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Hydrogen peroxide seed scarification of New Mexico collections of *ribes cereum*

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Summary

Ribes cereum is an early-colonizing shrub species being evaluated for disturbed-land revegetation in New Mexico. Along with embryo dormancy, seed coat dormancy is thought to occur in this species, but acid scarification has failed to improve germination consistently. To examine the efficacy of scarification using hydrogen peroxide, seeds from six *R.* cereum collections from throughout New Mexico were soaked in 3% hydrogen peroxide for 0, 4, 8, or 16 hours and then stratified for 0, 60, 90 or 120 days. For all collections, a four-hour soak improved germination. The combination of a four-hour hydrogen peroxide soak followed by 120 days of stratification was the optimal treatment combination for most collections. With the exception of the southernmost collection, stratification was more effective than hydrogen peroxide scarification in promoting *R.* cereum germination. For this small-seeded species, a four-hour soak in 3% peroxide effectively degrades seed coat tissues without causing embryo damage. The choice between sulfuric acid and hydrogen peroxide as chemical scarification agent is discussed in terms seed size, seed coat thickness and seed susceptibility to damage.

Introduction

Ribes cereum Dougl. (*Grossulariaceae*) is a shrub species occurring throughout the western United States, including ponderosa pine and mixed conifer forests of New Mexico. *Ribes cereum* is a valuable reclamation species because it grows well on all soil types, inhabits open forests, forest edges and shrub communities and occurs across a range of temperatures and precipitation amounts (Marshall and Winkler, 1995). *Ribes cereum* has been observed to be an early colonizer of disturbed sites within Douglas fir communities, providing canopy favorable for the establishment of Douglas fir seedlings (Marshall and Winkler, 1995).

Several dormancy mechanisms are suspected to occur in *Ribes* species – primarily embryo dormancy resulting from a rudimentary embryo – but also seed coat dormancy controlled by growth inhibitors and an impermeable seed coat (Pfister, 1974; Goodwin and Hummer, 1993). Embryo dormancy of most *Ribes* species can be broken by a long stratification period ranging from 60 to 300 days, depending on species (Fivaz, 1931; Quick, 1936; Heit, 1971; Pfister, 1974; Young and Young, 1992; Goodwin and Hummer, 1993). Although most New Mexico collections of *R.* cereum showed increasing germination with increasing duration of stratification up to 120 days, germination of the southernmost

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collection was not improved by any duration of stratification compared to untreated seeds (Rosner *et al.,* 2001). These data confirm other reports that seed dormancy level in *Ribes* varies widely between and among seed lots (Pfister, 1974; Young and Young, 1992).

Seed coat dormancy in some *Ribes* species has been broken by acid scarification. Germination of *Ribes nigrum* Linn. was improved by a five-minute soak in 50% sulfuric acid (Adam and Wilson, 1967). A 35-minute soak in "commercial" sulfuric acid improved germination of *Ribes rotundifolium* Michx. (Fivaz, 1931). Germination of *Ribes lacustre* (Pers.) Poir. and *Ribes viscosissimum* Pursh. was improved by five-minute soaks in 2% to 10% sulfuric acid solution (Pfister, 1974). The effect of sulfuric acid in promoting germination of *R. cereum* can be variable. In a study examining *R. cereum* seed collections from throughout New Mexico, acid scarification improved germination for only half of the collections evaluated and the optimal scarification duration for those collections showing a positive response to treatment varied from two to eight minutes. (Rosner, 2000).

Published literature documenting the use of hydrogen peroxide as a seed pretreatment for any *Ribes* species is lacking. Hydrogen peroxide promotes germination in other species both by disintegrating hard seed coats (Chien and Lin, 1994) and oxidizing germination inhibitors in the seed coat (Ogawa and Masaki, 2001). Hydrogen peroxide is also commonly used to surface-sterilize seeds (Prochazkova, 1996; Barnett *et al.,* 1999). Hydrogen peroxide scarification has been used successfully to promote germination of Rocky Mountain shrub species *Purshia tridentata* Pursh, *Purshia glandulosa* Curran and *Cowania mexicana* D.Don. (Young *et al.,* 1981). Treatment of seeds in hydrogen peroxide at concentrations of 1% to 30% has been employed; using weaker solutions and longer incubation durations may reduce the risk of seed damage (Young, Budy and Evans, 1984).

