Seed-coat anatomy and proanthocyanidins contribute to the dormancy of *Rubus* seed

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*Rubus* seed has a deep double dormancy that restricts germination due to seed coat structure and chemical composition. Improved germination of diverse *Rubus* species required for breeding improved blackberry and raspberry cultivars is partly dependent on the seed coat structure. This study evaluated the seed coat structure of three species with thin (*R. hoffmeisterianus* Kunth & C. D. Bouché), medium (*R. occidentalis* L) and thick (*R. caesius* L) seed coats. The three species exhibited distinctive seed-coat cell composition. The very thin testa (0.086 mm) of *R. hoffmeisterianus* had little exotesta (surface) reticulation; with the meso- and endotesta composed of sclereids of homogenous shape and size. *R. occidentalis* had a thick testa (0.175 mm) and a highly reticulate exotesta; the meso- and endotesta were composed of several diverse types of sclereids. *R. caesius* had the thickest seed coat (0.185 mm) but only moderate exotesta reticulation; the meso- and endotesta were composed of large, irregular, loosely arranged sclereids. *R. occidentalis*, a medium size seed, was the most heavily lignified with seed-coat thickness similar to *R. caesius*, the largest seed. Proanthocyanidins (PAs) from dry seed of six *Rubus* species were extracted and quantified by high performance liquid chromatography. *R. hoffmeisterianus*, a thin only slightly hard seed, had half the PA (0.45 μg/seed) of *R. occidentalis* with a thick, extremely-hard seed coat and diverse sclereids (1.07 μg/seed). PA content and sclereid composition both appear contribute to seed coat hardness and resulting seed dormancy. The effectiveness of sulfuric acid for *Rubus* seed scarification is likely due to degradation of PAs in the testa.

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1. Introduction

Commercially produced *Rubus*, blackberries and raspberries, are vegetatively propagated; however plant breeders use seeds of wild species to introduce new traits and produce improved cultivars. Improving scarification protocols and breaking the deep double dormancy of these seeds requires better knowledge of seed coat structure, thickness and chemical composition.

The seed coat (testa) plays an important role in the plant life cycle by controlling the development of the embryo and determining seed dormancy and germination (Moise et al., 2005). Consequently, seeds with thin or permeable seed coats (or pericarp) lose viability more quickly. The mature seed coat is usually divided into three regions. An exotesta or outer seed-coat layer(s), a mesotesta or middle seed-coat layer(s), and an endotesta or inner seed-coat layer(s); the sclerified tissue of the endocarp can have different origins (Cutler et al., 2007). Thick, sclerified seed coats serve in mechanical protection against physical, chemical and biological damage. Sclerenchyma cells are thick-walled dead cells, variable in size and shape that give rigidity to the plant (Esau, 1977; Metcalfe and Chalk, 1979). Because of their highly variable size and unique shape, sclereids in a plant are often characteristic of the species and could have taxonomic value (Barua and Dutta, 1959). Sclereid types include short, isodiametric brachysclereids (stone cells), elongated rod-like macrosclereids, bone-shaped, columnar osteosclereids, star shaped astrolesclereids, long, slender filiform sclereids, and branched trichosclereids (Blotch, 1946; Nicolson, 1960).

Botanically the fruit of the genus *Rubus* is a drupecetum; an aggregate fruit containing a number of fleshy drupelets attached to a single receptacle. Each drupelet consists of one pyrene; that includes the seed and its surrounding sclerenchymatous endocarp, a fleshy mesocarp and a thin exocarp (Ny bom, 1980; Tomlik-Wyremblewska et al., 2010). All *Rubus* seeds are enclosed by hard sclerenchymatous endocarp tissues that impede water imbibition and restrict the oxygen needed for germination (Reeve, 1954; Rose, 1919). Corner (1976) reported that the genus *Rubus* seed is unitegmic with a connate integument and cited Topham (1970)
on the seed-coat morphology of two Rubus species, R. fruticosus L., agg. and R. ideus L.; "integument is six cells thick, the persistent seed coat of thin-walled cells, the middle layer is crushed, and the endosperm is six cells thick".

