

## AN ABSTRACT OF THE THESIS OF

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Abstract approved: \_\_\_\_\_

Kim E. Hummer

*Rubus* spp. seeds have a thick protective endocarp and testa (seedcoat) that can restrict germination even under favorable conditions. The effect of pregermination treatments were studied in *Rubus chamaemorus* L., *R. eustephanos* Focke ex Diels, *R. idaeus* 'Amber' L., *R. leucodermis* Douglas ex Torrey & A. Gray, *R. multibracteatus* A. Leveille & Vaniot, *R. parviflorus* Nutt., and *R. ursinus* Cham. & Schldl.. Scarifying agents such as, sulfuric acid ( $H_2SO_4$ ), sodium hypochlorite ( $NaOCl$ ), calcium hypochlorite ( $CaO_2Cl_2$ ), driselase, liquid nitrogen ( $LN_2$ ), and others, were used as pregermination treatments. Sulfuric acid significantly increased percent germination in most of the *Rubus* spp. tested. Sodium hypochlorite above 2.6% injured seedlings of several species whereas lower concentrations were effective for scarification without seedling injury. Driselase did not significantly increase percent germination. This may have been due to little or no scarification of the endocarp at the 1-3% concentration range used. Liquid nitrogen did not significantly increase percent germination, however, it did not reduce overall percent germination as compared to the control. No loss of viability of *Rubus* seeds was found even with repeated immersions in  $LN_2$ . Cryogenic storage may thus be an effective way for preservation of *Rubus* seeds. The recommended

pregermination treatments for most of the *Rubus* species studied, ranked in order of effectiveness, were concentrated  $\text{H}_2\text{SO}_4$  followed by a 1%  $\text{CaO}_2\text{Cl}_2$  solution with an excess of  $\text{Ca}(\text{OH})_2$ , concentrated  $\text{H}_2\text{SO}_4$ , 0.5%  $\text{NaOCl}$  or 2.6%  $\text{NaOCl}$  if the particular *Rubus* species does not show a sensitivity to high concentrations of  $\text{NaOCl}$ , and no treatment.

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*Rubus* spp. Seed Germination and Morphology

by

Derek N. Peacock

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APPROVED:

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Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Derek N. Peacock, Author

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## Contribution of Authors

Mr. Jay Goodwin was involved in the design of the driselase experiment. The driselase scarification was performed in the laboratory of Dr. Pat Breen, who also assisted in the interpretation of data for most of this document. Dr. Kim Hummer was involved in the analysis and writing of each manuscript.

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## Preface

Several of the terms in this document may be ambiguous, and need clarification, they are:

**Endogenous dormancy**<sup>1</sup>. Dormancy imposed on a seed due to inherent properties which require physiological changes to relieve. Under *endogenous* control.

**Exogenous dormancy**<sup>1</sup>: A type of dormancy generally related to physical propitious of the seed coat in which an essential germination component (e.g. water, light, or temperature) is restricted or the enlarging embryo is restrained. Under *exogenous* control.

**Moist Prechill**: (please see stratification).

**Pretreatment**<sup>2</sup>: To subject to a chemical or physical process or application (before germination is evaluated).

**Pyrene**: A small hard nutlet (seed) and its surrounding stony endocarp.

**Scarification**<sup>1</sup>. The process of mechanically or chemically abrading a seed coat to make it more permeable to water.

**Stratification**<sup>1</sup>. The practice of exposing imbibed seeds to cool (3-10C) or warm temperature conditions for a few days (to months) prior to germination in order to break dormancy.

**Treatment**: (please see pretreatment).

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<sup>1</sup> Taken from, McDonald M.B., and L.O. Copeland. 1985. Principles of Seed Science and Technology. Burgess Publishing Company. pp. 321.

<sup>2</sup> Taken from, Webster's II, New Riverside University Dictionary. 1984. Riverside Publishing Company.

## *Rubus* spp. Seed Germination and Morphology

### 1. Introduction

Raspberries and blackberries are classified in the *Rubus* genus of the Rosaceae family. *Rubus* fruit have a high commercial value, not only in the fresh market, but especially by processing companies for the making of jams, juices, and pies. To carry out genetic investigations and breeding practices, breeding programs find it necessary to propagate this small fruit crop by means of seeds, although cultivar clones are maintained through vegetative propagation. Genetic recombination through sexual reproduction provides a way for acquiring desirable combinations in small fruit breeding. Seeds contain new combinations of genes for potential superior cultivars.

#### 1.1 Problem Definition.

Breeders, trying to improve upon the qualities of *Rubus*, are often hampered by the poor and irregular germination of *Rubus* seeds. Dale and Jarvis (1983) believe breeding programs would become more efficient if seed germination was more reliable and rapid. The germination of greater numbers of vigorous seedlings could greatly accelerate the rate of genetic advancement in *Rubus* breeding, however, germination studies are often time consuming and difficult: Attempts to accelerate and improve germination pretreatments have not been very successful. Reported pretreatments include scarification with sulfuric acid, sodium hypochlorite, and other chemical agents, mechanical removal of the seed coat, and stratification treatments of up to 9 months long. Seed testing organizations have not established any official germination protocols.



## 1.2 Statement of Purpose

The purpose of this study was to enhance our understanding of *Rubus* germination, and assist the development of new and improved pregermination treatments to increase percent germination. An array of scarifying agents, including sulfuric acid ( $\text{H}_2\text{SO}_4$ ), calcium hypochlorite ( $\text{CaO}_2\text{Cl}_2$ ), sodium hypochlorite ( $\text{NaOCl}$ ), driselase, liquid nitrogen ( $\text{LN}_2$ ), and others, were used to determine the optimum germination procedure for a range of *Rubus* spp.

I explored the use of the macerating enzyme, driselase, to see if this enzymatic scarifying agent will work for *Rubus* seeds. Driselase, a crude extract isolated from the basidiomycete fungus *Irpex lacteus* Fr. has activities similar to amylase, cellulase, and pectinase, and has been shown to improve *Rosa multiflora* Thunb. achene germination (Yambe and Takeno, 1992) which is the same family as *Rubus*.

I was also interested in determining if  $\text{LN}_2$  could be an effective mechanical scarifying agent for *Rubus*. Pritchard et al. (1988) examined  $\text{LN}_2$  as a pretreatment for the removal of legume hardseededness and reported that the mechanical freeze-thaw effect due to contraction and subsequent expansion of the testa improved germination. If *Rubus* seed viability was not altered by  $\text{LN}_2$  treatment, cryogenic preservation may be an effective way for long term preservation of *Rubus* seeds.

## 2. Review of the Literature

### 2.1 Description of *Rubus*

The genus *Rubus* includes the commercially grown blackberries and raspberries and more than 400 described species with habits of deciduous or evergreen leaves, often prickly canes, in either erect or trailing shrubs or vines (Heit, 1967). *Rubus* was first described by Focke in the early 1900's and has been further divided into 11 subgenera (Jennings, 1988).

Most *Rubus* plants have perfect flowers which bloom in the spring or summer and set fruit in aggregates of small, usually succulent, unevenly ripening drupelets (Brinkman, 1974). However, some *Rubus* such as *R. chamaemorus* L. and *R. ursinus* Cham. & Schldl. are dioecious, usually with abortive-anthered stamens and numerous pistils. Drupelets, by definition, contain a single hard-pitted seed (nutlet) enclosed in a stony endocarp and surrounded by flesh (Smith, 1977). Many western blackberries tip layer for vegetative propagation, while eastern blackberries and raspberries propagate by root suckers. Seeds provide the opportunity for genetic diversity and colonization (Nesme, 1985). Birds and mammals are chiefly responsible for the natural dispersion of *Rubus* seeds (Turcek, 1961, cited by Brinkman, 1974).

Studies have been conducted, usually in cooperation with breeding programs, for the production of improved varieties of blackberries and raspberries (Heit, 1967). Dale and Jarvis (1983) believed that breeding programs would become more efficient if seed germination was more reliable and rapid.

## 2.2 Seed Anatomy

*Rubus* fruit is an aggregate of drupelets which develop independently and adhere to a common receptacle. Each drupelet consists of one pyrene, (i.e. seed or nutlet), a fleshy mesocarp, and a thin dermatous exocarp (Nybom, 1980) (Fig. 2.1). The single hard-pitted pyrene of the drupe averages approximately 2 mm in length (Young and Young, 1992) but can range from 1.2 mm for *R. eustephanos* Focke ex Diels to 6.1 mm for *R. megalococcus* Focke (Hummer and Peacock, 1994). Pyrene weight ranges from 24.2 mg for *R. megalococcus* to 0.3 mg for *R. eustephanos* (Hummer and Peacock, 1994).

Most *Rubus* pyrenes have a hard, often ridged, outer endocarp covering an inner seed coat. This endocarp consists of two layers: an outer layer that is variable in thickness of longitudinal lignified fibers, and an inner region of 4 or 5 layers of lignified transverse fibers (Rose, 1919).

In *Malvaceous* seeds, the time of intense lignification correlates with the onset of impermeability and darkening of the seed coat (Egley, 1989). The precise role of lignin in impermeability has not been determined, but in prickly sida the least lignified seed coat region was the site where impermeability broke down first (Egley, 1989). Moore et al. (1974) reported a significant correlation between seed weight and endocarp thickness.

The testa or seed coat, located just inside of the endocarp, has four regions: 1) one layer of cushion-shaped cells with lignified walls, 2) four layers of collapsed cells with cellulose walls, 3) one layer of collapsed cells with thick pectinized walls, and 4) one layer of cells with cellulose walls which appear as a thickened outer wall of the endosperm (Rose, 1919). *Rubus* seeds have a small amount of endosperm with all of the nutrients being derived from the cotyledons (Young and Young, 1992; Brinkman, 1974).

The endosperm, which serves mainly as storage tissue, is composed of polyhedral parenchymatous cells with thin, largely cellulose, walls devoid of pits but possessing plasmodesma strands (Lasheen and Blackhurst, 1956). The layer of endosperm surrounding the embryo varies considerably in thickness depending on the individual seed and the location within the seed.

The mean thickness of this endosperm layer is approximately two cells (Kerr, 1954). However, four to five layers of cells are usually present along the flat side of the cotyledons and frequently the endosperm cells at the base of the cotyledons have been re-absorbed so that the embryo is in contact with the seed coat.

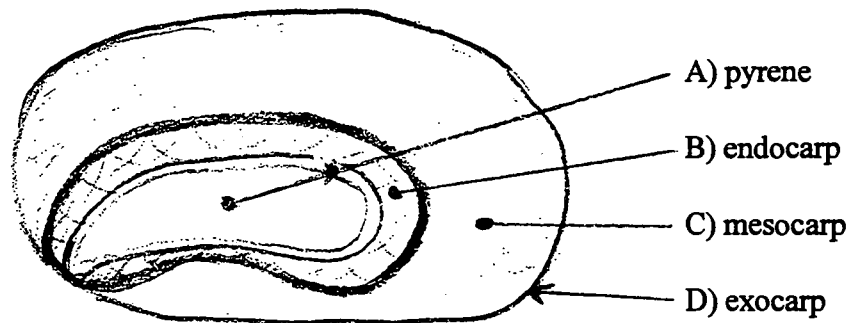


Fig. 2.1. Representative drawing of a *Rubus* drupelet. Each drupelet consists of, A) one pyrene, B) sclerenchymatous endocarp, C) a fleshy mesocarp, and D) a thin dermatous exocarp.

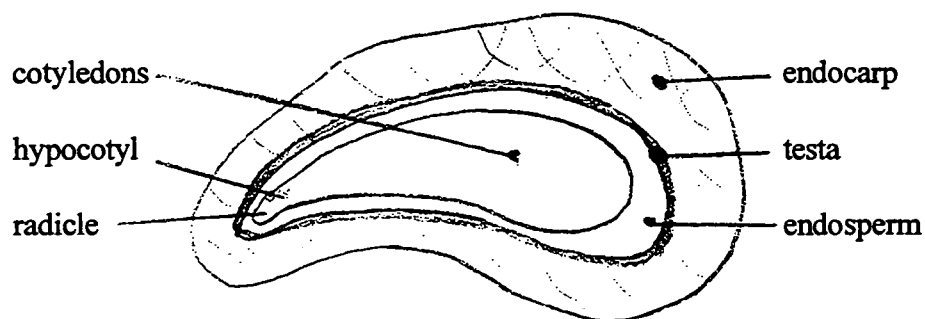


Fig. 2.2. The internal components of the *Rubus* seed.