The aim of this research was to examine the effectiveness of scarification in 3% hydrogen peroxide as an alternative to acid scarification and to assess variability in response to hydrogen peroxide treatments (in combination with stratification treatments) in terms of latitude and elevation of seed collection.

Materials and methods

Seeds were collected at six locations throughout New Mexico (table 1). Berries were collected from a minimum of five plants and varying plant heights at each location. Collection locations were selected to encompass both a range of latitudes within New Mexico and a range of elevations at Molycorp mine in Questa, New Mexico. Before cleaning, fruits were soaked overnight in tap water (floating seeds were discarded), fermented for 48 hours, mashed and dried. Seeds were dislodged from pulp in a rubbing box and separated from pulp in a Dakota blower. Cleaned seeds were stored dry within paper envelopes in a walk-in cooler maintained at 5°C for almost two years until the beginning of the experiment.

This experiment utilized a completely randomized design with a factorial treatment structure. Factors were seed collection, stratification length and duration of hydrogen peroxide soak. Stratification treatments were imposed after scarification treatments were completed. Six collections were used. Hydrogen peroxide soak durations were 0, 4, 8 and 16 hours. Stratification treatment levels were 0, 60, 90 and 120 days. All treatment

Table 1. Seed collections used in Ribes cereum germination studies.

Seed Source	Latitude	Location	Elevation	Collection Date
Capulin	36°42' N	Questa, NM	2987 m	Oct. 10, 1997
Raspberry Ridge	36°42' N	Questa, NM	2987 m	Oct. 12, 1997
Pinon Knob	36°42' N	Questa, NM	2896 m	Oct. 21, 1997
Mahogany Hill	36°42' N	Questa, NM	2774 m	Oct. 13, 1997
Rociada	35°50' N	Rociada, NM	2377 m	Oct. 17, 1997
Gila	34°06' N	Gila National Forest, NM	2499 m	Oct. 21, 1997

combinations were tested with four replications of 100 seeds. Germination data were analyzed as a six (collection) by four (stratification) by four (scarification) factorial and then separately by collection.

Hydrogen peroxide treatments involved submersing seeds in 20 ml of hydrogen peroxide (VWR 3% Stabilized) at room temperature. Seeds were placed in the hydrogen peroxide and stirred vigorously for 30 seconds. Following treatment, the seeds were removed from the hydrogen peroxide and rinsed under running tap water for one minute. Prior to stratification, scarification control seeds were surface sterilized by a one-minute soak in hydrogen peroxide (VWR 3% Stabilized). Scarification control seeds not undergoing stratification were surface sterilized prior to the start of germination testing. After surface sterilization, seeds were rinsed for one minute in tap water.

Stratification treatments were imposed by placing seeds between filter papers (VWR 9.0 cm Qualitative Grade #3) moistened with distilled water. Filter papers were placed in 100 mm petri dishes sealed in 15 × 16 cm self-sealing poly bags within a walk-in cooler. Cooler temperatures fluctuated from a mean daily low of -1.2°C to a mean daily high of 5.4°C. Stratification start dates were staggered so that all seeds completed stratification at the same time.

For germination testing, seeds were maintained between filter papers in petri dishes within poly bags. Petri dishes in poly bags were placed directly on greenhouse benches under natural light (filtered through shade cloth) with fluctuating temperatures. A one-foot border on all sides of each bench was left empty in order to minimize temperature differences between samples. Thermostat settings in the greenhouse were set to maintain daytime highs near 30°C and nighttime lows near 15°C. Daytime high temperatures averaged 34.1+/-0.49°C and nighttime low temperatures averaged 15.2 +/- 0.26°C during the experiment. Germination testing took place from May 14, 1999 through June 11, 1999.