A recent scanning electron microscopy (SEM) study of eight Rubus species seed, including coat histology and surfaces, indicated that the endocarp structure of all the European species studied was generally similar and not substantially different from R. strigosus as described by Reeve (Reeve, 1954; Tomlik-Wyremblewska et al., 2010). Great variation in the seed coats of a more diverse group of Rubus species and distinct differences in seed-coat surface structure, seed-coat thickness and seed size were noted in our earlier studies (Wada and Reed, 2008, 2010). Sulfuric-acid scarification ranging from 0.5 h to over 3 h was required to degrade the seed coats before germination could occur (Wada, 2009). Further improvements in Rubus seed scarification requirements were noted in additional studies and were related to seed coat thickness and hardness (Wada and Reed, 2011, submitted for publication).

The seed coats of Rosaceae, to which Rubus belongs, are impermeable to gases, and hydrogen cyanide (HCN) formed after water uptake builds up to inhibitory concentrations and remains present until the seed coat and endosperm pellicle are damaged or partially removed (Hess, 1975). The seed coat also synthesizes a wide range of compounds that may serve the plant in defense and also in control of seed development. Marbach and Mayer (1974) found that the permeability of seed coats to water is related to the content of phenolic compounds in the seed coat, and to their level of oxidation. The oxidation of phenolics may cause structural changes in seed coats. Phenolic compounds are found in the Rubus ideus L. testa and are implicated in reducing oxygen availability to the embryo (Nesme, 1979, 1985), as well as being inhibitors of plant growth (Kefeli and Kadyrov, 1971) and seed germination (Stom, 1982). Flavonoids are also responsible for strengthening seed-coat imposed dormancy and longevity (Debeaujon et al., 2000). Proanthocyanidins (PAs), condensed tannins, are colorless polymeric flavonoid compounds composed of flavan-3-ol subunits. PAs reduce water uptake and imbibition damage by solute leakage and possibly act as a mechanical barrier through protein binding (Bell et al., 1992; Debeaujon et al., 2001; Kantar et al., 1996). PAs also have an indirect preventive role in seed germination by testa hardening that hinders radicle protrusion from the integuments. In Arabidopsis, flavonoids accumulate in the mature testa in the endothelium layer (Albert et al., 1997; Devic et al., 1999) and in the crushed parenchymal layers adjacent the endothelium (Debeaujon et al., 2001). The large differences in dormancy and germination in mutant Arabidopsis seeds may be due to the seed-coat structure or phenolic content of the seed coat. Study of a large collection of mutants with testa defects indicates that these pigments and structural components affect germination of Arabidopsis seeds (Debeaujon et al., 2000). This study suggests that testa permeability and thickness are changed by the chemical compounds and structural alterations in the mutants, and that this affects germination and dormancy.

The objectives of this study were to investigate the structure of the Rubus seed coat, especially the layers and tissues relating to the hard texture; to define the unique seed-coat characteristics of three species representing thin, medium and thick seed coats; and to determine the PA content six species seed and relate it to seed-coat hardness and germination.

2. Materials and methods

2.1. Plant materials

Fully mature Rubus fruit were collected from plants in the USDA-ARS small fruit breeding program research field at the Oregon State University, Lewis Brown Farm (Corvallis, OR). Fruit was soaked in few drops of pectinase (Novozymes, Fresno, CA), mashed, and held for 24 h at room temperature. After treatment with pectinase, the solution was blended for one min in a blender with plastic-covered blades, then poured through a strainer and rinsed in tap water as the seeds were rubbed against the mesh of the strainer. The cleaned seed was scraped off the strainer and spread onto labeled paper towels, dried 5 days at room temperature, and then dried in a desiccator for 24 h using Drierite (W.A. Hammond Drierite Company, Xenia, OH).

