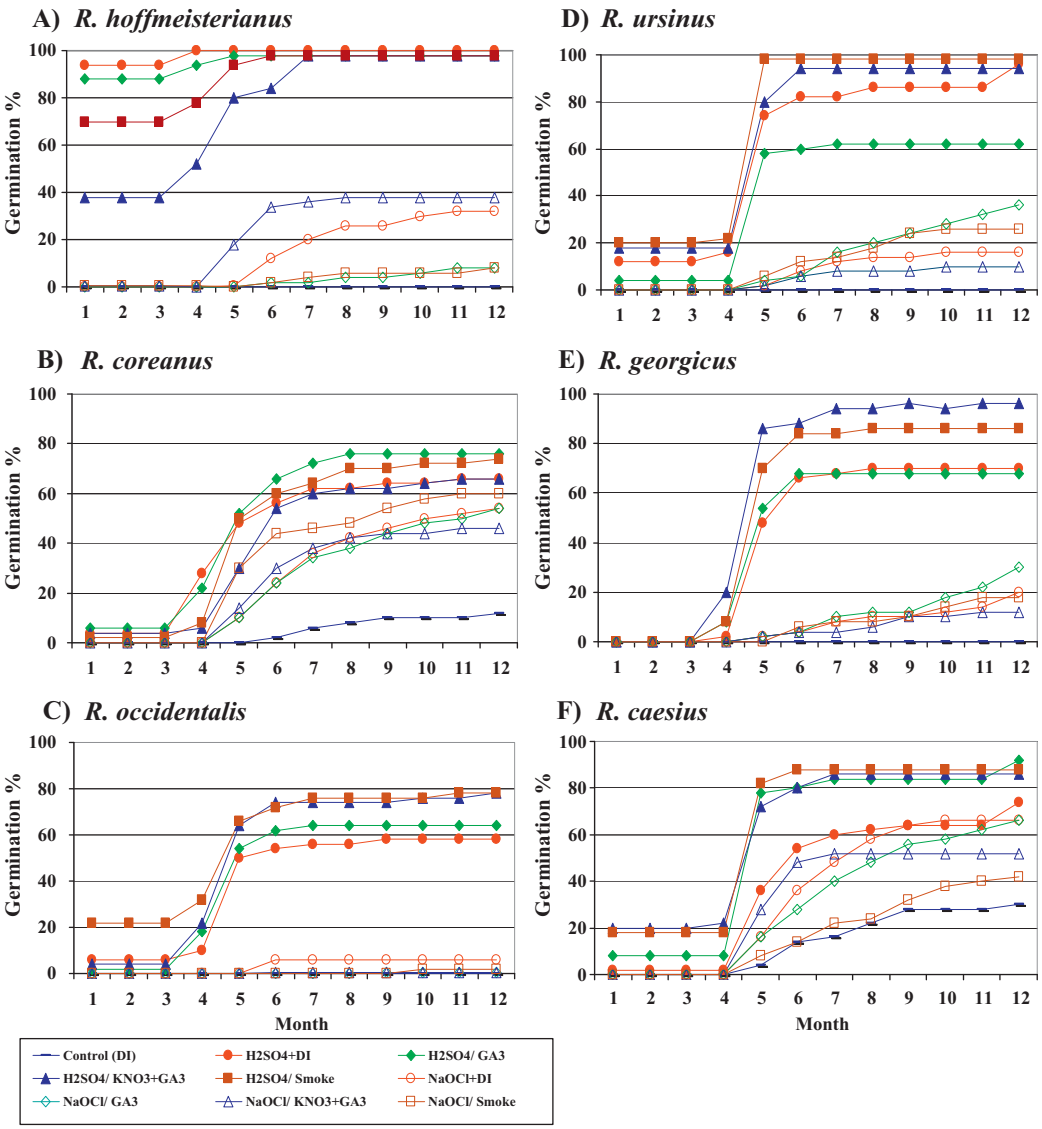

Fig. 2. Time course of germination of six species after two scarification techniques and four germination treatments ($n = 200$). Controls (DI water) were not scarified. All were germinated with 1 month warm stratification, 3 months cold stratification and 8 months in a germinator. Subg. *Idaeobatus* species were scarified for 0.5 h in H_2SO_4, except *R. occidentalis* with 3 h and all scarified for 6 h in NaOCl. Subg. *Rubus* species were scarified either for 3 h in H_2SO_4 or 20 h in NaOCl.

a better way to determine the appropriate scarification duration. TZ tests indicated that *R. occidentalis*, *R. ursinus*, and *R. georgicus* seed remained highly viable with 3 and 3.5 h H_2SO_4 scarification, but a 4 h scarification destroyed seed-coat structure and killed the embryos. We recommend TZ testing at a range of time points as an effective method for determining the optimum scarification duration for untested *Rubus* seed. We also found this effective with cold-stored *Rubus* seed from germplasm collections (Wada and Reed, 2011).