The mature embryo occupies almost the entire seed. The two cotyledons are usually three or four times as long as the hypocotyl. No plumule is present. The epicotyl is only a slight tissue protuberance between the cotyledons (Kerr, 1954). Vascular strands extend the entire length of the cotyledons, branching several times just before reaching the epidermis. Nutrients enter the embryo chiefly through the tips of the cotyledons. The endosperm between the embryo and the chalazal region does not contain any reserve food at maturity. Both dry and germinating *Rubus* seeds are acidic when immersed in water (Rose, 1919).

### 2.3 Seed Preparation

Seed extraction can be achieved by macerating the fruit in water and then floating off the pulp and empty, nonviable seeds. Several consecutive changes of water followed by pouring off the floating material yield cleaner seed (Brinkman, 1974). Dry, clean seed can be stored in air tight containers at low temperatures either in a refrigerator or freezer for many years without much germination loss (Brinkman, 1974).

### 2.4 Exogenous Dormancy

Galletta et al. (1989) believed that *Rubus* seeds are among the “doubly dormant” class of seeds, where germination is restricted by both a hard seed covering and a dormant embryo. In *Alisma*, seeds are held in a dormant condition because the force of the expanding contents is insufficient to rupture the seed coat (Rose, 1919). *Rubus* seeds are similar to those of *Alisma* in two ways: they germinate readily once the endocarp is removed, and they absorb water readily with the endocarp intact (Rose, 1919).

Occasionally ungerminated seeds with the endocarp show no break in the coat. This suggests that the inner pectinized layer of the coat may be involved in the delay, either by limiting water or oxygen absorption, or both (Rose, 1919). Rose (1919) felt that *Rubus* dormancy is probably due to the high breaking strength of the endocarp. The endocarp may also restrict seed germination by excluding oxygen and water (Moore et al., 1974).

Scarification, which is the cutting or softening of a hard seed coat to hasten germination, can result in reducing the mechanical resistance of the endocarp, making it more permeable thereby increasing germination (Moore et al., 1974).

Ourecky (1975) reviewed several papers indicating possible conditions inhibiting germination of raspberry seeds. These include the embryonic requirements of minimum quantities of air, moisture, and temperature. These requirements are withheld due the mechanical resistance of the seed coat to embryo swelling and impermeability to air and water (Ourecky, 1975). Dale and Jarvis (1983) reported that exogenous dormancy in raspberry seeds must first be broken for the seeds to germinate.

Scarification can be mechanical or chemical.

#### 2.4.1 Mechanical

Mechanical scarification impacts weak areas on the seed coat causing cell separation and breaks in impermeable barriers (Egley, 1989). Egley listed several types of mechanical treatments to artificially break impermeability including: impactation against hard surfaces, heating under wet conditions, freezing at ultra low temperatures, pressure changes caused by plunging seeds in hot water, or immersion in liquid nitrogen. He proposed that exposure

of hard seeds to several weeks of diurnal soil temperatures may break impermeability.

Thus, artificially alternating temperatures (about 15°/60°C) for several weeks may cause gradual expansion and contraction of cells.

Nesme (1985) experimented with four different types of seed coat (endocarp, testa, and endosperm) treatments: (1) nicking only the outer endocarp at the radicle pole leaving the inner testa and endosperm intact, (2) nicking the three seed coats at the radicle pole so as to expose the radicle, (3) nicking the three seed coats to expose only part of the cotyledons and (4) completely removing the seed coats resulting in a naked embryo. The germination curve for the fully-nicked-seeds and the naked embryo was identical, indicating that only a small portion of the embryo had to be exposed to the external environment for germination equivalent to seed coat removal.

Germination of nutlets where only the outer endocarp was nicked resulted in 15% germination with no increase after one year until further nicking of the remaining two layers occurred (Nesme, 1985). Intact testa and endosperm may be strong inhibitors of germination. Nesme (1985) obtained 100 percent germination in less than 15 days for raspberry seeds that had the endocarp, testa, and endosperm removed (resulting in naked embryos) or if each of the layers were nicked. Seeds that only had the outer endocarp nicked and not the inner two seed coat layers did not germinate until the testa and endosperm were injured. The intact nutlet group never germinated (Nesme 1985).

Nesme (1985) concluded from his mechanical scarification experiments that *R. idaeus* has no embryonic dormancy and that germination is physically inhibited by the testa and/or endosperm.

### 2.4.2 Chemical Scarification

Many different types of artificial chemical scarifications have been tried (Rose, 1919). He mentioned Alexander von Humboldt in 1793 as the first investigator to use chemicals as “forcing agents”. Since then, many investigators have used a great variety of both organic and inorganic agents to artificially promote seed germination. Rose (1919) stated, “the range of substances used is more interesting than the results obtained”.

Heit (1967) reported that some of these, vinegar soaking, draino, vitamin B<sub>1</sub> or potassium nitrate treatments, as recommended by some workers were unsuccessful. Rose (1919) listed hydrochloric acid, potassium hydroxide, ferric chloride, hydrogen peroxide, various nitrogen compounds especially nitrites and nitrates such as potassium nitrate and magnesium nitrate as possibilities. Some other treatments that have had varied degrees of success in overcoming impermeability include soaking in organic solvents and hydrolytic enzymes (Egley, 1989).

#### 2.4.2.1 Sulfuric Acid

Endocarp scarification with concentrated sulfuric acid is frequently used in blackberry breeding programs (Moore et al., 1974). Concentrated sulfuric acid rapidly desiccates tissues and causes stress resulting in cell separations and fissures in the seed coat (Egley, 1989). When working with sulfuric acid, Ourecky (1975) indicated that the seed coat must be absolutely dry or the heat generated by the treatment will kill the embryo. Small lots of seed must be used and stirred frequently for the same reason.



Percent blackberry germination was increased by prolonging sulfuric acid treatments in ice baths, with the greatest percent increase occurring after 3 hours of scarification (17.4% average) (Moore et al., 1974). Treatment within an ice bath prevented over-heating but also reduced acid activity so that longer treatment periods were required for satisfactory scarification.. Increased duration of acid treatment decreased endocarp thickness and seed weight (Moore et al., 1974). They observed a significant negative correlation between endocarp thickness and percent germination. They believed that, “the use of an ice bath to control acid temperature combined with longer treatment duration is superior to short term treatment with uncontrolled temperatures”. Moore et al. (1974) reported earlier and more uniform seedling emergence results, and that most seedlings emerge within 5 weeks of planting following a 3 hour sulfuric acid/ice bath treatment

Heit (1967) recommended sulfuric acid treatments for 15 minutes up to 2 hours to eliminate the hard or leathery seed coat. However, he mentioned care must be taken when acid treating many hybrids and varieties of raspberries. Injury to the seeds occur with treatments of more than 30 minutes, and the temperature of the acid during treatment must be watched for overheating. In the sand blackberry *R. cuneifolius*, Cambell and Erasmus (1988), demonstrated that a carbonized layer of seed covering adheres to the seed after scarification with sulfuric acid and inhibits germination. They were able to overcome this inhibition either by mechanical notching (nicking) of the seed coat, by removal of a portion of the carbonized seed coat, or by substituting an NaOCl seed soak prior to stratification (Cambell and Erasmus, 1988). Rose (1919) found that the carbonized endocarp can be removed by rubbing the treated seed on filter paper.

Heit and Slate (1950) worked with the blackberry *R. allegheniensis* and reported a definite injury to the seed in the 1.5 hour sulfuric acid treatment at room temperature. This treatment not only reduced germination but produced weak, poorly rooted seedlings. They recommended that seeds be treated with concentrated sulfuric acid for 1 hour at room temperature, sown in peat moss in flats during August, and be placed in cold frames until the following March to produce the greatest number of seedlings.

Jennings and Tulloch (1965) developed a pregermination method where seeds were soaked in concentrated sulfuric acid for 20 minutes, immersed for seven days in a calcium hypochlorite solution with excess calcium hydroxide (1 percent available chlorine), and are placed in moist-chill stratification for six weeks.

#### 2.4.2.2 Sodium Hypochlorite

Galletta et al. (1989) believed pregermination treatment of *Rubus* seeds with sodium hypochlorite (NaOCl) is easier than nicking the seed coat. Therefore, they initiated studies to determine the effect of NaOCl concentration and treatment duration on seed germination of *Rubus* species and hybrids. Preliminary experiments indicated that reagent grade NaOCl levels of either 5 or 12 percent produced good germination for several raspberry and blackberry species in 4–6 weeks following a 24–48 hours pregermination seed soak. They reported that the ratio of volume-to-seed was equally or more important than NaOCl concentration. Commercial chlorine bleach was used as a source of NaOCl since it was approximately the same concentration as reagent grade sodium hypochlorite, was very economical and readily available.

Other studies indicate that (NaOCl) treatments ranging from 0.5–15.0 percent have varying degrees of success. Cambell and Erasmus (1988) found that the concentration of NaOCl and the length of application affect the structure of the endocarp using scanning electron microscopy. Four days in 1% NaOCl limited endocarp scarification whereas 18 hours in 15% NaOCl removed a considerable portion of the endocarp. After 22 days the germination of hand-notched and NaOCl treated seed did not differ significantly, indicating that NaOCl is a substitute for tedious manual scarification (Cambell and Erasmus, 1988). Scarification with NaOCl may have increases permeability of the seeds or allowed oxidization of growth inhibitory substances (Cambell and Erasmus, 1988).

But some sensitivity to NaOCl was reported by Scott and Ink (1957) and Jennings and Tulloch (1965). Scott and Ink (1957) found 1% NaOCl for 1 to 2 weeks worked best, but if that soak was extended to three weeks germination was lowered. Jennings and Tulloch (1965) found that all treatments with 1% NaOCl were inferior to treatment with 0.5%, with germination being frequently delayed or prevented at higher NaOCl concentrations. It was of interest that the cultivar 'Lloyd George' was much more sensitive than the 'Norfolk Giant' to the adverse effects of higher NaOCl concentrations (Jennings and Tulloch, 1965). They suggested that an interaction between growth inhibitors and promoters govern germination capacity and that higher concentrations of such chemicals as 0.5% NaOCl, 1% calcium hypochlorite, or 1% thiourea adversely affected the relationship.

#### 2.4.2.3 Macerating Enzymes

Lester (1985, cited by Egley, 1989) increased germination of hard seeds of wild tomato by etching the spermoderm with cellulytic enzymes. Brant et al. (1971, cited by Egley, 1990) slightly reduced the impermeability of crownvetch seeds by incubating the seeds in hemicellulase or pectinase. Generally, maceration of plant tissues and disintegration of cell walls are associated with pectinase activity and cellulase (or hemicellulase) activity (Noguchi et al., 1978).

Driselase, another such macerating enzyme, has activities similar to amylase, cellulase, and pectinase (Noguchi et al., 1978). Driselase is a product of the Basidiomycete fungus, *Irpex lacteus* Fr., which produces various kinds of polysaccharide saccharifying enzymes (Noguchi et al., 1978; Yambe and Takeno, 1992). Two percent driselase (w/v) is the equivalent of 340 units per milliliter of hemicellulase and 400 units per milliliter of cellulase activity (Chen and Boss, 1990). The optimum pH ranges for the tissue macerating activity of Driselase was between 4.0 and 6.0 (Noguchi et al., 1978). They also found a optimum temperature range of 40°–45°C.

Yambe and Takeno (1992) used driselase dissolved in 10 mM 2-(N-morpholino)-ethansulfonic acid buffer (pH 5.0) at varying ranges from 0.05 percent to 2 percent (w/v) to improve rose achene germination. The 1% solution for 36 hours was optimum for *Rosa multiflora* achenes. Percent germination reached a maximum in 9 days. Yambe and Takeno (1992) reported that the seeds germinated more rapidly the longer the treatment period with 1 percent driselase, but that the final percent germination was almost the same regardless of the treatment duration between 12 and 48 hours.

Driselase treatment caused splitting of the *Rosa multiflora* pericarp along the suture lines (Yambe and Takeno, 1992). Cellulase onozuka has also improved germination even at 0.05 percent, but at 2 percent concentration the final germination decreased significantly (Yambe and Takeno, 1992). Pectinase caused maximum germination at 0.2 percent concentration and the optimum cellulase concentration of 1 percent produced 50 percent germination in *Rosa multiflora* compared to the nontreated achenes (control group) which had less than 10 percent germination success within 20 days (Yambe and Takeno, 1992).

Macerating enzymes may loosen the bond between cells along the suture by degrading the cementing substances and the cell walls (Yambe and Takeno, 1992). This loosening of bonds between cells apparently forced the splitting of the pericarp of *Rosa multiflora* causing the removal of physical barriers that were inhibiting germination. However, prolonged treatment or an excessively high concentration of the enzymes actually suppressed germination, suggesting that excessive enzyme treatment may have degraded the seeds themselves as well as the pericarp (Yambe and Takeno, 1992).

Lester (1985) used driselase to etch the cell walls of hard seeds and reported the softening of the seed coats permitted water to enter.