Germinated seeds were counted and removed when samples were taken out of stratification and after 7, 14, 21 and 28 days of incubation. Filter papers were remoistened as needed. Seeds were considered germinated when the radicle became visible to the naked eye. There were some problems with fungal infestation of petri dishes. Rotten seeds were counted and removed from the petri dish. Ungerminated seeds not counted as rotten were left in the petri dish and returned to the greenhouse bench.

Categorical analysis of variance using SAS Proc Catmod (SAS Institute 1989) was used to determine treatment differences in germination using a factorial treatment structure. This procedure is a generalization of the chi-square (X^2) test of homogeneity, which uses the "logit" –the natural log of the ratio of germinated to non-germinated seeds for each treatment– as the response. Maximum-likelihood analysis was used to calculate X^2 test statistics. Observed significance levels less than α=0.05 were considered significant. Percentages and standard errors were calculated for main effects and interaction combinations. Approximate pairwise Z-statistics were used to conduct pairwise comparisons of main treatment effects, both overall and within collection, using a conservative alpha value of 0.05 divided by the total number of comparisons. Pairwise comparisons of combinations of treatment factors were informally tested; means were considered different if the higher mean minus its standard error did not overlap the lower mean plus its standard error.

Two-factor interactions were tested in the presence of a three-factor interaction. This approach can be justified when testing an environmental factor not controllable in ordinary practice and when the interaction is weak – there is no crossover effect (Murray, Sterling and Schroeder, 1999). For reclamation purposes, seed collection is an environmental factor that is not always controllable. Main effects were uninformative relative to two-way effects and are not discussed.

Results

Stratification, collection, hydrogen peroxide soak and all interactions of these factors impacted germination (table 2). Although a three-factor interaction was present, there was relative consistency among collections in response to combinations of stratification and hydrogen peroxide treatments (figure 1). Except for the southernmost collection (Gila), collection germination tended to improve with increasing stratification duration, although not by the same differential, and for all collections, germination tended to be highest following a four-hour peroxide soak, but also not by the same differential.

With the exceptions of the Capulin and Gila collections, the optimal combination of hydrogen peroxide soak and stratification was four hours/120 days (figure 1). The Capulin collection germinated equally well following the combination of eight hours/120 days as it did following four hours/120 days, but the Gila collection germinated best following a four-hour peroxide soak without stratification. In all but the southernmost collection (Gila), stratification was more effective than hydrogen peroxide scarification in promoting *R. cereum* germination.

The relative consistency of the three-way effect justifies examination of two-way effects. A four-hour soak in hydrogen peroxide improved germination at all stratification levels, but degree of improvement varied (figure 2). At all stratification levels, germination decreased as hydrogen peroxide soak duration was increased beyond four hours, but by varying amounts. Germination improvement resulting from a four-hour peroxide soak was least when a 90-day stratification treatment was used and greatest following a 120-day stratification treatment.

The effect of hydrogen peroxide treatment was consistent across collections, with a four-hour soak increasing germination by 6 to 12 percentage points depending on

Table 2. Categorical analysis of variance table for *Ribes cereum* germination response to stratification, seed collection and hydrogen peroxide soak.

Source of Variability	df	Chi-Square	Observed Significance Level
Strat[1]	3	1202	<.0001
Coll[2]	5	745	<.0001
H_2O_2[3]	3	134	<.0001
Strat × Coll	15	1516	<.0001
Strat × H_2O_2	9	136	<.0001
Coll × H_2O_2	15	256	<.0001
Strat × Coll × H_2O_2	45	245	<.0001

[1] Strat = Stratification
[2] Coll = Collection
[3] H_2O_2 = Hydrogen Peroxide Scarification

collection and germination falling as treatment duration was increased (figure 3). The effect of stratification varied across collections (figure 4). For all collections except the southernmost (Gila), germination increased with each increase in stratification duration. For Gila, no level of stratification improved germination.