2.2. Seed weight

Dry seed for each species was weighed in 3 lots of 100 seeds each. Three seed sizes were studied for each of two subgenera: for subg. Idaeobatus, small – R. hoffmeisterianus Kunth & C. D. Bouché (0.04 g/100 seed), medium – R. coreanus Miq. (0.10 g), and large – R. occidentalis (0.19 g). In subg. Rubus, small – R. ussiris Cham. & Schltdl. (0.12 g), medium – R. georgicus Focke (0.26 g), and large – R. caesius L. (0.37 g).

2.3. Seed-coat thickness and hardness

Seed-coat thickness was measured for 10 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). The seed coat thickness was measured before and after scarification treatments. Scarification treatment was H2SO4 (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 CaCl2 (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. 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 seed samples 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 (Wada and Reed, 2011).

2.4. Seed-coat anatomy

Dried seeds of R. hoffmeisterianus, R. occidentalis, and R. caesius were soaked in distilled water for 48 h before fixation. Seed samples were fixed in FAA (formaldehyde 38%, 10 mL; ethyl alcohol (ETOH) 95%, 50 mL; glacial acetic acid, 5 mL; and distilled water, 35 mL/100 mL solution) for 48 h (Baker, 1966). After fixation, the samples were dehydrated through the ethanol series; 50% ETOH for 2 days; 70% ETOH for 2 days; 95% ETOH for 3 days; all the samples in a dehydration procedure were kept at 4 °C. For histological infiltration, a glycolmethacrylate embedding kit (Technoviz 7100, Kulzer, Germany) was used following standard procedures (2:1, 1:1 and 1:2 ratios of 95% ETOH to the cold-curing resin solution). Two seeds were embedded in each resin block (1 × 0.5 × 0.5 mm) for sectioning. A Spencer rotary microtome (Spencer Lense Co., NY) was employed for the longitudinal sectioning (5 μm) of seed 8 seeds for each genotype. Sections were stained with toluidine blue O (0.05% equivalent) for 1 min and washed in running water, then oven dried at 56 °C for 2 h and mounted with synthetic mounting medium (Polymount, PA).

2.5. Microscopy

A Nikon compound microscope (Eclipse 55i, Japan), Infinity camera and imaging capture software (Lumenera, Canada) were
Table 1
Seed-coat thickness and number of seeds per gram of six Rubus species.

<table>
<thead>
<tr>
<th>Rubus species</th>
<th>Subgenus</th>
<th>Seed hardness* (1–5)</th>
<th>Seed-coat thickness* (mm)</th>
<th>Seed number (per gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. caesius</td>
<td>Rubus</td>
<td>3</td>
<td>0.185 ± 0.006</td>
<td>280</td>
</tr>
<tr>
<td>R. coreanus</td>
<td>Idaeobatus</td>
<td>2</td>
<td>0.135 ± 0.004</td>
<td>884</td>
</tr>
<tr>
<td>R. georigorius</td>
<td>Rubus</td>
<td>5</td>
<td>0.174 ± 0.004</td>
<td>413</td>
</tr>
<tr>
<td>R. hoffmeisterianus</td>
<td>Idaeobatus</td>
<td>2</td>
<td>0.086 ± 0.003</td>
<td>1396</td>
</tr>
<tr>
<td>R. occidentalis</td>
<td>Idaeobatus</td>
<td>5</td>
<td>0.175 ± 0.004</td>
<td>672</td>
</tr>
<tr>
<td>R. ursinus</td>
<td>Rubus</td>
<td>4</td>
<td>0.145 ± 0.004</td>
<td>603</td>
</tr>
</tbody>
</table>

* Hardness rating of 1; soft; 2; slightly hard; 3, hard; 4, very hard and 5, extremely hard.
* Mean of 10 seeds ± SE (mm).

Fig. 1. Seed surface morphology of three Rubus species: shape, relative size, color and reticulation on the seed coat as revealed by low power (20×) microscopy. (A) Rubus hoffmeisterianus (1.0 mm length × 0.5 mm width × 0.4 mm thickness). (B) R. caesius (2.0 mm L × 1.0 mm W × 0.8 mm T). (C) R. occidentalis (1.5 mm L × 0.8 mm W × 0.6 mm T).