## 2.5 Endogenous Dormancy

The seeds of many woody plant species are usually physiologically dormant until a period of after-ripening. Dormant seeds have a dramatically slowed metabolism. They can “wait” until favorable environmental conditions exist and then germinate, increasing their

chances of survival. However, their metabolism is only slowed, not completely stopped, thus endogenous dormant seeds have a finite life span.

Lasheen and Blackhurst (1956) found dormancy of blackberry seed to be of a dual nature. Dormancy was caused by the external coverings of the testa and hard endocarp and could be broken by scarifying with sulfuric acid or by removing the seed coverings. Secondly, embryo dormancy was associated with definite biochemical and biophysical changes occurring during an after-ripening period at low temperatures. Long duration's of moist-chill stratification (an after-ripening process) are necessary to overcome embryonic dormancy for proper germination in many *Rubus* species (Lasheen and Blackhurst, 1956). This may parallel the influence of temperate zone weather cycles upon fallen seeds after their maturation in early summer.

Lasheen and Blackhurst (1956) indicated that dormancy of the *Rubus* embryo is associated with biochemical and biophysical changes occurring during an after-ripening period at low temperature. Specifically, as the after-ripening process progressed in the seed, the water content, acidity, catalase, peroxidase, lipase activity, and sucrose content increased, but the level of fats, proteins and starch decreased.

Carbohydrate transformation took place very slowly when the seeds were after-ripened at room temperature, but became greatly accelerated by stratification at  $3^{\circ} \pm 2^{\circ}\text{C}$ . The initial rate of starch breakdown was accelerated by treatment of seeds with sulfuric acid for 45 minutes, followed by moist-chilling. As after-ripening progressed, catalase, peroxidase, and lipase enzyme activity increased, reaching a maximum at five months (Lasheen and Blackhurst, 1956).

The relative concentration of growth-inhibiting material was highest in the endosperm, lower in the testa and lowest in the embryo (Lasheen and Blackhurst 1956). The inhibiting substance(s) were largely acidic in nature and disappeared during low temperature stratification of the seeds under moist conditions. This disappearance correlated with the breaking of dormancy and the ability of seeds to germinate. Seeds stored dry at room temperature for the same period of time remained dormant and still contained the inhibiting material. Therefore, embryonic dormancy in blackberry seed can be broken by stratification at low temperatures. Stratification, which is considered to affect the germination potential of the embryo, allowed for more rapid germination by biochemical changes within the embryo rather than physical structural changes of the endocarp (Cambell and Erasmus, 1988).

Many studies have found that a period of cold, moist stratification is required to improve the germination of many *Rubus* species (Rose, 1909; Lasheen and Blackhurst, 1956), Scott and Ink, 1957), but Dale and Jarvis (1983) studied several alternative germination pretreatments to determine what factors influence this stratification need. Seeds harvested from mature ripe fruits and air-dried overnight before being treated with concentrated sulfuric acid and calcium hypochlorite germinated without being stratified, but seeds similarly treated but harvested from unripe fruits did not (Dale and Jarvis, 1983). They showed that raspberry seeds can germinate without a prior stratification treatment. Dale and Jarvis (1983) stated that:

the embryos of freshly harvested raspberry seeds probably resemble blackberry in that dormancy is physically imposed on them by the testa and endosperm and in that the germination inhibitors are present initially in these tissues. Their chemical pretreatments probably facilitate the removal of some

or all of the inhibitors which have reached the seed. However, when the seed is stored dry, sufficient inhibitors will have moved into the embryo to induce embryo dormancy, which can only be broken by low temperatures.

Dale and Jarvis (1983) recommended that fresh seed be harvest from ripe fruit, allowed to air-dry overnight, be scarified with sulfuric acid and sown immediately to achieve maximum germination.

Phenolic compounds in the testa, and lipids in the endosperm play a role in the inhibition of germination of many species (Nesme, 1985). He explained that phenolic compounds are capable of trapping oxygen and reducing the amount that reaches the embryo, while lipids may constitute a hydrophobic barrier to the water supply of the embryo.

The trapped gas between the endosperm and cotyledons of *R. chamaemorus* was found to contain harmful cyanogenetic compounds (Warr et al., 1979). Cyanogenetic compounds like amygdalin in seeds are known to break down when soaked in water due to the action of emulsion producing cyanide, benzaldehyde and glucose (Warr et al., 1979). Bakeapple seeds (*R. chamaemorus*) are known to contain amygdalin. Exogenous application of high concentrations of cyanide inhibit germination in lettuce (Mayer et al., 1957) Because the seed coat and the endosperm pellicle are impermeable to gases and cyanide, nicking of the testa and endosperm allow these inhibitory compounds to escape (Warr et al., 1979).

Where dormancy exists in the embryo itself, temperatures slightly above freezing have been found effective in hastening after-ripening (Rose, 1919). *Rubus odoratus* L. and *R. idaeus* seeds may germinate after a prolonged prechilling stratification of 120 days or



more, but that most other species require a warm stratification (86°–68°F) followed by prolonged prechilling (36°–41°F) for at least 90 days (Brinkman, 1974). This parallels and replicated the natural conditions of temperate zone weather cycles upon fallen seeds after their maturation in early summer. Seeds held moist in flats in the greenhouse for two months prior to stratification yielded more seedlings than those not given this treatment (Scott and Ink, 1957).

Cambell and Erasmus (1988) studied the effects of altering stratification times and incubation temperature along with alternate methods of scarification to optimize germination. They scarified seeds for 0, 9, 12, or 18 hrs in 15% NaOCl, maintained at  $6^{\circ} \pm 2^{\circ}\text{C}$  for 0, 4, 12, or 20 weeks and then incubated them in the light for 6 weeks at  $10^{\circ}/20^{\circ}\text{C}$ . The longer stratification times, 12 and 20 weeks, when NaOCl was used for a minimum of 6 hours, produced the highest percent germination (Cambell and Erasmus, 1988).

Heit (1967) found that prechilling was necessary to overcome dormancy in *Rubus* embryos following scarification treatments. But Heit (1967) also found that blackberry seed (*R. allegheniensis* Porter) could, without special treatment, germinate over a period of several years. No seedlings appeared the first year but the maximum germination occurred in the third year (Heit, 1967). Because *R. idaeus* fruits do not have the extremely hard seed coat found in blackberry, a warm-and-cold stratification period gives satisfactory germination.

Heit (1967) studied prechilling temperatures to determine the optimum to overcome seed dormancy and obtain the most uniform, maximum germination. Seeds were prechilled within temperature ranges of  $0^{\circ}\text{--}3^{\circ}\text{C}$  (32°–36°F),  $3^{\circ}\text{--}6^{\circ}\text{C}$  (36°–41°F), and  $6^{\circ}\text{--}9^{\circ}\text{C}$  (42°–

46°F). The 6°–9°C (42°–46°F) range in temperature resulted in poor germination (Heit, 1967). Their prechilling studies indicated that the optimum prechilling temperature range for *Rubus* species is 2°–4°C (34°–38°F) (Heit, 1967).

Care must be taken to maintain an adequately moist, but not wet, condition throughout the stratification period or secondary dormancy may result (Ourecky, 1975). He cited several possible conditions that inhibit germination of raspberry seeds. These included the embryonic requirements of minimum quantities of air, moisture, and temperature. These requirements triggered certain physiological conditions, such as the mechanical resistance of the seed coat to embryo swelling, and the seed coat's impermeability to air and water (Ourecky, 1975). Dormancy in raspberry seeds must first be broken for the seeds to germinate (Dale and Jarvis (1983).

## 2.6 In Vitro

Embryos of 10 *Rubus* crosses germinated in vitro on a modified Lepoivre medium, pH 5.2, without growth regulators (Ke et al., 1985). Seeds were extracted from fresh fruit, disinfected, cut in half, and both halves placed directly onto tissue culture medium. Germination occurred within a few days, without cool-moist stratification, indicating that drying bramble seeds may tend to induce dormancy (Ke et al., 1985). The half seed treatments germinated from 56.8 percent to 81.1 percent. They believe that bramble geneticists may find the in vitro system useful to reduce generation time. Viable seeds often germinated within 8 to 12 days, producing a germination time savings that might be enough to ensure well-rooted seedlings capable of surviving field transplanting during the

same year that crosses are made (Ke et al., 1985). However, tissue culture is a labor intensive operation. Therefore only seed of critical crosses with a low set ( e.g. interspecific hybridization's) should be germinated in vitro. Other systems are more appropriate for more routine germination (Ke et al., 1985).

## 2.7 Germination

*Rubus* germination has been reported to be very poor and irregular (Ourecky, 1975). Satisfactory germination and seedling production was harder to obtain with blackberry seed lots than with raspberries (Heit, 1967). Germination of *Rubus* was epigenous, i.e., the hypocotyl elongates and raises the cotyledons above the ground (Brinkman, 1974).

Usually the radicle was the first to emerge from the seed, but Warr et al. (1979) observed that cloudberry cotyledons always broke through the nicked seed before the radicle emerged.

Galletta et al. (1989) hypothesized that a weakened seed covering permits embryo expansion. Moore et al. (1974) encourage *Rubus* breeders to improve germination of their seed lots by selecting genetic clones with high germinability for seed parents in crosses. Other factors besides endocarp characteristics may be involved in germination. Clones with similar seed size and endocarp thickness differ greatly in percent germination (Moore et al., 1974). Varieties differ in optimum germination temperature ranges. Some varieties germinated better under cooler 5°C–10°C conditions, while others do better at 30°C–35°C. The optimum temperature where each did equally well was 20°C (Nesme, 1985). Alternating temperatures of 10°/20°C and 15°/30°C resulted in the greatest germination

(Cambell and Erasmus, 1988). Germination was increased by pretreatment of seed with 500 ppm gibberellic acid after a chilling requirement and before sowing (Jennings and Tulloch, 1965). Gibberellins are present in most germinating seeds, and show specific responses in the germinating process (Warr et al., 1979). Galletta et al. (1989) reported that germination under a mist in a greenhouse appeared more promising than germination in petri dishes. Recommended planting substrata include: moist vermiculite or finely shredded sphagnum moss (Ourecky, 1975) and cotton, filter paper, and quartz sand (Rose, 1919). Seeds when sown should not be covered with more than 2-8 mm of the media because light favorably affects germination of at least some species (Jennings and Tulloch, 1965; Scott and Ink, 1957).

Ourecky (1975) indicated that seedlings may be “pricked out” into individual pots or containers just as the second true leaf begins to develop. Transplanting very young seedlings is difficult because the main root is often injured. A more fibrous root system on older seedlings makes them easier to establish. A fungicide drench after pricking out is often beneficial by eliminating the damping-off disease complex (Ourecky, 1975). For nurseries and fields, Brinkman (1974) believes that best emergence will follow a late summer or early fall sowing, 1/8 to 3/16 inch deep, of scarified seeds in a row. He also recommended a spring planting of a prescarified seed that has undergone stratification.

Lundergan and Carlisi (1984) stated that germination of freshly harvested seed in areas where blackberry fruits ripen early in the growing season, can shorten the time between generations and accelerate the rate of genetic advance in blackberry breeding.

## 2.8 References

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### 3. *Rubus* Pregermination Treatments with Driselase or Sodium Hypochlorite

#### 3.1 Abstract

*Rubus* seeds have a thick protective endocarp and seed coat that restrict germination under favorable conditions. Two scarifying agents, sodium hypochlorite (NaOCl) and driselase (a macerating enzyme), were applied as pregermination treatments to improve *R. ursinus* L. and *R. parviflorus* Nutt. germination. Driselase was applied in concentrations of 1, 2, and 3 percent over 24, 36, and 48 hours, did not significantly improve germination. Sodium hypochlorite at 2.6 percent (diluted household bleach) significantly improved germination of *R. parviflorus*, achieving 33 percent within 60 days. *Rubus ursinus*, however, responded negatively and showed a sensitivity response to 2.6% NaOCl exposure: the embryonic axis of the seedlings failed to develop after cotyledon emergence. Another pregermination treatment, 2 months of moist-warm stratification followed by 3 months of moist-chill stratification, resulted in 34 percent germination for *R. parviflorus* but only 1.5 percent for *R. ursinus*. For *Rubus* species that do not show a sensitivity response similar to that of *R. ursinus*, 2.6% NaOCl is an effective scarifying agent for improving percentage of germination. For those that show sensitivity, a lower concentration may be effective.

#### 3.2 Introduction

Some woody plant seeds have a thick protective endocarp and seed coat that impedes germination even under favorable conditions. Removal or scarification of these barriers is needed before seeds germinate. For *Rubus*, these barriers are removed in nature by the digestive system of birds, by fungal degradation in the soil, or by weatherization (Galletta et al., 1989). To propagate *Rubus* for research, breeding or in the



nursery, mechanical scarification is used but is often tedious and cumbersome. I wondered if an enzyme extract would be an effective scarifying agent in *Rubus*.