As stratification duration was increased, the proportion of seeds germinating during stratification treatment imposition also increased (figure 5). For seeds undergoing 120 days of stratification, over two thirds of the observed germination occurred in stratification. Hydrogen peroxide treatment, however, did not further increase germination during stratification treatment imposition (figure 6).

A four-hour soak in 3% hydrogen peroxide followed by stratification did not significantly increase the number of seeds rotting during the course of the experiment (1.8%) compared to seeds undergoing only stratification (1.4%). However, 6.1% of seeds undergoing an eight-hour scarification treatment rotted and 16.7% of seeds undergoing a 16-hour scarification treatment rotted.

Discussion

Variability among collections in response to hydrogen peroxide treatments was low. For every collection, germination increased by 6 to 12 percentage points following a four-hour treatment. Variability in response to stratification can be explained by latitude of collection; the southernmost collection was the only collection failing to benefit from stratification. This latitudinal gradient has been observed previously in New Mexico populations of *Cercocarpus montanus* Raf (unpublished data) as well as *R. cereum* (Rosner *et al.*, 2001).

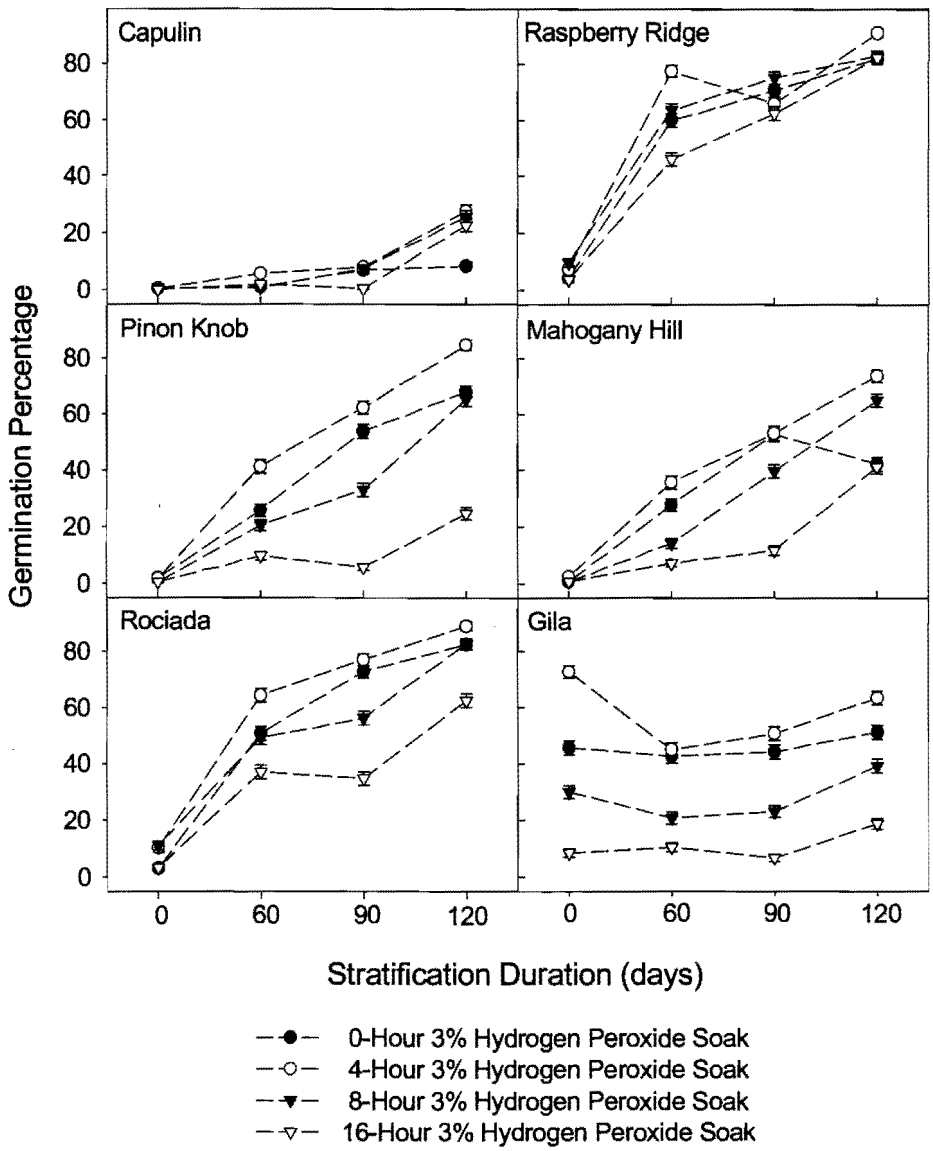

Figure 1. Effect of interaction between seed collection, stratification duration, and duration of soak in 3% hydrogen peroxide on *Ribes cereum* germination.

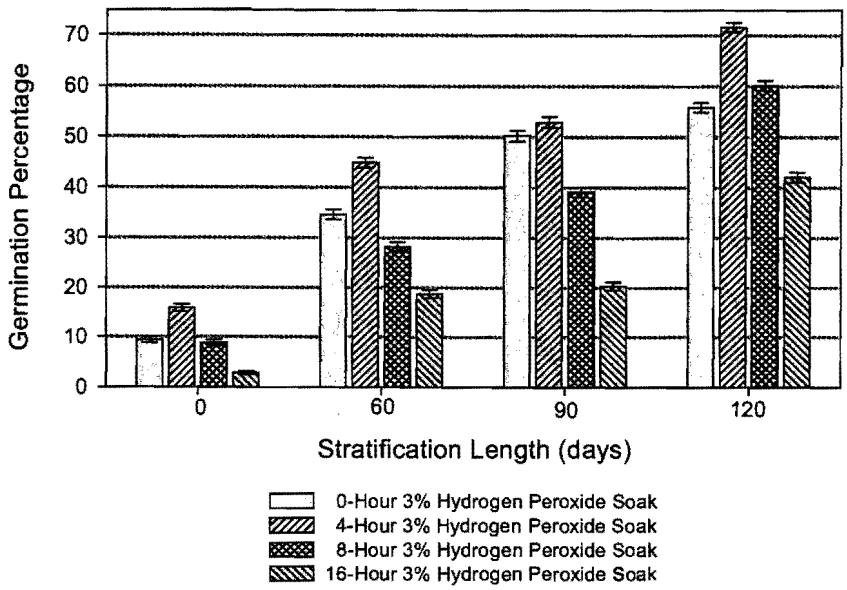

Figure 2. Effect of interaction between hydrogen peroxide soak duration and stratification length on *Ribes cereum* germination for data averaged over all seed collections.