After effective scarification is applied, seed germination treatments can also impact the rate and amount of germination for many types of seeds. For fresh seed in the current study, treatment with GA_3 and KNO_3 was significantly better ($P \leq 0.05$) than GA_3 alone for three species following H_2SO_4 scarification and for one species after NaOCl (Table 3). GA_3 alone was better than the combination or the control for three other NaOCl scarified species. GA is commonly used as a seed germination treatment, and GA_3 also induced significantly more germination of nicked seed of *R. chamaemorus* (71%) than water treatments (44%) (Warr et al., 1979). Recent studies of ABA and GA interactions in Arabidopsis seed dormancy found that H_2O_2 increased NO production that in turn increased the catabolism of ABA, a germination inhibitor, and increased activity of GA biosynthesis genes, resulting in better germination (Liu et al., 2010). It is possible that H_2O_2 or directly administered NO would provide a more direct germination response in the seeds.

KNO_3 is commonly used as a seed treatment in seed-testing laboratories to overcome dormancy, but until recently the mode of action was not known. A range of nitrogen-containing compounds, including KNO_3, break Arabidopsis seed dormancy through effects on phytochrome A, with seeds germinating after short exposure to red light (Bataka et al., 2002). Further studies found that nitrogen compounds break dormancy in Arabidopsis through NO production that results in expression of an NO-associated gene in the aleurone layer and embryo and of GA_3 oxidase genes in the embryo (Bethke et al., 2007b, 2005). The combination seed treatment with GA_3 and KNO_3 together may be exploiting two modes of action, resulting in improved germination of some *Rubus* species. Our earlier study with germination treatments of stored *Rubus* species seeds found that GA_3 and KNO_3 was the best overall treatment for improving germination (Wada and Reed, 2011).

Treatment with smoke gas solution produced early seed germination for *R. hoffmeisterianus*, *R. caesius*, *R. occidentalis* and *R.*

ursinus after H_2SO_4 scarification (Fig. 2) and produced high germination percentages in all species by 6 months (Table 3). Smoke was as effective, or more effective than the other germination treatments in this study of fresh seed, while in stored seed it was often less effective than GA_3 and KNO_3 combined (Wada and Reed, 2011). Smoke derived from burning cellulose increases germination of a wide range of plant species from Australia, North America, and South Africa, including *Rubus* (Brown and van Staden, 1997; DiTomaso et al., 2006; Merritt et al., 2006; Nuzzo et al., 1996). One active compound, butenolide, is stable at high temperatures, water-soluble, active at a wide range of concentrations and capable of inducing germination in a wide range of fire-following species (Flematti et al., 2004). Gardener et al. (2001) found that germination induced by either red light or smoke was reversed by far-red light, and that gibberellin biosynthesis inhibitors decreased smoke-treated seed germination, leading to the speculation that gibberellin synthesis was stimulated by the smoke. Germination of *Nicotiana* seed was induced by several forms of GA and inhibited by GA biosynthesis inhibitors paclobutrazol and abscisic acid, as well as terpenes from unburned litter (Schwachtjea and Baldwina, 2004). In the same study, ABA pools decreased sharply in smoke-treated seeds. Three active compounds in smoke, designated karrikins and considered a new class of phytohormones, stimulate germination in dormant Arabidopsis seed (Nelson et al., 2009). These compounds enhance expression of two GA biosynthesis genes during imbibition. It is not known which of the compounds in smoke gas are effective for *Rubus* seed germination. The acidity of the germination treatment solution may affect the response of seeds to GA, smoke and nitrates; buffered acidic (pH < 6) solutions produced a greater response than neutral solutions in *Emmenanthe penduliflora* (Keeley and Fotheringham, 1998). Liquid smoke solution at pH 3 was highly effective for red rice seed germination, but the response decreased significantly at pH 5–7; buffer controls did not germinate at any pH (Doherty and Cohn, 2000). Our germination treatment solutions (other than pH 6.5–7.0 DI water) were all at acidic pH and ranged from 2.8 (smoke gas), to 4.8 (GA_3) and 4.9 (GA_3 and KNO_3), similar to the low pH solutions effective in earlier studies. The use of acidified water may produce a different result for the DI water treatments of *Rubus*, as *Emmenanthe* seeds germinated at 60% with pH 5 water and not at all with pH 6 water (Keeley and Fotheringham, 1998). This may also relate to the less successful germination of seeds scarified with NaOCl since residual base may have neutralized the germination-treatment acidity.

Most of the freshly collected moist seed of 17 diverse *Rubus* species did not germinate, confirming that primary dormancy occurs in the genus *Rubus*. Germination requirements for the dry seed of six *Rubus* species varied, depending on seed-coat thickness and hardness; but optimal scarification time could be easily determined by TZ testing. NaOCl-scarified seed germinated more slowly and at significantly lower percentages for most species than H_2SO_4 scarified seed. An overall recommendation for *Rubus* seed would be scarification with H_2SO_4 and treatment during stratification with $GA_3 + KNO_3$ or smoke gas. This protocol was effective for all six species and produced 60–100% germination by 6–8 months.

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