Driselase (Sigma Chemical Co.; St. Louis), a crude extract isolated from the basidiomycete fungi *Irpex lacteus* Fr. has several activities similar to amylase, cellulase, pectinase, and others (Noguchi et al., 1978). Yambe and Takeno (1992) greatly increased germination in achenes of *Rosa multiflora* Thunb. after using a 1% driselase treatment. Sodium hypochlorite (household bleach) is an effective scarifying agent (Campbell et al., 1988; Galletta et al., 1989; Jennings and Tulloch, 1965; Scott et al., 1975). The objective of this study was to see if driselase or sodium hypochlorite could scarify the tough outer endocarp of *Rubus ursinus* L. and *R. parviflorus* Nutt and increase percent seed germination.

### 3.3 Materials and Methods

*Rubus ursinus* and *R. parviflorus* seed lots, collected in 1987, were donated by the USDA Forest Research Laboratory, Corvallis, Oregon. Nine treatments with four 50-seed replicates were counted with an automatic counter (Seedburo<sup>TM</sup>, Chicago, Ill, USA). Seeds were surface sterilized with 2.6% sodium hypochlorite for 15 min, then soaked in distilled water for 20 min, with the water changed twice. Fifty seeds were placed in a 9-mm test tube, then 10 ml of one of the treatment solutions was added: water, 2.6% sodium hypochlorite, or 1% (w/v) driselase in 10 mM 2-(N-morpholinyl)-ethanesulfonic acid buffer (pH 5.0).

The tubes were placed in a rack and set on a 20-rpm rotor at room temperature for the treatment duration. Treatment times were 24, 48 and 72 h. producing a 3 x 3 factorial design. The room temperature, which can affect the scarifying process, ranged from 19.5C–27C over the 72-hour treatment period. The seeds were then rinsed with dH<sub>2</sub>O for 1 min and placed in a randomly assigned spot in a germination tray containing sterile moist sand. The trays were put into the germinator in a randomized block design, with

each block assigned to a different shelf. The germinator provided 12 h of fluorescent white light and had alternating temperatures of 10C for 14 h and 25C for 10 h.

Germination was counted for 90 consecutive days.

Germination was defined by the emergence of the radicle 5 mm from the seed (when only the of cotyledons appeared, it was scored as simply emergence and not complete germination). Germinated seeds were removed from the box and transplanted into a seedling peat mixture for evaluation of plant normality.

A fourth treatment not included in the 3 x 3 factorial was 2 months of warm stratification at room temperature (approx. 26C) followed by 3 months cold stratification at 3C and then placed in the germinator for 90 days.

Analysis of variance ( $p=0.01$ ) was calculated on the mean number of emerged seeds and significant means were separated using Tukey's comparison method.

*Experiment two.* Four 50-seed replicates of *R. parviflorus* and *R. ursinus* were randomly assigned to one of nine different treatments. The seeds were surface sterilized with 2.6% sodium hypochlorite for 15 min and soaked in distilled water for 20 min with the water changed twice. The seeds were put into 9-mm test tubes, to which were added 5 ml of the predetermined driselase solution, 1, 2, or 3% w/v driselase in 10 mM 2-(N-morphonlino)-ethansulfonic acid buffer (pH 5.7).

The test tubes were placed in a water incubator adjusted to 30C, with the rotor set at 20 rpm. The sample tubes were removed at 24, 48, and 72 h. Seeds were removed from the test tube, rinsed with distilled water for 10 min, put into plastic germination boxes filled with sterilized sand, and placed into a germinator. The germinator was set as described above. Observations were taken for 90 days. Statistical analysis was performed as in experiment one.

### 3.4 Results and Discussion

The 2.6% NaOCl pretreatment significantly improved both the germination rate and percentage of *R. parviflorus* ( $p < 0.01$ ). *Rubus ursinus*, however, showed only significant cotyledon emergence, with none of the seedlings fully germinating ( $p < 0.01$ ). After cotyledon emergence, the *R. ursinus* seedlings died because the embryonic axis failed to elongate and develop. The driselase pretreatment did not improve germination significantly ( $p > 0.01$ ). The stratification pretreatment resulted in a significant 34-percent germination for *R. parviflorus*, but a nonsignificant 1.5-percent for *R. ursinus* (Table 3.1). The *R. parviflorus* seedlings grew normally.

Table 3.1. Results of experiment one, the 3 x 3 factorial and warm-cold stratification treatment, according to species and treatment.

| Treatment      | <i>R. parviflorus</i>              |                         | <i>R. ursinus</i>                  |                         |
|----------------|------------------------------------|-------------------------|------------------------------------|-------------------------|
|                | Average initial germination (days) | Average germination (%) | Average initial germination (days) | Average germination (%) |
| Water control  |                                    |                         |                                    |                         |
| 24 h           | 39                                 | 7                       | 0                                  | 0                       |
| 48 h           | 25                                 | 9                       | 0                                  | 0                       |
| 72 h           | 41                                 | 9                       | 0                                  | 0                       |
| 2.6% NaOCl     |                                    |                         |                                    |                         |
| 24 h           | 20                                 | 32                      | 28                                 | 32 <sup>z</sup>         |
| 48 h           | 16                                 | 37                      | 26                                 | 34 <sup>z</sup>         |
| 72 h           | 16                                 | 17                      | 27                                 | 16 <sup>z</sup>         |
| 1% driselase   |                                    |                         |                                    |                         |
| 24 h           | 39                                 | 9                       | 0                                  | 0                       |
| 48 h           | 41                                 | 10                      | 32                                 | .05                     |
| 72 h           | 40                                 | 9                       | 0                                  | 0                       |
| Stratification | 175                                | 34                      | 180                                | 1.5                     |

<sup>z</sup>Emergence of cotyledon only, embryonic axis failed to develop, so not complete germination.

In agreement with previous research (Campbell et al., 1988; Galletta et al., 1989; Jennings and Tulloch, 1965; Scott et al., 1975), sodium hypochlorite was an effective scarifying agent. *Rubus parviflorus* treated with 2.6% NaOCl showed a significant improvement in the percentage of germination compared to the control ( $p < 0.01$ ). *Rubus ursinus*, however, showed a negative response to 2.6% NaOCl exposure: the embryonic axis of the seedlings failed to develop after emergence (Fig 3.1 and 3.2). This type of mixed response to NaOCl has been reported before (Jennings and Tulloch, 1965; Scott and Ink, 1957). Jennings and Tulloch (1965) found the cultivar 'Lloyd George' much more sensitive than 'Norfolk Giant' to high concentrations of NaOCl. They suggested that an interaction between growth inhibitors and promoters govern germination capacity and that higher concentrations of NaOCl adversely affect the relationship.

A 5-month stratification was as successful for *R. parviflorus* germination (34%) as the NaOCl scarification, but for *R. ursinus*, it produced only 1.5% germination. However, the stratification pretreatment took three times as long to accomplish the same result as the NaOCl scarification pretreatment.

*Experiment two.* All of the driselase scarifying pretreatments failed to significantly improve percent germination for the two species (Table 3.2). Analysis showed no significant difference between any of the concentrations, or any of the time variables ( $p > 0.01$ ). No significant interaction was found between time and concentration ( $p > 0.01$ ).

Lack of increased percent germination may have been due to little or no scarification of the endocarp by this macerating enzyme at the concentrations used. This is different than what Yambe and Takeno (1992) found with *Rosa multiflora*. They reported that driselase split the pericarp along the suture line. But in *Rubus*, driselase apparently failed to loosen or dissolve the tough endocarp enough to affect germination. This may have been due to the presence of lignin and its protection of the seedcoat cells.

Although there is no significant difference in treatments, a general trend toward improvement in germination occurred with increased concentration of driselase.

Table 3.2. Experiment two, the 3 driselase solution factorial, results according to species and treatment.

| Treatment    | <i>R. parviflorus</i>              |                         | <i>R. ursinus</i>                  |                         |
|--------------|------------------------------------|-------------------------|------------------------------------|-------------------------|
|              | Average initial germination (days) | Average germination (%) | Average initial germination (days) | Average germination (%) |
| 1% driselase |                                    |                         |                                    |                         |
| 24 h         | 48                                 | 12                      | 0                                  | 0                       |
| 48 h         | 37                                 | 20                      | 0                                  | 0                       |
| 72 h         | 42                                 | 8                       | 0                                  | 0                       |
| 2% driselase |                                    |                         |                                    |                         |
| 24 h         | 32                                 | 12                      | 0                                  | 0                       |
| 48 h         | 42                                 | 12                      | 0                                  | 0                       |
| 72 h         | 32                                 | 12                      | 0                                  | 0                       |
| 3% driselase |                                    |                         |                                    |                         |
| 24 h         | 37                                 | 20                      | 0                                  | 0                       |
| 48 h         | 37                                 | 20                      | 0                                  | 0                       |
| 72 h         | 37                                 | 16                      | 0                                  | 0                       |

Further studies should examine increased driselase concentrations and/or increased exposure times. The recommended treatment for *R. parviflorus* is 2.6% sodium hypochlorite for 36 h. No treatment for *R. ursinus* can yet be recommended, but perhaps a lower concentration, such as 0.5% NaOCl can be tried.



Fig. 3.1. Control (dH<sub>2</sub>O): normal development of the seedling.



Fig. 3.2. Sensitivity response to 2.6% NaOCl: embryonic axis failed to develop after emergence.

### 3.5 References

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#### 4. Pregermination Studies with Liquid Nitrogen and Sulfuric Acid on Several *Rubus* Species

##### 4.1 Abstract

Many *Rubus* species have a seed coat imposed exogenous dormancy. My objective was to contrast the effect of liquid nitrogen (LN<sub>2</sub>), sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) and a untreated control on the germination of six *Rubus* species. I was also interested to determine if LN<sub>2</sub> could be an effective mechanical scarifying agent for these species. Three replicates of 100 seeds of each species were treated with three 3-min dips in LN<sub>2</sub> with alternating 10 min thaws; with 30 min H<sub>2</sub>SO<sub>4</sub>; or were left untreated. The percent germination of *R. multibracteatus* A. Leveille & Vaniot, *R. parviflorus* Nutt., *R. eustephanos* Focke ex Diels, *R. leucodermis* Douglas ex Torrey & A. Gray, *R. ursinus* Cham. & Schldl., and *R. chamaemorus* L., treated with LN<sub>2</sub> was not significantly different than the control. Germinated seedlings from the LN<sub>2</sub> treatment of each species showed normal development upon planting indicating that long term cryogenic preservation of these *Rubus* species seeds may be possible. The H<sub>2</sub>SO<sub>4</sub> treatment significantly increased the rate and percentage of germination in *R. parviflorus*, *R. eustephanos*, *R. leucodermis*, and *R. ursinus*, over that of the control and the LN<sub>2</sub> treatment. The alternative LN<sub>2</sub> application techniques attempted did not significantly improve *Rubus* seed germination as compared with that of the control.



## 4.2 Introduction

Some *Rubus* seeds have a thick endocarp and testa (seed coat) that can impede germination (Ourecky, 1975; Rose, 1919). Pregermination treatments such as scarification or stratification of these seeds are usually needed before they will germinate (Lasheen and Blackhurst, 1956). Mechanical scarification can be tedious and cumbersome (Galletta et al., 1989). Endocarp scarification with concentrated sulfuric acid ( $\text{H}_2\text{SO}_4$ ) is used in blackberry breeding programs (Moore et al., 1974). Unfortunately, this acid can be unsafe to work with and over exposure can lead to seedling damage. Therefore, less toxic, alternative scarification techniques are being sought.

Liquid nitrogen ( $\text{LN}_2$ ) was examined as a germination pretreatment for legume seeds (Pritchard et al., 1988). Legume hardseededness can be removed by a mechanical freeze-thaw effect due to contraction and subsequent expansion of the testa (Boyce, 1989; Pritchard et al., 1988; Stout, 1990). I thought that this technique could be applied to improve *Rubus* germination.

My objectives were to contrast the effect of  $\text{LN}_2$  and  $\text{H}_2\text{SO}_4$  on *Rubus* seed germination and to determine if  $\text{LN}_2$  could be used as a mechanical scarifying agent for *Rubus*.

### 4.3 Materials and Methods

*Rubus* seed for this experiment was obtained from the USDA-ARS National Clonal Germplasm Repository, Corvallis where it was stored for 2-10 years in aluminum foil lined paper envelopes at -18C. Nine lots of 100 seeds each of *R. multibracteatus* (Corvallis accession #1642), *R. parviflorus* (Cor. #1738), *R. eustephanos* (Cor. #1638), *R. leucodermis* (Cor. #692), *R. ursinus* (Cor. # 1739), and *R. chamaemorus* (Cor. #1728), were obtained with an automatic counter (Seedburo, Chicago, Ill., USA). Each lot was assigned to one of three treatments: LN<sub>2</sub>, H<sub>2</sub>SO<sub>4</sub>, and a control, based on a randomized complete block design. Each treatment had three 100-seed replications.