2.6. Proanthocyanidin content

2.6.1. HPLC analysis

Proanthocyanidins (PAs) from seeds of the six Rubus species were extracted and quantified by high performance liquid chromatography (HPLC) after acid-catalyzed degradation in the presence of excess phloroglucinol (phloroglucinolysis) using a previously described method (Kennedy and Jones, 2001). Extraction and chromatography solvents (acetone, methanol and ascorbic acid) were HPLC grade, purchased from VWR (Radnor, PA), and phloroglucinol from Sigma–Aldrich (St. Louis, MO).

2.6.2. Instrumentation

An Agilent 1100 HPLC (Palo Alto, CA) consisting of a vacuum degasser, autosampler, quaternary pump, diode array detector, and column heater was used. A computer workstation with Chemstation software was used for chromatographic analysis. An Innova model 2300 platform shaker from New Brunswick Scientific (Edison, NJ), Labconco centrifrip concentrator (Kansas City, MO), and Büchi model R-205 rotary evaporator (New Castle, DE) were used for the extraction and concentration of PAs.

2.6.3. PA determination

One gram of each species seed was counted and the seed numbers per gram recorded (Table 1). One gram of seed was ground to a powder using a mortar and pestle under liquid nitrogen; 15 mL of acetone: distilled-deionized (DI) water (2:1) were added to the powder, covered with aluminum foil, and placed at ambient temperature (ca. 20 °C) for 22 h on a platform shaker at 150 rpm. After extraction, samples were centrifuged to remove gross particulates, filtered, the acetone was removed by rotary evaporation (35 °C), and the residue was brought to 25 mL with DI water, and stored at −80 °C until analyzed. For analysis, 5 mL of each aqueous extract was evaporated to dryness in a centrifugal concentrator, and then reconstituted in 2 mL methanol. Phloroglucinolysis reagent was prepared as described previously (Kennedy and Jones, 2001), although the concentrations of phloroglucinol, ascorbic acid, and HCl were doubled to compensate for dilution. For reaction, 1 mL methanolic extract was combined with 1 mL reagent and reacted at 50 °C for 20 min. To stop the reaction, one volume of this solution was then combined with five volumes of 40 mM aqueous sodium acetate.

The reversed-phase HPLC method used to analyze the proanthocyanidins following phloroglucinolysis (Taylor et al., 2003) consisted of two Chromolith RP-18e (100 × 4.6 mm) columns connected in series and protected by a guard column containing the same material, all purchased from EM Science (Gibbstown, NJ, USA). The method utilized a binary gradient with water containing 1% v/v aqueous acetic acid (mobile phase A) and acetonitrile containing 1% v/v acetic acid (mobile phase B). Eluting peaks were monitored at 280 nm, and the elution conditions were as follows: column temperature 30 °C; 3.0 mL/min; 3% B for 4 min, a linear gradient from 3% to 18% B in 10 min, and 80% B for 2 min. The column was washed with 3% B for 2 min before the next injection. Proanthocyanidin
concentration, and composition were determined as previously described (Kennedy and Jones, 2001).

2.7. Data analysis

Data were analyzed for standard error (SE) using SAS (2008).

3. Results

3.1. Seed-coat anatomy

Variation was apparent in seed size as well as seed-coat thickness and hardness of the six species studied (Table 1). The thin seed coat of the small seeded R. hoffmeisterianus had low surface sculpturing (Fig. 1A); R. caesius seed was large and the seed coat was very thick with moderate sculpturing (Fig. 1B) while R. occidentalis seed was moderate sized with a thick seed coat with deep surface relief (Fig. 1C). A schematic structure of a Rubus seed coat is shown in Fig. 2. Each species exhibited three very distinct layers of heavily lignified sclerenchymatous cells in the seed coat (Fig. 3). R. hoffmeisterianus had two layers of exotesta cells, mostly stacked trachearies and vascular bundles, and sometimes large, empty sclerenchymatous cells (Fig. 3A). The mesotesta included regularly stacked macrosclereids, six or seven layers of 40–50 μm long, slender cells, closely attached to an adjacent stack of macrosclereids. Some pits were observed in the macrosclereids and druses (spherical aggregates of prismatic crystals) were seen in the vacuoles. The endotesta consisted of uniformly globular, isodiamic, macrosclereid layers arranged perpendicular to the mesotesta. R. hoffmeisterianus had the thinnest testa of the three species and the least exotesta reticulation with the meso- and endotesta composed of homogeneous shape and size sclereids.

