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

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Abstract. We contrasted the effect of liquid nitrogen (LN₂), sulfuric acid (H₂SO₄), and a nontreated control on the germination of six *Rubus* species. We also were interested in determining if LN₂ could be an effective mechanical scarifying agent for these species. Seeds of each species were treated with three 3-minute dips in LN₂ with alternating 10-minute thaws, with H₂SO₄ for 30 minutes, or 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 LN₂ was not significantly different than the control. Germinated seedlings from the LN₂ treatment of each species showed normal development upon planting, indicating that long-term cryogenic preservation of these *Rubus* species seeds may be possible. The H₂SO₄ 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 LN₂ treatment. The alternative LN₂ application techniques that have been attempted thus far have not significantly improved *Rubus* seed germination compared with that of the control.

Some *Rubus* seeds have a thick endocarp and testa (seedcoat) that can impede germination (Ourecky, 1975). 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 (H₂SO₄) is used in blackberry breeding programs (Moore et al., 1974). However, 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 (LN₂) was examined as a germination pretreatment for legume seeds (Pritchard et al., 1988). Legume hard-seededness 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). We thought that this technique could improve *Rubus* germination.

Our objectives were to contrast the effect of LN₂ and H₂SO₄ on *Rubus* seed germination and to determine if LN₂ could be used as a mechanical scarifying agent for *Rubus*.

Materials and Methods

Rubus seed for this experiment was obtained from the U.S. Dept. of Agriculture–Agricultural Research Service National Clonal Germplasm Repository, Corvallis, where it was stored for 2 to 10 years in aluminum foil-lined paper envelopes at –18C. Nine lots of 100 seeds each of *R. multibracteatus* (Corvallis accession no. 1642), *R. parviflorus* (Cor. no. 1738), *R. eustephanos* (Cor. no. 1638), *R. leucodermis* (Cor. no. 692), *R. ursinus* (Cor. no. 1739), and *R. chamaemorus* (Cor. no. 1728) were obtained with an automatic counter (Seedbuco, Chicago). Each lot was assigned to

one of three treatments: LN₂, H₂SO₄, and a control, based on a randomized complete block design. Each treatment consisted of three 100-seed replications.

The LN₂-treated seeds were placed in paper envelopes. A plastic cooler was filled to a depth of ≈3 cm with LN₂. The upright seed packets were immersed in the LN₂ for 3 min, removed, and allowed to thaw at ≈26C for 10 min. This procedure was repeated three times. Liquid nitrogen was replenished to maintain a constant volume.

For treatment with H₂SO₄, seeds were placed in 13-mm test tubes with enough concentrated acid (2 to 4 ml) to cover them (Moore et al., 1974). After 30 min, the H₂SO₄ 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 were rinsed with water for 5 min.

Seeds of the LN₂-treated, H₂SO₄-treated, and nontreated control groups were then placed directly onto moistened sterilized sand in germination boxes. Boxes were put in a Hoffman UE 650 germinator (Albany, Ore.) in a randomized complete block design with each shelf considered a block. The germinator had alternating temperatures of 10C for 14 h and 25C for 10 h and provided 10 h of fluorescent white light during the 25C period plus 2 h more during the 10C 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.) for 6 weeks at 3C in darkness (cold stratification period), and finally was returned to the germinator under the original conditions for an additional 12 weeks (germination period). Radicle emergence was counted during 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 ($P \leq 0.05$).

Results and Discussion

After the first 8 weeks of warm stratification, only three species, *R. eustephanos*, *R. multibracteatus*, and *R. parviflorus*, showed some germination in the control treatment (Table 1). The LN₂-treated seeds were not significantly different from the control. Two species, *R. multibracteatus* and *R. parviflorus*,

Table 1. Germination percentages of six *Rubus* species after warm (8 weeks) and warm-cold-warm treatments (total 26 weeks).

Species	Avg germination (%) by treatment					
	Warm			Warm-cold-warm		
	LN ₂	H ₂ SO ₄	Control	LN ₂	H ₂ SO ₄	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 ^a	51 a	1 b	25 b	75 a	12 b
<i>R. multibracteatus</i>	74 b	86 a	74 b	84 a	88 a	85 a
<i>R. chamaemorus</i>	0	0	0	39 a	26 b	32 ab
<i>R. parviflorus</i>	3 b	26 a	5 b	17 b	45 a	21 b

^aMean separation within rows by LSD at $P \leq 0.05$.

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Fig. 1. Germination curves for *R. multibracteatus* and *R. parviflorus* seeds treated with sulfuric acid (H_2SO_4), liquid nitrogen (LN_2), and a nontreated control. Mean germination percentages (three replications of 100 seeds each) are plotted throughout the 26-week germination period. Warm, 25°C; cold, 3°C.

germinated following LN_2 treatment without cold stratification (Table 1). The H_2SO_4 treatment significantly improved germination rate (Fig. 1) and germination percentage (Table 1) in these species. This significant response to acid scarification during a warm stratification period indicated that exogenous rather than endogenous embryo dormancy prevented nontreated seed of these species from germinating.

After the final 12 weeks, germination for the LN_2 treatment was not significantly different from that of the control for each of the six species. Exposure to LN_2 presumably caused a drastic temperature shift in the seedcoat, 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 LN_2 was planted and had normal development and vigor. Liquid nitrogen did not significantly alter germination positively or negatively. The LN_2 treatment did not damage the embryo, suggesting that LN_2 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 germ-

plasm; but, to our knowledge, no methods have been published for *Rubus*.

In agreement with findings on other *Rubus* species (Jennings and Tulloch, 1965; Moore et al., 1974), H_2SO_4 significantly improved the final germination percentage in four of the six species (Table 1). The *R. multibracteatus* germination percentages were similar for the three treatments after the final germination period. The final germination percentage of LN_2 -treated *R. chamaemorus* was significantly higher than for the H_2SO_4 treatment; however, neither differed from that of the control. Both *R. chamaemorus* and *R. multibracteatus* have a similar endocarp : embryo ratio by weight (Peacock, unpublished), but the endocarp of *R. multibracteatus* could be cut more easily 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. Scarification of this species is unnecessary because 85% of the control group germinated. The germination rate for *R. multibracteatus* and *R. parviflorus* were initially increased with H_2SO_4 over that of LN_2 or the control (Fig. 1).

Sulfuric acid was the most effective scarifying treatment in this study; however, overexposure can result in blackberry seed injury (Moore et al., 1974). We also investigated a modified LN_2 application as a scarifying agent with six and nine repeated immersions (spraying the seedcoat with water immediately before LN_2 immersion) and more extreme temperature fluctuations (using an oven set at 38°C for the thaw period). None of these protocols have shown a significant germination increase (data not shown) as compared with the nontreated control. We conclude that LN_2 is not an effective scarifying agent for *Rubus* seeds. However, our study suggests that long-term cryogenic preservation of *Rubus* seeds should be investigated for germplasm preservation.

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