The LN<sub>2</sub>-treated seeds were placed in paper envelopes. A plastic cooler was filled to a depth of approximately 3 cm with LN<sub>2</sub>. The upright seed packets were immersed in the LN<sub>2</sub> for 3 min, removed, and allowed to thaw at about 26C for 10 min. This procedure was repeated three times. Liquid nitrogen was replenished to maintain a constant volume.

For treatment with H<sub>2</sub>SO<sub>4</sub>, seeds were placed in 13mm test tubes with enough concentrated acid to cover them (approx. 2-4 ml). After 30 min the H<sub>2</sub>SO<sub>4</sub> was decanted, seeds were rinsed with running water for 5 min, and then excess sodium bicarbonate was added to neutralize any remaining acid, and the seeds again rinsed with water for 5 min.

Seeds of the LN<sub>2</sub>-, H<sub>2</sub>SO<sub>4</sub>- treated, and untreated control groups were then placed directly onto moistened sterilized sand in germination boxes. Boxes were put in a Hoffman UE 650 germinator (Albany, Ore., USA) in a randomized complete block design with each shelf considered as a block. The germinator had alternating

temperatures of 10C for 14 h and 25C for 10 h and provided 12 h of fluorescent white light during the 25C period. Each box remained in the germinator for 8 weeks (warm stratification period) then was moved into a walk-in cooler (Vollrath, River Falls, Wis., USA) for 6 weeks at 3C (cold stratification period), and finally returned to the germinator for an additional 12 weeks (germination period). Radicle emergence was counted during both the warm stratification and germination periods. Selected seedlings were planted and grown out in the greenhouse to observe their growth. Analysis of variance was calculated on the mean number of emerged seeds. Significant means were separated using Least Significant Difference ( $p=0.05$ ).

#### 4.4 Results and Discussion

After the first 8 week warm stratification period, only three species, *R. eustephanos*, *R. multibracteatus*, and *R. parviflorus*, showed some germination in the control treatment (Table 4.1). The LN<sub>2</sub> treated seeds were not significantly different than the control. Two species *R. multibracteatus* and *R. parviflorus*, germinated following LN<sub>2</sub> treatment without cold stratification (Table 4.1). The H<sub>2</sub>SO<sub>4</sub> treatment significantly improved both germination rate (Fig. 4.1) and percent germination (Table 4.1) in these three species. This significant response to acid scarification during a warm stratification period, indicated that exogenous rather than endogenous embryonic dormancy prevented untreated seed of these species from germinating.

Table 4.1. Germination percentages of six *Rubus* species after an 8 week warm stratification and the final germination period which included eight weeks warm, six weeks cold, and twelve weeks warm treatment.

| Species                   | Average germination (%) by treatment |                                |         |                   |                                |         |
|---------------------------|--------------------------------------|--------------------------------|---------|-------------------|--------------------------------|---------|
|                           | Warm treatment                       |                                |         | Final germination |                                |         |
|                           | LN <sub>2</sub>                      | H <sub>2</sub> SO <sub>4</sub> | Control | LN <sub>2</sub>   | H <sub>2</sub> SO <sub>4</sub> | Control |
| <i>R. ursinus</i>         | 0                                    | 3                              | 0       | 1 b               | 48 a                           | 3 b     |
| <i>R. leucodermis</i>     | 0                                    | 0                              | 0       | 2 b               | 44 a                           | 1 b     |
| <i>R. eustephanos</i>     | 0 b <sup>z</sup>                     | 51 a                           | 1 b     | 25 b              | 75 a                           | 12 b    |
| <i>R. multibracteatus</i> | 75 b                                 | 86 a                           | 74 b    | 84                | 88                             | 85      |
| <i>R. chamaemorus</i>     | 0                                    | 0                              | 0       | 39                | 26                             | 32      |
| <i>R. parviflorus</i>     | 3 b                                  | 26 a                           | 5 b     | 17 b              | 45 a                           | 21 b    |

<sup>z</sup> Means followed by the same letter within rows are not significantly different at P<0.05.

After the 12 week germination period, the LN<sub>2</sub> treatment was not significantly different than the control for each of the six species. Exposure to LN<sub>2</sub> presumably caused a drastic temperature shift in the seed coat but no indication of endocarp disruption was noted either physically or through an increase in germination.

A sample of seeds that germinated after exposure to the LN<sub>2</sub> treatment were planted and observed to have normal development and vigor. Liquid nitrogen did not significantly alter germination positively or negatively. The LN<sub>2</sub> treatment did not damage the embryo, suggesting that LN<sub>2</sub> preservation of *Rubus* seed may be possible.

Stanwood and coworkers (Stanwood and Bass, 1981; Stanwood and Roos, 1979) consider cryogenic storage of seeds to be valuable for the long term preservation of germplasm, no methods have been published for *Rubus* seeds.

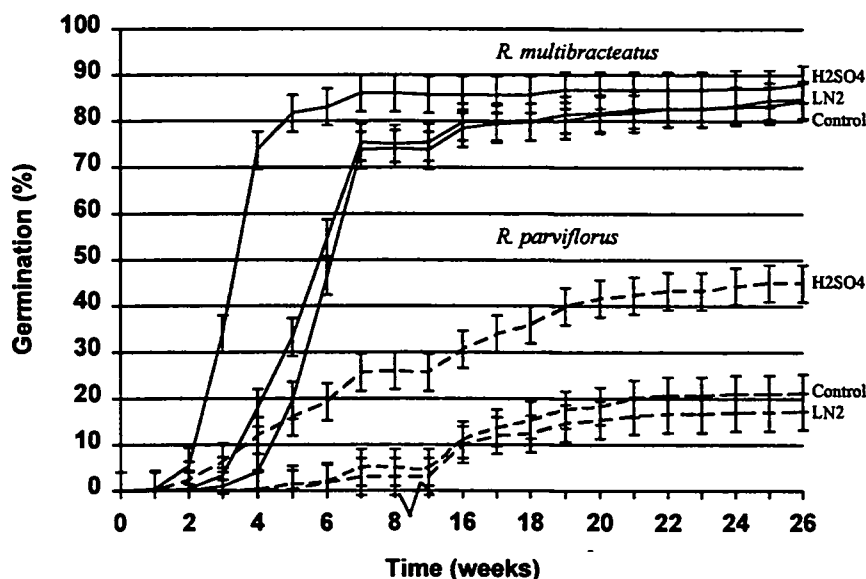


Fig. 4.1. Germination curves for *R. multibracteatus* and *R. parviflorus* seeds treated with sulfuric acid, liquid nitrogen, and an untreated (water) control are shown. Mean germination percentages (3 replicates of 100 seeds each) are plotted throughout the 26 week germination period. Bars are SE of the mean.

In agreement with findings on other *Rubus* species (Jennings and Tulloch, 1965; Moore et al., 1974) the H<sub>2</sub>SO<sub>4</sub> treatment significantly improved germination percent in four of the six species (Table 4.1). The *R. multibracteatus* germination percentages did not significantly differ in the three treatments after the final germination period.

The final germination percentage of  $\text{LN}_2$  treated *R. chamaemorus* was significantly higher than that of the  $\text{H}_2\text{SO}_4$  treatment, however neither differed from that of the control. Both *R. chamaemorus* and *R. multibracteatus* have a similar endocarp to embryo ratio by weight (Appendix B), but the endocarp of *R. multibracteatus* could more easily be cut with a scalpel. *Rubus chamaemorus* may require longer periods of exposure to acid to scarify its tough endocarp (Warr et al., 1979). *Rubus multibracteatus* appears to have no exogenous dormancy. I believe that scarification of this species is unnecessary because 85 percent of our control group germinated. However, the germination rate for *R. multibracteatus* and *R. parviflorus* were initially increased with  $\text{H}_2\text{SO}_4$  (Fig. 4.1), as it was in the other species, except for *R. chamaemorus*.

Sulfuric acid was the most effective scarifying treatment in this study, however, overexposure can cause seedling damage (Moore et al., 1974). Additional research has investigated a modified  $\text{LN}_2$  application as a scarifying agent with six and nine repeated immersions, spraying the seed coat with water immediately before  $\text{LN}_2$  immersion, and more extreme temperature fluctuations using an oven set at  $38^\circ\text{C}$  for the thaw period. Thus far none of these varying protocols have shown a significant germination increase (chapter 5) as compared with the untreated control. I conclude that  $\text{LN}_2$  is not an effective scarifying agent for *Rubus* seeds, however, this study suggests that long term cryogenic preservation of *Rubus* seeds should be investigated for germplasm preservation.

#### 4.5 References

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## 5. *Rubus* Pregermination Treatment Comparison

### 5.1 Abstract

Many *Rubus* pregermination treatments have been reported. I was interested in comparing several pregermination treatments for a wide range of *Rubus* spp. and to see if scarification with lower concentrations of sodium hypochlorite (NaOCl) would produce a sensitivity reaction observed in several species from previous experimentation using higher NaOCl concentrations.

Three replicates of 100 seeds of each species were treated with one of five pregermination treatments: a non-scarified control; 0.5% NaOCl; sulfuric acid (H<sub>2</sub>SO<sub>4</sub>); H<sub>2</sub>SO<sub>4</sub> followed by 1% calcium hypochlorite (CaO<sub>2</sub>Cl<sub>2</sub>) with an excess of calcium hydroxide (CaOH); and liquid nitrogen (LN<sub>2</sub>). *Rubus chamaemorus* L., *R. idaeus* L. 'Amber', *R. multibracteatus* A. Leveille & Vaniot, *R. parviflorus* Nutt., and *R. ursinus* Cham. & Schldl., were examined under these pretreatments to look for significant increases in percent germination.

Percent germination of *R. multibracteatus*, *R. parviflorus*, and *R. ursinus* was significantly increased by 0.5% NaOCl, H<sub>2</sub>SO<sub>4</sub>, and H<sub>2</sub>SO<sub>4</sub> with 1% CaO<sub>2</sub>Cl<sub>2</sub>. For *R. idaeus* 'Amber' only the H<sub>2</sub>SO<sub>4</sub> with 1% CaO<sub>2</sub>Cl<sub>2</sub> treatment significantly increased germination. *Rubus chamaemorus* did not show a response to any of the treatments. Tetrazolium staining indicated that this seedlot may have been nonviable. The LN<sub>2</sub> treatment did not significantly increase percent germination in any species.



The 0.5% NaOCl concentration improved germination significantly over that of the control groups, with *R. ursinus* and *R. multibracteatus* showing no sensitivity reaction at this concentration as they had in previous experiments. The other species also did not show any type of sensitivity reaction. Germinated seedlings from each of the 5 treatments showed normal development after planting.

For most *Rubus* species the recommended treatments ranked in order of effectiveness would be  $H_2SO_4$  with 1%  $CaO_2Cl_2$ ,  $H_2SO_4$ , 0.5% NaOCl, and no treatment. Liquid nitrogen can not be recommended as a scarifying agent for these *Rubus* species. These tests indicate no loss of viability of *Rubus* seeds upon repeated immersion in  $LN_2$ . Cryogenic storage may be an effective way for long term preservation of *Rubus* seeds.

## 5.2 Introduction

*Rubus* germination is difficult. These seeds may have both an external seed coat imposed exogenous dormancy and internal embryonic endogenous dormancy and therefore require both scarification of the seed coat followed by several weeks of cold stratification. A number of pregermination techniques have been reported to improve *Rubus* germination (Galletta et al., 1989; Jennings and Tulloch, 1965; Moore et al., 1974; Scott and Ink, 1957).

Endocarp scarification with concentrated sulfuric acid ( $H_2SO_4$ ) is frequently used in blackberry breeding programs (Moore et al., 1974). Scott and Ink (1957) used a 1% calcium hypochlorite solution with and excess of calcium hydroxide that reportedly worked well. Jennings and Tulloch (1965) reported that combining these two treatments,  $H_2SO_4$  and 1%  $CaO_2Cl_2$  solution, was the best for improving germination.

In an effort to avoid using sulfuric acid, sodium hypochlorite (NaOCl) has been used with some success (Galletta et al., 1989; Jennings and Tulloch 1965; Scott and Ink, 1957). But NaOCl sensitivity has been previously demonstrated (Chapter 3) in several species: the embryonic axis of the seedlings failed to develop after cotyledon emergence. Jennings and Tulloch (1965) also found some cultivars were much more sensitive to the adverse effects of higher NaOCl concentrations. It was suggested that for those species that show sensitivity, a lower NaOCl concentration may be effective..