R. caesius had two to five layers of exotesta cells, mostly composed of large, sclerenchymatous holes, trachearies and vascular bundles (Fig. 3B). The outer mesotesta consisted of irregular-shaped macrosclereids. About 12 layers of short, round or polygonal cells of 5–10 μm to more than 60 μm long filiform cells were loosely intermingled within this layer. Some pits were observed in the macrosclerid cells and druses in the vacuoles. The endotesta consisted of uniformly globular, isodiamic, macrosclereid layers that were arranged perpendicular to the mesotesta. R. caesius had moderate to little exotesta reticulation and was composed of somewhat irregular cells, but the largest sclereid cells were loosely arranged in the meso- and endotesta.

R. occidentalis had two to five layers of exotesta cells composed of stacked trachearies and vascular bundles with more large, empty sclerenchyma holes than R. hoffmeisterianus (Fig. 3C). The mesotesta consisted of more than 12 layers of irregularly intermingled macrosclereids, and osteosclereids of mixed sizes. Some sclereids were less than 5 μm while others were 10–20 μm with many different shapes and sizes. Sometimes this layer also had 10–20 thin filiform sclereids stacked in layers. Pits and druses were often observed in the macrosclereids. The endotesta consisted of globular, triangular or polygonal shaped sclereids and
the macroserdied layers were arranged perpendicular to the mesotesta. The endotesta of *R. occidentalis* was further divided into two layers of differentially blue-stained cells and an inner layer with intensive phenolics possibly remaining from the embro-
onic tissues. In the micropylar region of *R. occidentalis*, plug-like sclerenchyma tissue was observed (Fig. 3D). The inner micropyl-
lar region had numerous green-dyed cells indicating phenolics (stained green by tolidine blue O) (Baker, 1966). *R. occidentalis* had the thickest and most reticulate exotesta of the three species (Fig. 1) and the meso- and endotesta were composed of various shaped sclereids; macro-, oste- and filiform-sclereids as well as densely packed short, polygonal and globular sclereids.

These three *Rubus* species showed unique cell composition, but all possessed three clearly distinguishable testa sections. Those sections included a thickened outer epidermis (exotesta), under which there were two perpendicular multi-cell layers of macrosclere-
renchyma cells, the mesotesta and endotesta, lying at right angles to each other (Fig. 3A–C). An exception was observed at the micropy-
lar region (Fig. 3D) where the macrosclerenchyma cell layers were in one direction, blocking the microplre. The seed-coat anatomy of the three species showed distinct differences. The thin mostly flat exotesta (Fig. 1A) of *R. hoffmeisterianus* was composed mostly of two cell layers (Fig. 3A) while the thick slightly textured exotesta (Fig. 1B) of *R. caesius* varied from 2 to 5 cells thick (Fig. 3B). The structure of the outer-most exotesta layer that formed the deep seed-coat reticulations (Fig. 1C) was multiple-cells thick on the very-thick seed coat of *R. occidentalis* (Fig. 3C). Similar seed-coat thickness was found in the medium sized *R. occidentalis* seed and the large *R. caesius* seed, but the *R. occidentalis* seed coat had the most heavily lignified and intricate sclereid composition. Seed-coat reticulations of the three Rubus species revealed by low power light microscopy (Fig. 1) are consistent with the histological sections (Fig. 3).

3.2. PA analysis

Analysis of the seed coats indicated proanthocyanidin peaks in all of the six species (Table 2, chromatograms not shown). The only peak of significance was consistent with a catechin extension subunit (Fig. 4). The two species with thin, slightly-hard seed coats, *(R. coreanus, R. hoffmeisterianus)* had <0.5 μg PA (catechin equiva-
 lent)/seed, while the four harder-seeded species had from 0.9 to 2.8 μg PA/seed (Table 2).

