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Optimized scarification protocols improve germination of diverse *Rubus* germplasm

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

Seed collections of the wild relatives of cultivated blackberry and raspberry (*Rubus* species) are maintained at the National Clonal Germplasm Repository, Corvallis, OR. Information on wild species germination requirements is rarely available, and germination may be poor or slow, making it difficult for scientists to use them for breeding improved cultivars. Eight diverse *Rubus* species in 6 of the 12 *Rubus* subgenera from seed stored at −20 °C for 1–23 years were studied. Seed weight, seed-coat thickness and hardness varied widely. Scarification with sulfuric acid (98% H_2SO_4) or sodium hypochlorite (14% NaOCl) was followed by germination treatments of deionized water (DI), smoke gas or a combination of gibberellic acid (2.03 mg/L GA_3) and potassium nitrate (34 mg/L KNO_3) during stratification. The commonly used scarification protocols were not effective for many species; but effective scarification exposure was established based on the amount of embryo damage seen with 2,3,5 triphenyl tetrazolium chloride (TZ) viability testing. H_2SO_4 scarification followed by a treatment with KNO_3 and GA_3 during stratification was highly effective for the most species. Two species in subgenus *Anoplobatus* had a hilar-end hole that allowed rapid germination of unscarified seed. Some species with extremely hard seed coats had little or no germination, and longer scarification times are suggested based on seed size, seed-coat thickness and hardness and viability testing.

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1. Introduction

Germplasm collections are valuable to plant breeders as a source of new genes or traits to improve cultivated crops. The National Clonal Germplasm Repository (NCGR) in Corvallis, OR preserves more than 1300 wild-collected *Rubus* seed accessions at −20 °C. These seeds are available to plant breeders for blackberry and raspberry crop improvement, however little is known about the germination requirements of most of the species. Often they do not germinate using the commonly used protocols found in the literature. In addition the seed collections were made by many plant collectors over many years and information on the seed maturity and initial viability is often not known. The genus *Rubus* is very diverse, includes over 750 species in 12 subgenera, and is found on all continents except Antarctica (Finn, 2008). The deep dormancy of many *Rubus* species seeds makes it difficult to use wild germplasm for blackberry and raspberry breeding programs (Clark et al., 2007; Daubney, 1996). Seed germination of the resulting hybrids is one of the largest challenges in blackberry breeding as there is large variation in germination based on genotype (Clark et al., 2007).

Rubus seed germination is constrained by both physical and physiological dormancy. An impermeable seed coat imposes mechanical resistance to growth and may contain chemical inhibitors; slow germination may also be due to the slow maturation of the dormant embryo (Zasada and Tappeiner, 2003). The endosperm and testa of dormant blackberry seeds contain growth inhibitors that degrade or are leached out during stratification under moist conditions, resulting in breaking dormancy and germination after about 5 months (Lasheen and Blackhurst, 1956). Polyphenols in the seed coat may also be linked with seed dormancy and longevity (Werker et al., 1979). Heit (1967b) noted that a hard seed coat or the combination of a hard seed coat and a dormant embryo will inhibit germination.

Sulfuric acid (98% H_2SO_4) scarification and a long stratification period, first warm and then cold, are required for satisfactory germination of hard-seeded species (Heit, 1967a). A scarification procedure using concentrated H_2SO_4 neutralized with calcium hypochlorite ($Ca(ClO)_2$) and calcium hydroxide ($Ca(OH)_2$) described by Jennings and Tulloch (1965) is a widely used protocol for *Rubus* seed germination. H_2SO_4 scarification of 30 min is recommended for the small seeded raspberries (*Rubus idaeus* L., *Rubus occidentalis* L.) and up to 3 h for the larger seeded

* 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 (B.M. Reed).
[1] Tel.: +1 541 738 4218.

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Table 1
Taxonomy, year collected, origin and identifying numbers of cold-stored seed of 8 *Rubus* species from the USDA-ARS National Clonal Germplasm Repository collections.

Rubus species	Subgenus	Year collected	Origin	Local number	PI number
R. arcticus subsp. stellata	*Cyclactis*	1996	U.S.A.	1881	606514
R. chamaemorus L.	*Chamaemorus*	2001	Russian Federation	2156	638202
R. leucodermis Douglas ex Torrey & A. Gray	*Idaeobatus*	1985	U.S.A.	651	553681
R. niveus Thunb.	*Idaeobatus*	2007	Brazil	2354	653286
R. odoratus L.	*Anoplobatus*	2003	U.S.A.	2214	638246
R. parviflorus Nutt.	*Anoplobatus*	2007	U.S.A.	719	553805
R. sanctus Schreber	*Rubus*	1989	Pakistan	1053	553879
R. urticifolius Poir.	*Lampobatus*	1990	Ecuador	1290	548933