Liquid nitrogen (LN<sub>2</sub>) can remove legume hardseededness by a mechanical freeze-thaw effect due to the contraction and subsequent expansion of the testa (Boyce, 1989; Pritchard et al., 1988; Stout, 1990). I thought that a modified LN<sub>2</sub> technique may be applied to improve *Rubus* germination.

The objective of this experiment was to determine the optimal pregermination treatments for a wide range of *Rubus* species.

### 5.3 Materials and Methods

*Rubus* seed for this experiment was obtained from the USDA-ARS National Clonal Germplasm Repository, Corvallis, where it was stored for 1-9 years in aluminum foil lined paper envelopes at -18C. Fifteen lots of 100 seeds each of *R. chamaemorus* (Cor. #1774), *R. ideaus* 'Amber' (Cor. #777), *R. multibracteatus* (Cor. #1642), *R. parviflorus* (Cor. #1738), and *R. ursinus* (Cor. # 1739), were obtained with an automatic counter (Seedburo, Chicago, Ill., USA).

Each lot was randomly assigned to one of three treatments: control, 0.5% NaOCl,  $\text{H}_2\text{SO}_4$ ,  $\text{H}_2\text{SO}_4$  followed by 1%  $\text{CaO}_2\text{Cl}_2$ , and  $\text{LN}_2$ . Each treatment had three 100-seed replications.

The control group were surface sterilized with 0.5% NaOCl for 20 minutes on a shaker at 50 rpm then rinsed and shaken in  $\text{dH}_2\text{O}$  for 20 minutes. Surface sterilization was not performed on the other treatments because it would have been redundant in the NaOCl treatment, the two  $\text{H}_2\text{SO}_4$  treatments required dry seed to avoid heat damage, and the  $\text{LN}_2$  treatment may have fractured the embryo if the seeds had been allowed to imbibe water.

The NaOCl treated seeds were exposed to a 0.5% solution for 7 days while on a shaker set at 50 rpm. The species were covered with approximately 5 ml of solution, except for *R. chamaemorus* which received 10 ml because it is a significantly larger seed. The seeds were then rinsed with water for 5 min, and shaken again at 50 rpm in water for an additional 20 min.

For the two  $\text{H}_2\text{SO}_4$  treatments, seeds were placed in 13mm test tubes with enough concentrated acid to cover them (approximately 2-4 ml) and stirred with a glass rod every couple of min. After 30 min the  $\text{H}_2\text{SO}_4$  was decanted, seeds were rinsed with running water for 5 min, and excess sodium bicarbonate was added to neutralize any remaining acid. The seeds were rinsed again on a shaker at 50 rpm for 20 min in distilled water ( $\text{dH}_2\text{O}$ ). One of the two  $\text{H}_2\text{SO}_4$  treatments underwent additional exposure to 1% calcium hypochlorite ( $\text{CaO}_2\text{Cl}_2$ ) solution with an excess calcium hydroxide ( $\text{CaOH}$ ) for 7 days while on a shaker set at 50 rpm.

The LN<sub>2</sub> treated seeds were placed in paper envelopes, sprayed with a light mist of water from a spray bottle (1 spray) and then dipped into approximately 3.0 cm of LN<sub>2</sub>. The upright seed packets were immersed in the LN<sub>2</sub> for 3 min, removed, and thawed in an oven set at 38C. This procedure was repeated 4 times except for the water spray, which was only done twice (before the first and third dips).

Seeds of the 5 treatment groups were then placed directly onto 100 x 15mm petri dishes with 2 tablespoons of sterilized sand moistened with 15 ml of dH<sub>2</sub>O and wrapped with parafilm to seal in moisture. Petri dishes were put into a walk-in cooler (Vollrath, River Falls, Wis., USA) for a 6 week 3C cold stratification period. Each petri dish was then moved to a Hoffman UE 650 germinator (Albany, Ore, USA) in a randomized complete block design with each shelf considered as a block.

The germinator had alternating temperatures of 10C for 14 h and 25C for 10 h and provided 12 h of fluorescent white light, 10 of which was provided during the 25C period. Each box remained in the germinator for a 12 week period. The unit to be counted was one petri dish with one replication of seed where radicle emergence was recorded during the germination period. Selected seedlings were planted and grown out in the greenhouse to observe their growth. Analysis of variance was calculated on the mean number of emerged seeds. Significant means were separated using Least Significant Difference ( $p=0.05$ ).

#### 5.4 Results and Discussion

After the 12 week germination period, 4 of the 5 species responded significantly to the  $\text{H}_2\text{SO}_4$  with 1%  $\text{CaO}_2\text{Cl}_2$  treatment, and 3 to the other  $\text{H}_2\text{SO}_4$  treatment (Table 5.1). The 0.5% NaOCl treatment significantly increased germination in three of the five species. Liquid nitrogen showed mix responses, increasing germination in *R. multibracteatus* as compared to the control, not significantly changing that of *R. ursinus*, and reducing germination of *R. parviflorus*. *Rubus chamaemorus* failed to respond to any of the treatments.

Table 5.1. Average percent germination of 5 *Rubus* species following several different treatments.

| Species                   | Control          | NaOCl | $\text{H}_2\text{SO}_4$ | $\text{CaO}_2\text{Cl}_2$ | $\text{LN}_2$ |
|---------------------------|------------------|-------|-------------------------|---------------------------|---------------|
| <i>R. chamaemorus</i>     | 0                | 0     | 0                       | 0                         | 0             |
| <i>R. idaeus</i> 'Amber'  | 0 b <sup>z</sup> | 0 b   | 1 b                     | 6 a                       | 0 b           |
| <i>R. multibracteatus</i> | 53 c             | 79 ab | 84 a                    | 81 a                      | 70 b          |
| <i>R. parviflorus</i>     | 19 b             | 38 a  | 44 a                    | 36 a                      | 8 c           |
| <i>R. ursinus</i>         | 1 c              | 24 b  | 15 b                    | 43 a                      | 2 c           |

<sup>z</sup> means followed by the same letter within rows are not significantly different at  $p < 0.05$ .

The lack of a response in *R. chamaemorus* indicates it may have been a non viable seed lot. Although collected in 1992 and received in 1993 (Cor. #1774) a tetrazolium chloride (TZ) test showed negative results with the excised embryos being completely

white, giving no indication of a pink color. Thus no embryo metabolic activity was apparent since there was no color change.

Another possible explanation for a lack of a germination response or positive TZ color change in *R. chamaemorus* could be due to an extremely deep embryonic dormancy. This experiment lacked a warm stratification period which was included in previous experiments and had a slightly shorter cold stratification period (6 weeks as opposed to 8-12) (Galletta et al., 1989). Perhaps this seed lot of *R. chamaemorus* would have benefited from a prolonged warm and/or cold stratification period. Most likely the seedlot was nonviable.

In previous experiments (Chapter 3) *R. ursinus* and *R. multibracteatus* have shown a sensitivity response to 2.6% NaOCl. In this experiment, germination of both species at a lower NaOCl concentration level (0.5%) was significantly better than the control, and neither showed any sign of a sensitivity reaction. This indicates that low concentrations of NaOCl may be substituted for the harsher sulfuric acid treatments but perhaps with slightly less overall germination percentages

The LN<sub>2</sub> treatment did not significantly increase percent germination for any species except in *R. multibracteatus*, which had a lower than expected control percent germination, so the author thinks this LN<sub>2</sub> significance is an artifact. The lower percent germination in LN<sub>2</sub> treated *R. parviflorus* could also be experimental error, or was perhaps due to the extreme freeze-thaw method used, where the seeds were slightly wetted, immersed, and then thawed at a high temperature. This method differed from previous experiments which

showed no significant difference between the control and LN<sub>2</sub> treatment in both the pre-wetting and higher thaw temperature. These two additions were done in an attempt to increase the effectiveness of the LN<sub>2</sub> treatment.

For most *Rubus* species the recommended treatments ranked in order of effectiveness would be H<sub>2</sub>SO<sub>4</sub> with 1% CaO<sub>2</sub>Cl<sub>2</sub>, H<sub>2</sub>SO<sub>4</sub>, 0.5% NaOCl, and no treatment. Liquid nitrogen can not be recommended as a scarifying agent for these *Rubus* species. These tests indicate no loss of viability of *Rubus* seeds upon repeated immersion in LN<sub>2</sub>. Cryogenic storage may be an effective way for long term preservation of *Rubus* seeds.

### 5.5 References

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## 6. Conclusion

### 6.1 Summary

*Rubus* seed germination is difficult. Both an external seed coat imposed exogenous dormancy and an internal embryonic endogenous dormancy combine to make a double dormancy that is hard to overcome with high a percentage of success. In this thesis, a number of pregermination techniques have been reported to improve germination (Chapters 3, 4, and 5), and of those tried, the recommended ranking in order of effectiveness would be, concentrated  $\text{H}_2\text{SO}_4$  followed by a 1%  $\text{CaO}_2\text{Cl}_2$  solution with an excess of  $\text{CaOH}$ , concentrated  $\text{H}_2\text{SO}_4$ , 0.5%  $\text{NaOCl}$  or 2.6%  $\text{NaOCl}$ , and then no treatment. With the exception of a few species which apparently have little endogenous dormancy, such as *R. multibracteatus*, these treatments should be followed by at least 6 weeks of stratification before being placed in the germinator.

Germination differences between species can partially be explained by differences in levels of exogenous dormancy. This exogenous dormancy is seed coat imposed and appears to be related to degree of mechanical resistance. Some species, such as *R. chamaemorus*, have an extremely hard seed coat, while others such as *R. multibracteatus*, have a softer one. Both species have a similar endocarp-to-embryo ratio by weight (Appendix B), but the endocarp of *R. multibracteatus* is more easily cut with a scalpel.



The macerating enzyme driselase did not significantly increase *Rubus* germination rate or percent (Chapter 3). This failure may have been due to little or no scarification of the endocarp at the 1-3% concentrations used. Driselase apparently failed to loosen or dissolve the endocarp enough to affect germination. It is believed that the lignified fibers in the seed coat protected the seed from any driselase enzymatic action(s).

Liquid nitrogen can not be recommended as a scarifying agent for these *Rubus* species. Liquid nitrogen did not significantly increase *Rubus* germination, however, it did not diminish overall percent germination either (Chapters 4 and 5). Experimentation indicates no loss of viability upon repeated immersions in LN<sub>2</sub>. Cryogenic storage may be an effective way for long term preservation of *Rubus* seeds.

One of the problems encountered was in an aspect of the experimental design: The number of replications used, and the number of seeds per replication, were inconsistent and too small. For accurate statistical analysis, it is important to have a large number of countable units. In chapter 3, I used replications of only fifty seeds, and since germination was averaged 10 to 30 percent, that meant only 5 to 15 seeds were germinating and being counted per replication. This is not a high number of countable units, and I had less confidence in my results than I liked. Later experiments, such as in chapters 4 and 5, used 100 seeds per replication. Ideally, this number should have been even greater, but with the small size of the National Clonal Germplasm Repository seed lots, the total number of seed I could use was limited.

## 6.2 Recommendation for Future Research

When I first started my research, one of my fellow graduate students, Duangporn Suwanagul, joked that since I studied *Rubus* seed germination, I also needed to study the anatomy of a bird. I told myself that sulfuric acid duplicated the natural acid of a bird's stomach. But in retrospect, the scarifying process of a bird's digestive system on a *Rubus* seed goes is, obviously, a much more complicated picture. Besides stomach acids (which may in themselves work differently than sulfuric acid), there is mechanical scarification in the gizzard and/or intestines, digestive enzymes are present, and many more factors including the depositing of the seed within feces that may act as a fertilizer source.

The concept of "the bird" brings about the idea of a synergistic effect. Two or more variables coming into play during a sequence of events, working together to bring about a greater end result than either one acting alone. I should have used multiple factors together in a single treatment, such as the addition of nutrient sources (e.g. potassium nitrate or magnesium nitrate), or other chemicals (e.g. gibberellic acid, potassium hydroxide, thiourea), or altering environmental influences (like increasing temperature and amount of light), along with the primary scarification factors such as sodium hypochlorite, sulfuric acid, or driselase.

It is believed that enzymatic specific scarification, such as was attempted with driselase, is worth continued pursuit. Since the *Rubus* seed coat consists of lignified fibers, perhaps a "ligninase" based enzyme may work. White rot fungi, *Phlebia subserialis* and *Phanerochaete chrysosporium*, among others, produce lignin peroxidases that degrade lignin. Crude extracts could be isolated from cultures which may contain enough ligninase to scarify the tough *Rubus* endocarp thereby increasing germination.