4. Discussion

The seed coats of the *Rubus* species studied had unique internal and surface anatomy that followed the basic plan of the Rosaceae, but also was specific to each species. Cutler et al. (2007) emphasized that comparative studies of seeds can yield taxonomic characters of significance. The taxonomic importance of *Rubus* seed surface reticulations was noted for a range of species and cultivars (Tomlik-Wyremblewska et al., 2010; Wada and Reed, 2008, 2010).

Observation of cross sections of the seed coat under 200× magnification provided clear comparisons of the relative seed-
coat thickness and structure of the three species. The seed of these three *Rubus* species showed unique cell composition, but all possessed a thickened outer epidermis (exotesta), and two per-
dependicular multi-cell layers of macrosclerenchyma cells lying at right angles to each other (Figs. 2 and 3A–C). This perpendicular or a diagonal orientation was also seen in European *Rubus* species (Tomlik-Wyremblewska et al., 2010). The alternating orientation provides strengthening of the seed coat; the more diverse the cell types, the more strengthening these layers provide. This pattern is very apparent in the differences seen between the thin testa of *R. hoffmeisterianus* and the thick, hard, multilayered testa of *R. occidentalis* (Fig. 3A and C).

Endocarp sclereids in *Rubus* are similar to those in other drup-
aceous fruits. The histology of three species in this study (Fig. 3) showed common features in the testa similar to Reeve’s findings from *R. strigosus* (Reeve, 1954). The mature sclereid walls were lignified and had many tiny pit canals and druses were frequently seen near the vascular traces. However, in our study we did not observe sclereids over 500 μm long as noted by Reeve. We used only fully mature seeds for this investigation, so the integument structure was crushed and not observable. Many druses were noted in the macrosclereids of the exotesta; pits and cell lumina were visible in sclereids of the meso- and endotesta (Fig. 3). European species had endotesta layers three to six cells thick, consisting of long tightly packed sclereids with small lumens and numerous pits (Tomlik-Wyremblewska et al., 2010).

In a prior *Rubus* seed germination study, these three species exhibited unique germination behaviors (Wada, 2009; Wada and Reed, submitted for publication). *R. hoffmeisterianus* germinated well (98%) after a 0.5 h sulfuric-acid scarification with no require-
ment for cold stratification. This is clearly consistent with the thin seed coat noted in this study (Fig. 3A). *R. caesius* was unique in that even with a very thick seed coat (Fig. 3B) all seed, even unscarified seed, germinated well (89.5%) by 12 months. The large, loosely-packed sclerenchymatous meso- and endotesta of *R. caesius* may allow more water penetration, resulting in the leaching of germina-
tion inhibitors known to occur in the seeds. *R. occidentalis* was scarified in sulfuric acid for 0.5 h with little (<10%) germination, then for 2 h with low germination (20%) and for 3 h with good germination (>70%) (Wada and Reed, submitted for publication). This long scarification time is very consistent with the hard, thick, mul-
tilayered seed coat and the scleried plug and phenolics noted in the micropyple region (Fig. 3C and D). Published germination data on *R. occidentalis* seeds with 0.5 h sulfuric-acid scarification was based on
treatments for other species in the subg. *Idaeobatus* and resulted in low or no germination (Clark and Moore, 1993; Zasada and Tappeiner, 2003). The seed-coat structure of *R. occidentalis*, with thick layers of sclereids clearly indicates a strong, extremely-hard seed-coat and likely resistance to short scarification procedures resulting in low germination.