blackberries (*Rubus* spp.) (Hummer and Peacock, 1994). Scarification with sodium hypochlorite (15% NaOCl) is recommended for sand blackberry seeds (*Rubus cuneifolius* Pursh) (Campbell et al., 1988). Due to the great diversity of *Rubus* species, a single standard protocol is unlikely to be useful for germinating seed of wild germplasm. This genus is especially difficult due to the diversity in seed-coat thickness and structure present in the genus (Daubney, 1996). In standard protocols *Rubus* seed are usually stratified and germinated in sand, sphagnum or soil in a greenhouse (Clark et al., 2007; Hummer and Peacock, 1994), but can also be germinated *in vitro* (Mian et al., 1995) or on blotters (Wada, 2009). After effective scarification is completed, additional treatments can also impact the rate and amount of germination. Some *Rubus* species germinate well with only water added to the medium, while others have increased germination when smoke from cellulose fires or gibberellic acid and potassium nitrate are added during stratification and germination (Wada, 2009; Wada and Reed, submitted for publication).

The objective of this study was to determine the germination response of wild-collected cold-stored *Rubus* germplasm held in the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), National Clonal Germplasm Repository (NCGR), Corvallis, OR. Seed attributes of eight species were delineated and commonly used H_2SO_4 and NaOCl scarification treatments were tested for reducing physical dormancy. We also tested the effects of germination treatments for overcoming physiological dormancy and improving germination.

2. Materials and methods

2.1. Plant materials

The NCGR *Rubus* seed-collection database was screened and 8 species belonging to six subgenera were selected from the germplasm collections stored for 1–23 years at −20 °C (Table 1). Accessions were selected with seed of variable sizes that had 30–95% TZ viability and a large amount of seed, so that seeds of the accession were still available for distribution. *Rubus leucodermis*, *Rubus niveus*, *Rubus odoratus*, and *Rubus parviflorus* were used for four germination treatments (750 seed each) and four species with fewer available seed (*Rubus arcticus*, *R. chamaemorus*, *Rubus sanctus*, and *Rubus urticifolius*) were used for two germination treatments (450 seed each).

2.2. Seed-coat thickness and hardness

Seed-coat thickness was measured for ten seed of each species. A Nikon SMZ 1000 stereomicroscopic Zoom Microscope (Nikon Instruments, Tokyo, Japan) was employed and measured with Infinity image capture and analysis software (Luminera Corporation, Ottawa, Canada). Measurements were taken in the center of the cut seed equidistant from the micropylar region and the hilar end. Hardness ratings of 1–5 were assigned after seed samples were soaked in deionized (DI) water for 2 days and hand sectioned with a scalpel. The subjective hardness grading was 1 soft, 2 slightly hard, 3 hard, 4 very hard (difficult to cut), and 5 extremely hard (very difficult to cut). This scale was developed based on sampling of a range of species.

2.3. Microscopy

Light microscope (LM) images were taken using a Nikon SMZ 1000 stereomicroscopic Zoom Microscope (Nikon Instruments, Tokyo, Japan). Scanning electron microscopy (SEM) images were taken using an AmRay3300 (Amray, Bedford, MA)

2.4. Scarification procedures

Due to the lack of specific guidelines for diverse species, scarification times were estimated for each accession based on the subgenus and seed weight. (1) Control–no treatment. (2) concentrated sulfuric acid (98+% H_2SO_4) in an ice bath for 30 min–3 h, then rinsed in running water for 1 h. Then seeds were soaked for 5 min in a solution of 3 g/L $Ca(ClO)_2$ completely dissolved in water with 3 g/L $Ca(OH)_2$ and rinsed for 5 min in running water. Seeds were rubbed against a strainer before stratification to remove the carbonized portions of testa. (3) Sodium hypochlorite (14% NaOCl) at room temperature for 2–8 h and rinsed for 1 h in running water. Scarified seeds were placed on germination blotters for stratification and germination.

2.5. Secondary scarification test

After poor response to the initial germination tests with 1 h scarification, seeds of *R. chamaemorus*, one of the largest, thickest and hardest seeds, were H_2SO_4 scarified for 0, 3, 3.5, 4, 4.5, 5.0, and 5.5 h to determine if TZ testing could be used to optimize scarification duration. TZ tests were performed on the scarified seed after each time point to determine viability of the embryo and observe any damage resulting from the scarification process.

2.6. Germination treatments

Germination blotters were placed in 20 mm × 100 mm petri dishes and fully soaked with DI water. Scarified seed were placed on the blotters and 5 mL the germination treatment solution was added. Control seeds were not scarified and were germinated with only DI water. NaOCl scarified seeds were treated with only DI water. H_2SO_4 scarified seed treatments were: (1) DI water, (2) gibberellic acid (2.03 mg/L GA_3) plus potassium nitrate (34 mg/L KNO_3), or (3) smoke gas solution (Super Smoke Plus, Claremont, South Africa) prepared by soaking 5 pieces of smoke infused paper in 250 mL DI water for 24 h. All treatments were replicated with 50 seeds per 9 cm plastic Petri dish and 3 dishes per accession (n = 150).