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## Appendices

## Appendix A. Seed Dimension and Weight of Selected *Rubus* Species

### A.1. Abstract.

Many lesser-known wild *Rubus* species from Ecuador, the People's Republic of China, and North America have been obtained on recent USDA plant collecting expeditions. The USDA-ARS National Clonal Germplasm Repository-Corvallis preserves these and additional species as seedlots and plants. In this study, the seed size of 43 *Rubus* species was measured. An 80-fold range in seed weight was observed within the genus. Asian species in the subgenera *Idaeobatus* and *Malachobatus* had the lightest seed, ranging from 0.3 mg (*R. eustephanus* Focke ex Diels) to 1.2 mg (*R. coreanus* Miq.). The seeds of about 80% of the species examined weighed less than 2 mg. Seeds of European species in the subgenera *Idaeobatus* and *Rubus* (formerly *Eubatus* of Focke) ranged from 1.3 to 3.0 mg. The South American *Orobatus* included several of the heaviest seeded species. The heaviest and largest seed was *R. megalococcus* Focke, subgenus *Rubus*, weighing 24.2 mg. Seed size was not related to ploidy level in wild species. Seed weight and length were positively correlated. Seed flatness was not related to seed length. Several of the smaller-seeded Asian species, such as *R. minusculus* A. Lev. & Van., *R. hirsutus* Thunb., and *R. eustephanus* had more drupelets per fruit than did those of larger-seeded species. This heritable trait may be useful in breeding for increased fruit size.



## A.2 Introduction

The USDA has recently sponsored expeditions to collect *Rubus* within Ecuador, the People's Republic of China, and North America. Many species collected during these trips represent taxa previously unavailable to American small fruit researchers (Ballington et al., 1991, 1993; Thompson, 1991, 1992). The USDA-ARS National Clonal Germplasm Repository-Corvallis preserves these and additional species as seedlots and plants. While fruit of these wild species are unsuitable for direct commercial production, they need to be evaluated for specific characters to determine which qualities may benefit *Rubus* improvement. The Repository will be collaborating with many scientists to evaluate these species and is beginning this effort by examining seed characteristics.

The seed size of cultivated *Rubus* is significant to both the processing industry and fresh market production (Darrow and Sherwood, 1931; Moore et al., 1973). Size can be measured by mass (Darrow and Sherwood, 1931; Petersen, 1921) or by length dimensions (Churchill et al., 1991, and 1992). Fruits with high pulp to seed ratios or with small, flat seeds are less objectionable for processing than thick-seeded fruits (Darrow and Sherwood, 1931). Large numbers of small seed are positively correlated with fruit firmness (Darrow and Sherwood, 1931).

The objective of this study was to determine seed weight and dimensions for a broad range of *Rubus* species and contrast these measurements with known North American and European blackberries, subgenus *Rubus*, and raspberries, subgenus *Idaeobatus*.

### A.3 Materials and Methods

From 1988 through 1993, seeds of 43 wild *Rubus* species were collected as ripe fruit, cleaned, packaged, labeled, and stored at -20C upon receipt. Five replicates of 100 seeds were counted and weighed, except for three species where less than 500 seeds were available. Analysis of variance was performed on the replicate weights. Mean separation was determined by least significant difference (LSD). The physical properties of drupelets were not examined because U.S. quarantine requirements state that foreign *Rubus* seed must be cleaned prior to entry into this country.

Seed size of economically important *Rubus* cultivars have been reported by Darrow and Sherwood (1931) and Moore et al. (1973). However, this information has not been compiled for many wild species. Seed dimensions were measured for 10 taxa representing the range of seed weight. Length (longitudinal axis), width, and thickness (transverse plane) were measured with electronic calipers on 10 randomly chosen seeds of each taxon. The seed length : thickness ratio ( $R_{lt}$ ) was calculated. Analysis of variance (ANOVA) and mean separation (determined by LSD) were performed on the seed dimensions and  $R_{lt}$ . Seed weight and length were correlated.

### A.4 Results and Discussion

The weight and length of seeds differed significantly among *Rubus* species (Table A.1, Fig. A.1). Seeds of two Ecuadorian species, *R. megalococcus* Focke, and *R. bogotensis* H.B.K., were significantly heavier and longer than those of other *Rubus*. The average weight of the heaviest-seeded species, *R. megalococcus*, was 24.2 mg; while the

next heaviest seeds (9.5 mg) were of those of *R. bogotensis* H.B.K. (Table A.1). Seeds of *R. nubigenus* H.B.K., from Ecuador, and *R. chamaemorus* L., from Alaska, were larger (7.4 mg) than those of most species examined. These seeds also were longer, wider, and thicker than those of other species (Fig. A.1). Seed length was correlated positively with seed weight ( $R=0.91$ ,  $P < 0.01$ ).

The average seed weight of European and North American blackberry and raspberry species ranged from 1.3 to 2.7 mg. Seeds of both the North American blackberry, *R. allegheniensis* Porter and *R. ursinus* Cham. & Schldl., and of the European blackberries, *R. procerus* Muller and *R. caesius* L., were larger than those of the European raspberry, *R. idaeus* L. (Table A.1). Peterson (1921) observed that seed weight of northeastern blackberry species ranged from 1.59 to 3.86 mg. *Rubus procerus* seeds were longer and thicker than those of *R. idaeus* (Fig. A.1).

*Rubus* ploidy ranges from diploid (Jennings, 1988) to 14-ploid (Nybom, 1986). Seed size is not related to ploidy level. Seeds of wild, diploid *R. allegheniensis* are lighter than seeds of the many cultivated tetraploid blackberry selections of the same species. Moore et al. (1973) reported that seeds of the cultivated blackberry weigh up to 4.3 mg. Similarly, seeds of diploid Arctic raspberry species are much lighter than those of the octaploid, *R. chamaemorus*, native to the same region (Table A.1). However, the seed weight of most other *Rubus* species is unrelated to ploidy. For example, many of the smallest-seeded species are diploid, but the second heaviest seed is also diploid. Both *R. roseus* and *R. nubigenus* are members of the subgenus *Orobatus*. Ploidy level for these species are unpublished. The seeds of the former weigh less than one third those of the

latter. Seeds of *R. glabratus*, another *Orobatus* whose ploidy is unknown, weigh less than one third those of *R. roseus*. Ploidy of the heaviest-seeded species, *R. megalococcus*, subgenus *Rubus*, is unreported.

The seeds of over 80 % of the species examined weighed less than 2 mg. Some of the smaller-seeded species included the Asian diploids, *R. eustephanus* Focke ex Diels, *R. hirsutus* Thunb., *R. minusculus* A. Lev. & Van, *R. rosifolius* Smith, and *R. corchorifolius* L. Darrow and Sherwood (1931) pointed out that black raspberries seem seedy because the seeds, though small, are numerous. They concluded that seed size is only one of the factors making up seediness, and that the proportion of seed weight to the total berry weight is more important than the seed size. These authors were referring to the cultivated raspberries and blackberries and not to the extremely large seeded species: *R. megalococcus*, *R. bogotensis*, *R. nubigenus* and *R. chamaemorus*.

We did not examine the seeds of *R. occidentalis* L. or *R. leucodermis* Dougl. ex Tor. & Gray, the North American black raspberries, but the small-seeded, numerous-drupelet Asian species of this study, were of similar "seediness". While these species would have limited use for processors or fresh market, they may be of significance to berry breeders. One breeding strategy to increase fruit size includes preliminary crosses with wild species to increase the number of drupelets per fruit. The small-seeded Asian species would be good initial candidates for such a project. Moore et al. (1975) have shown that seed size in blackberry is highly heritable, and that small seed size is partly dominant.

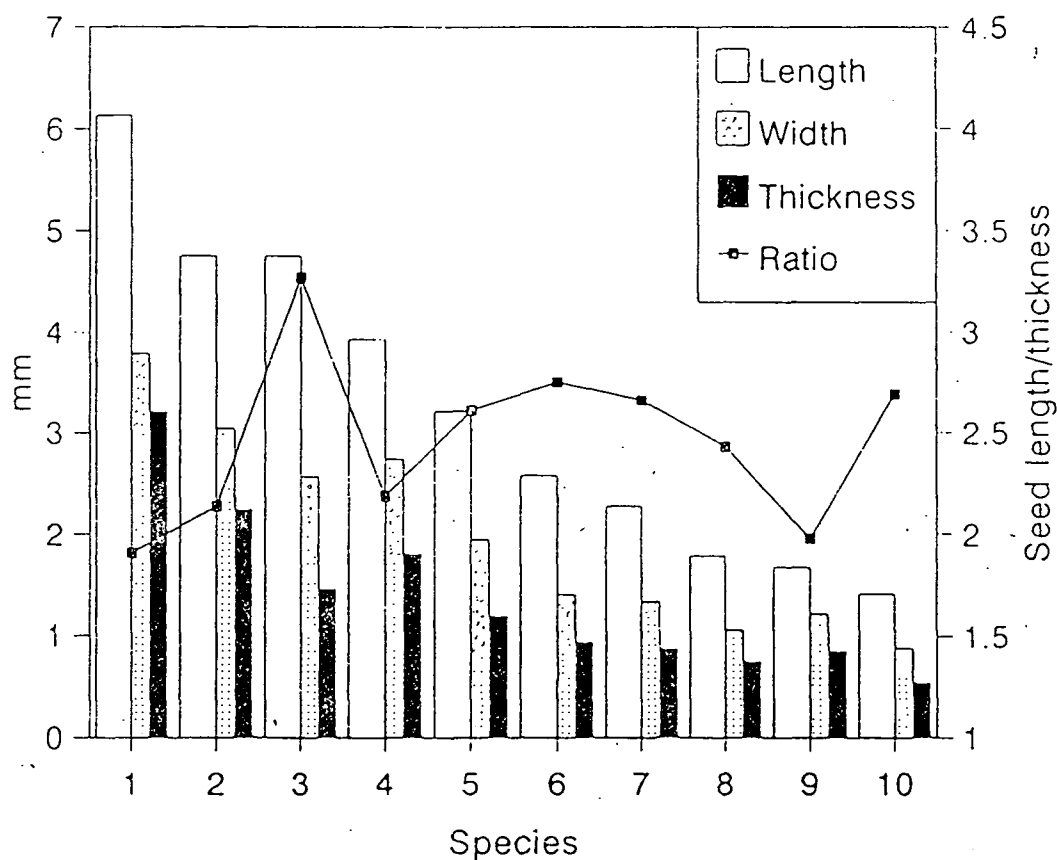


Fig. A.1. Seed length, width, and thickness (Y1 axis) and the ratio of seed length to thickness ( $R_{lt}$ , Y2 axis) for 10 *Rubus* species. Each bar or point represents the mean of 10 seeds. The species represented are: 1. *R. megalococcus*, 2. *R. bogotensis*, 3. *R. rubigemus*, 4. *R. chamaemorus*, 5. *R. procerus*, 6. *R. idaeus*, 7. *R. chingii*, 8. *R. ellipticus*, 9. *R. innominatus*, 10. *R. eustephanos*.