The methods for extracting, purifying and determining the subunit composition of proanthocyanidins were originally developed and used for grape tissues, but have also been applied to other plant tissues and products (Cortell et al., 2005; Koerner et al., 2009; Taylor et al., 2003). Compared to grape seeds, significantly lower amounts of PAs were detected from the *Rubus* seed. Seed tannins decrease as the fruit matures; grape seed polyphenols decrease dramatically during ripening with a 90% reduction in flavan-3-ol monomers and a 60% decrease in procyanidins (Kennedy et al., 2000). PAs isolated from fully-ripened plant tissues have lower conversion yields as much as 50–80% by mass (Kennedy et al., 2002). Proanthocyanidins are very susceptible to oxidative degradation. As seed matures, PAs oxidize to form anthocyanidins, resulting in species specific seed color and characteristic seed-coat pigmentation. In Arabidopsis, immature seeds are transparent; PAs are found in the endothelium of the seed coat where they give a brown color at maturity after oxidation (Debeaujon et al., 2001). Specifically PA was located in the cells of the inner integument of the seed, micropyle and in the chalaza (Debeaujon et al., 2003). In *Rubus* seeds we found evidence of phenolics on the interior of the testa and in the micropylar region of *R. occidentalis* (Fig. 3C and D), a similar position to that of the endothelium of Arabidopsis.

Arabidopsis mutants with seed flavonoid defects are pale yellow to pale brown depending on the accumulated intermediate flavonoids (Debeaujon et al., 2000) and usually have reduced dormancy and a high germination rate (Debeaujon et al., 2001). These pigmentation mutants were permeable to tetrazolium dyes while the wild type excluded the dye and appeared to have reduced permeability to oxygen and water (Debeaujon et al., 2000). Other mutants have defects in the number or type of cell layers or defects in mucilage production and testa surface structure that increase germination but decrease longevity (Debeaujon et al., 2000). Flavonoids are responsible for strengthening seed-coat imposed dormancy and longevity (Debeaujon et al., 2000). PAs may act as a mechanical barrier by binding the proteins that create harder seed coats (Debeaujon et al., 2001). *R. hoffmeisterianus* and *R. coreanus* both had light colored seed, low concentrations of PA (Table 2) and were also the easiest to germinate of the six species tested in this study, requiring only a 0.5 h sulfuric-acid scarification (Wada and Reed, submitted for publication). The testa structure of *R. hoffmeisterianus* was also the least robust of the three species examined and had no indication of phenolics in the histological sections (Fig. 3A). The darker, harder seeds required 3 h sulfuric-acid scarification for good germination (Wada and Reed, submitted for publication) and had correspondingly higher PA concentrations (Table 2).

One role of PA is to limit the exchange of gases and fluids between the environments and the seed, protecting and prolonging the life of the dormant embryo, as well as preventing germination until conditions are favorable (Haughn and Chaudhury, 2005). PAs are susceptible to oxidative degradation and can be degraded by H2SO4. In green tea, the most effective solvent pretreatment for releasing phenolic compounds is sulfuric acid (Kim et al., 2010). No similar degradative effect on PA is known for other scarification agents. It seems likely that the more proanthocyanidin present in the seed coat, the longer the duration of concentrated sulfuric-acid scarification required for breaking seed dormancy. Commonly used sulfuric-acid scarification recommendations are 0.5 h for raspberries and 3 h for the more thick-coated blackberries (Moore et al., 1974). These recommendations were accurate for seeds with thin and only slightly-hard seed coats that were low in PA like *R. hoffmeisterianus* and *R. coreanus*, but were inadequate for breaking dormancy and allowing germination of *R. occidentalis* (>3 h) or other seeds with thick, hard to extremely-hard seed coats (Wada and Reed, 2011, submitted for publication).

### 5. Conclusions

These *Rubus* species exhibited testa structure generally typical for the genus; however anatomical differences and unique composition of the cells, cell shapes, numbers and structure of layers, were noted among the species. These differences relate to the protective functions and germination inhibiting qualities of the seed coat. The structure and thickness of the testa layers of these species appear to explain variations noted in seed response to sulfuric-acid scarification and subsequent germination. PA analysis indicated a wide range of concentrations of PA in the *Rubus* seed, with two to five times as much in the harder, thicker seeds compared to the softer, thinner seeds. These PAs are likely involved in both physical and physiological dormancy mechanisms of *Rubus* species.

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