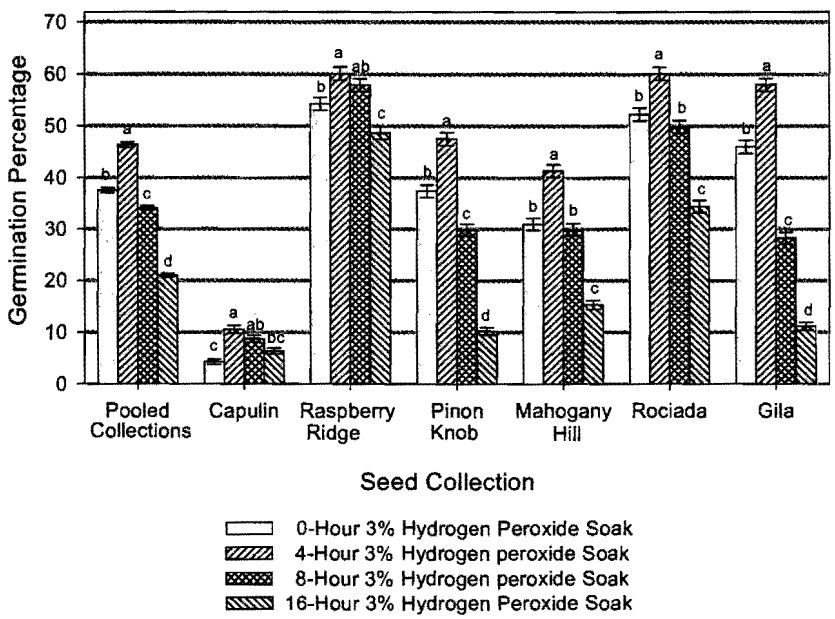

Figure 3. Effect of hydrogen peroxide soak duration, averaged across stratification treatments, on *Ribes cereum* germination by seed collection and averaged across collections—"Pooled Collections". Within each collection, means labeled with the same letter are not significantly different at α=0.0083 (0.05/6).

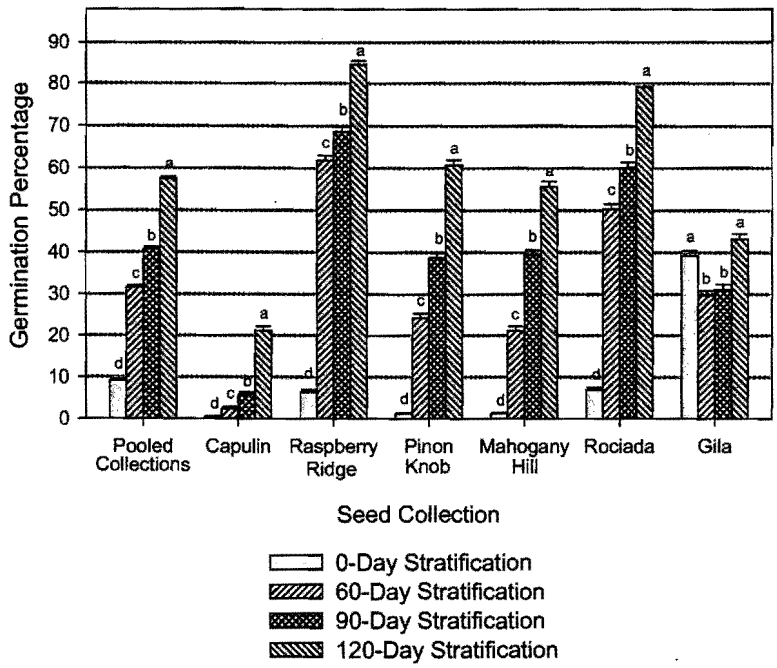

Figure 4. Effect of stratification treatment duration, averaged over scarification treatments, on *Ribes cereum* germination by seed collection and for "pooled collections". Within each collection, means labeled with the same letter are not significantly different at α=0.0083 (0.05/6).

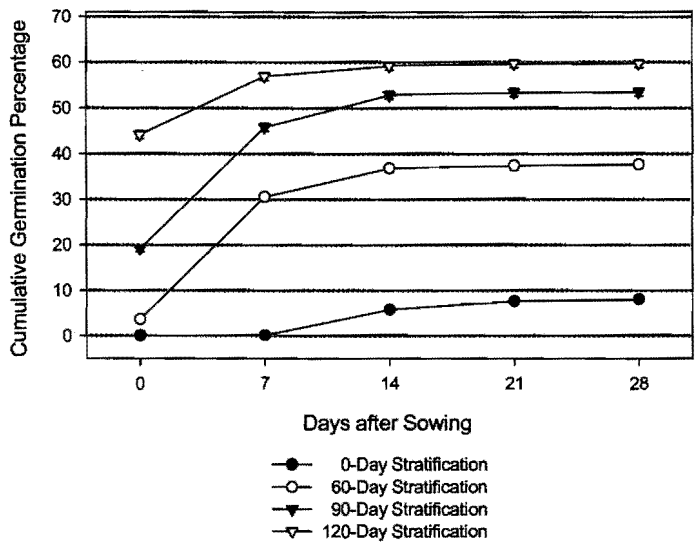

Figure 5. Effect of stratification treatment duration on the timecourse of *Ribes cereum* germination for data averaged over seed collections and hydrogen peroxide soak durations. Cumulative germination at day 0 accounts for all seeds germinated during stratification treatment.