Table A.1. Seed weight of selected *Rubus* species, ordered heavy to light.<sup>z</sup>

| Corvallis accession no. | Species                                   | Collection location | Seed Weight (mg/seed) |
|-------------------------|---|---------------------|-----------------------|
| 1268                    | <i>R. megalococcus</i> Focke <sup>y</sup> | Azuay, Ecuador      | 24.2 a                |
| 1280                    | <i>R. bogotensis</i> H. B. K.             | Loja, Ecuador       | 9.5 b                 |
| 1249                    | <i>R. nubigenus</i> H. B. K.              | Carchi, Ecuador     | 7.4 c                 |
| 1757                    | <i>R. chamaemorus</i> L.                  | Alaska, U.S.A.      | 7.4 c                 |
| 1262,1270               | <i>R. glabratus</i> Kunth                 | Carchi, Ecuador     | 3.7 <sup>x</sup>      |
| 397                     | <i>R. procerus</i> Muller                 | Oregon, U.S.A       | 3.0 d                 |
| 1234                    | <i>R. caesius</i> L.                      | Tajikistan          | 2.7 e                 |
| 495                     | <i>R. allegheniensis</i> Porter           | Wisconsin, U.S.A.   | 2.7 e                 |
| 1266                    | <i>R. roseus</i> Poir.                    | Carchi, Ecuador     | 2.4 e                 |
| 1252,1255,1258          | <i>R. coriaceus</i> Poir.                 | Carchi, Ecuador     | 2.3 <sup>w</sup>      |
| 185                     | <i>R. ursinus</i> Cham. & Schldl.         | Oregon, U.S.A.      | 2.1 f                 |
| 1248                    | <i>R. robustus</i> C. Presl.              | Carchi, Ecuador     | 1.8 g                 |
| 239                     | <i>R. idaeus</i> L.                       | Scotland, U.K.      | 1.7 gh                |
| 407                     | <i>R. glaucus</i> Benth.                  | Hawaii, U.S.A.      | 1.7 h                 |
| 399                     | <i>R. hawaiiensis</i> A. Gray             | Hawaii, U.S.A.      | 1.5 i                 |
| 1250                    | <i>R. adenothallus</i> Focke              | Carchi, Ecuador     | 1.5 ij                |
| 626                     | <i>R. sachalinensis</i> A. Leveille       | Jilin, China        | 1.5 ij                |
| 186                     | <i>R. parviflorus</i> Nutt.               | Oregon, U.S.A.      | 1.4 jk                |
| 822                     | <i>R. caucasicus</i> Focke                | Russian Federation  | 1.4 k                 |
| 1630,1634,1635          | <i>R. coreanus</i> Miq.                   | Guizhou, China      | 1.2 l                 |
| 1658                    | <i>R. parvifolius</i> L.                  | Guizhou, China      | 1.1 lm                |
| 1277                    | <i>R. urticifolius</i> Poiret             | Zamora, Ecuador     | 1.0 mn                |
| 1620,1035               | <i>R. chingii</i> Hu                      | Jiangsu, China      | 1.0 mn                |
| 1671                    | <i>R. swinhoei</i> Hance                  | Guizhou, China      | 1.0 mn                |
| 1064,1061               | <i>R. hoffmeisterianus</i> Knth & Bche    | Gilgit, Pakistan    | 0.9 no                |
| 1073,1068,1650          | <i>R. niveus</i> Thunb.                   | Swat, Pakistan      | 0.9 no                |
| 175                     | <i>R. kawakamii</i> Hayata                | Taiwan              | 0.8 op                |
| 178                     | <i>R. hayata-koidzumii</i> Naruhashi      | Taiwan              | 0.8 op                |
| 1645,1642               | <i>R. multibracteatus</i> A.Lev. & Van.   | Guizhou, China      | 0.7 pq                |
| 246                     | <i>R. lasiostylus</i> Focke               | Asia (through U.K.) | 0.7pqr                |
| 1039                    | <i>R. innominatus</i> S. Moore            | Jiangxi, China      | 0.7 qr                |
| 160,627                 | <i>R. crataegifolius</i> Bunge            | China               | 0.7 qr                |
| 158                     | <i>R. microphyllus</i> L.                 | Japan               | 0.7 qr                |
| 181,1183,1710           | <i>R. lambertianus</i> Ser.               | Jiangsu, China      | 0.7 qr                |
| 174                     | <i>R. formosensis</i> Kuntze              | Taiwan              | 0.6 rs                |
| 1695                    | <i>R. setchuenensis</i> Bureau & Fran.    | Guizhou, China      | 0.5 st                |
| 1665                    | <i>R. pinfaensis</i> A. Lev. & Van.       | Guizhou, China      | 0.5 st                |
| 394                     | <i>R. ellipticus</i> Smith                | Nepal               | 0.5 st                |
| 1628                    | <i>R. corchorifolius</i> L.               | Guizhou, China      | 0.5 tu                |
| 188                     | <i>R. rosifolius</i> Smith                | Java, Indonesia     | 0.3 uv                |
| 161                     | <i>R. minusculus</i> A. Lev. & Van.       | Japan               | 0.3 v                 |
| 1040,1037,1038          | <i>R. hirsutus</i> Thunb.                 | Jiangsu, China      | 0.3 v                 |
| 1638,1639               | <i>R. eustephanus</i> Focke ex Diels      | Guizhou, China      | 0.3 v                 |

<sup>z</sup> Mean of five replications of 100 seeds, mean separation by LSD,  $P \leq 0.05$ .<sup>y</sup> Mean of five replicates of 50 seeds.<sup>x</sup> Insufficient seed for analysis of variance (ANOVA); mean of 91 seeds.<sup>w</sup> Insufficient seed for ANOVA; mean of 80 seeds.

Seed flatness of 10 *Rubus* species was not related to seed length (Fig. A.1). Seed length varied from more than 3 to less than 2 times the thickness. *Rubus nubigenus* and *R. idaeus* had the largest  $R_{lt}$  values, i.e., the thinnest seeds for their length (Fig. A.1). While seeds of *R. nubigenus* had the highest  $R_{lt}$ , its thickness was larger than the length of most seeds tested. The  $R_{lt}$  of *R. eustephamus* (the smallest seed), *R. chingii*, and *R. procerus* was less than 3. In contrast, the  $R_{lt}$  of *R. megalococcus* (the largest seed), *R. innominatus*, *R. bogotensis*, and *R. chamaemorus* was about 2 (Fig. A.1). This second group of seeds were more "round" than "flat" in cross-section.

Generally, the heaviest seeds, were members of the South American subgenera, *Rubus* and *Orobatus*, although the monotypic, circumpolar-boreal subgenus, *Chamaemorus*, also had large seeds. Seeds of species in the economically important subgenus, *Rubus*, were slightly heavier than those of the European *Idaeobatus*. The Asian *Idaeobatus* and *Malachobatus* species had the lightest, shortest seeds.

In summary, the seeds of *R. megalococcus*, *R. bogotensis*, *R. nubigenus*, and *R. chamaemorus* were heavier and longer, than those of other *Rubus*. We observed an 80-fold range in seed weight among species within the genus. Seed of blackberry species were longer and thicker than those of raspberry species. Many Asian species had smaller seeds than did those of the European red raspberry, *R. idaeus*. While seed length and weight were correlated, seed length was not related to seed flatness. Seed weight is not related to the ploidy level of wild *Rubus* species.

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## Appendix B. Percent Endocarp

In order to overcome exogenous dormancy, the *Rubus* seed embryo must be able to expand, and push its way out of the seed coat. Percent endocarp is one way of relating how much resistance the expanding embryo has to proportionally overcome. *Rubus chamaemorus* is difficult to germinate and is an extremely heavy seed at 7.4 mg (Appendix A). *Rubus multibracteatus* germinates readily, and is significantly lighter at only 0.7 mg (Appendix A). The seed of *R. chamaemorus* has a much thicker endocarp overall than *R. multibracteatus* (data not shown), but I was interested in knowing if the ratio of the endocarp to the embryo was similar. Three replicates of 5 seeds had their dry weight recorded, the embryos were excised out of the endocarp, and then the endocarp was reweighed to determine percent endocarp for each species (Table B.1).

Table B.1. Percent endocarp of *R. chamaemorus* and *R. multibracteatus*. Weights are of the endocarp before the embryo was excised out and after.

| Species                   | Wt. before (g) | Wt. after (g) | % Endocarp | Mean (%) |
|---------------------------|----------------|---------------|------------|----------|
| <i>R. chamaemorus</i>     | 0.0421         | 0.0279        | 66.27      | 64.98    |
|                           | 0.0397         | 0.0249        | 62.72      |          |
|                           | 0.0411         | 0.0271        | 65.94      |          |
| <i>R. multibracteatus</i> | 0.0037         | 0.0025        | 67.57      | 68.93    |
|                           | 0.0039         | 0.0028        | 71.79      |          |
|                           | 0.0043         | 0.0029        | 67.44      |          |

The data shows the mean percent endocarp is approximately the same for both species. What the ratio doesn't show is the toughness of the endocarp: Based on subjective measurement, the endocarp of *R. multibracteatus* is significantly softer and easier to cut with a scalpel than the endocarp of *R. chamaemorus*.

## Appendix C. Seed Lot Viability and Tetrazolium Testing

With most of the *Rubus* species used in these experiments, poor germination was recorded. It is important to know why percent germination was not a perfect 100%. There may be several reasons why total germination might not be achieved. Viability may be lost due to the treatment, such as over-scarification with potentially damaging chemicals, sulfuric acid or sodium hypochlorite. Also, due to random censoring, which is fungal or mold contamination. Sterile technique eliminated most mold problems.

Total germination might not be achieved due to right censoring or premature termination of the germination tests. Duration for *Rubus* germination experiments could be lengthened from 90 days to several years and stratification cycles worth of observations and recordings!

Total germination might not be achieved due to the initial viability of the seed lot. Viability may be poor if during seed formation the ovule doesn't mature correctly becoming non-functional, or viability may also be lowered due to age, improper storage conditions, and unsuitable moisture content (McDonald and Copeland, 1985).

One way to predetermine the initial viability of the seed lot that is being worked with is by using a tetrazolium staining test. Tetrazolium staining is a color test to detect metabolic activity of an embryo and thus is a measure of a seed's viability (Moore, 1985). A water solution with 2,3,5-triphenyl tetrazolium chloride (TTC) salt penetrates embryonic tissue and becomes oxidized by hydrogen ions released from dehydrogenase enzymes forming red formazan.

Only living tissue will stain red. Patterns and intensity of the stain relate the degree of viability and possibly detect fractures and dead areas in the embryo. A properly stained embryo will be completely stained. There should be no necrosis or nonviable tissue which is observed as abnormal staining. The staining patterns are difficult to evaluate and require experience to do accurately.

The staining procedure for *Rubus* was to moisten 100 seed per species on sand media for 72 hours under warm stratification conditions. A scalpel cut longitudinally across the endocarp exposed the seed's tissue (Fig. C.1). Care must be taken to avoid cutting the embryonic axis to the basal end, while cutting full depth at the distal end, leaving one-half of the seed (Moore, 1985).

The cut seed was immersed in a 1% TZ solution and placed in an incubator overnight (18-24 hours) at 15/25C. The seed was removed from the incubator and four drops of lactic acid was used to hold the stain. After staining, the embryo and cotyledon was viewed by removal out of the endocarp. The testa (inner seedcoat) sometimes stayed around the embryo and also needed to be removed. Observation and evaluation under a dissection scope scored the seed based on stain intensity and patterns.

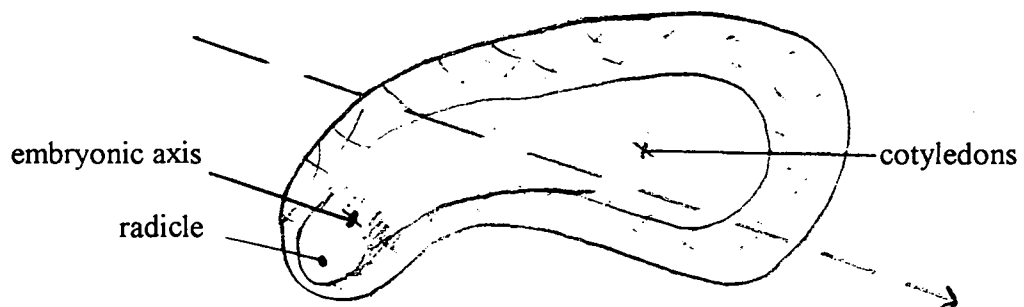


Fig. C.1. Longitudinal cut through entire length of the *Rubus* seed's midsection.

Most of the *Rubus* species used in this thesis underwent TZ staining to correlate seed lot viability with germination test results (Table C.1). The *R. chamaemorus* (Cor. #1774) seed lot did not germinate at all (chapter 5) and TZ tests showed it to be a completely nonviable seed lot since no embryonic staining was observed.

*Rubus multibracteatus*, which is believed to have no or little dormancy, closely matched the TZ estimated viability. The other 3 *Rubus* species, *R. idaeus* ‘Amber’, *R. ursinus* and *R. parviflorus*, had TZ viability estimates that were considerably higher than actual recorded percent germination. The difference between the two scores may be due to dormancy, either exogenous or endogenous. Improved germination pretreatments and/or longer test duration’s may be able to increase the final percent germination recorded.

Table C.1. Estimated viability of *Rubus* species by using tetrazolium (TZ) staining tests and comparing the results to the maximum percent germination achieved during experimentation.

| Species                       | TZ viability (est.) | Max. germination (%). |
|-------------------------------|---------------------|-----------------------|
| <i>R. chamaemorus</i> (#1774) | 0                   | 0                     |
| <i>R. idaeus</i> ‘Amber’      | 73                  | 6                     |
| <i>R. multibracteatus</i>     | 92                  | 88                    |
| <i>R. parviflorus</i>         | 67                  | 45                    |
| <i>R. ursinus</i>             | 70                  | 43                    |

But since *Rubus* can have a dormant embryo (endogenous dormancy) intensity of the staining was inconsistent and subjective evaluation was difficult on most of the species. General trends could be determined with some level of confidence, but exact percent of seed lot viability was unknown.

To make tetrazolium staining a better test for *Rubus* seed, it is recommended to increase the metabolic activity with a warm and/or cold stratification period of several days to months. Higher staining temperatures of 38-40C may also increase staining success. Tetrazolium test are not perfect, but can provide useful information about the potential viability of a given seed lot.

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