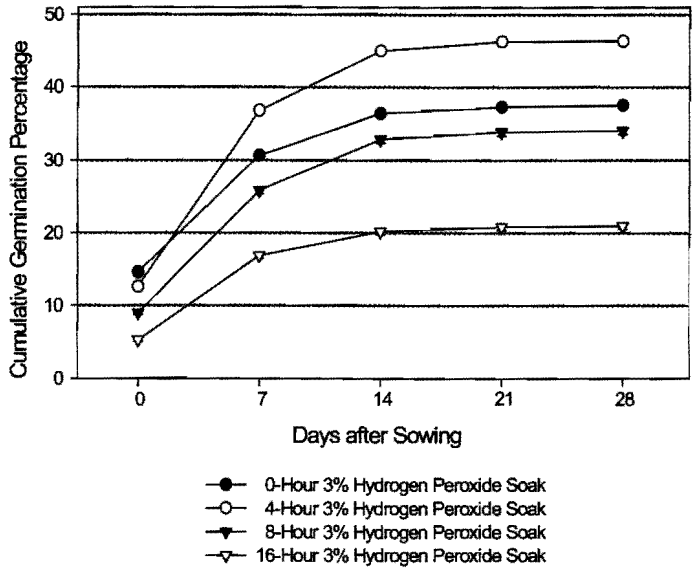

Figure 6. Effect of hydrogen peroxide soak duration on the timecourse of *Ribes cereum* germination for data averaged over stratification durations and seed collections. Cumulative germination at day 0 accounts for all seeds germinated during stratification treatment.

To be effective, a chemical scarification treatment must adequately degrade seed coat structures without causing damage to embryonic tissues. The choice of chemical scarification agent for a given species is influenced by seed size, seed coat thickness and seed susceptibility to oxidative damage. For species with hard, thick seed coats, a stronger agent such as concentrated sulfuric acid may be preferable, whereas a milder agent such as 3% hydrogen peroxide may be more effective for small-seeded species such as *R. cereum*. In a related study, *Symphoricarpos oreophilus* A. Gray seeds required a 30-minute sulfuric acid scarification treatment to obtain optimal germination, but seed weights for that species ranged from 5.2 to 10.2 mg per seed depending on collection (Rosner *et al.*, 2001). Seed weights for *R. cereum* in this study were 1.3 to 2.5 mg per seed depending on collection. In a separate study examining the effect of acid scarification on New Mexico collections of *R. cereum*, only half of the collections responded positively to treatment and optimal treatment duration was variable (Rosner, 2000). As the sulfuric acid soak duration was increased from two to eight minutes, the number of seeds rotting during stratification and germination testing increased from 17% to 35%. This increase in rotting is associated with an increase in seed damage, as seeds damaged or weakened by excessive treatment lose their ability to resist pathogenic deterioration (Murdoch and Ellis, 1992).

Success of four-hour soaks in 3% hydrogen peroxide in this study is partially attributable to low occurrence of seed damage as suggested by a low mean percentage of rotten seeds following treatment (1.8%). An increase in number of seeds rotting with increasing duration of hydrogen peroxide soak beyond four hours parallels the decrease in germination resulting from longer treatment.

Rapid germination following sowing increases the likelihood a seedling will resist disease and overcome the early emergence phase (Leadem, 1988). However, seeds germinating in stratification become susceptible to pathogens, are vulnerable to damage during handling and are less likely to survive sowing compared to non-dormant ungerminated seeds. In the present study, it was not possible to stratify seeds effectively without incurring a large number of seeds germinating in stratification. Hydrogen peroxide treatment, however, increased total germination (germination in stratification plus germination following stratification) without further increasing the number of seeds germinating in stratification. Methods to reduce the number of seeds germinating during stratification treatment are needed.

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