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Seed Treatments Enhance American Elderberry Germination

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Abstract

Sexual propagation of American elderberry [*Sambucus nigra* L. subsp. *canadensis* (L.) Bolli] can be difficult without specialized handling and storage of seeds after fruit harvest. A study was conducted to evaluate the effect of 3% hydrogen peroxide (H_2O_2), GA_{4+7} (Provide®) at 250 mg·L^{-1}, dilute sulfuric acid [1 H_2SO_4: 1 tap water (v/v)], and periods of cold stratification (90 and 150 d) on percent shoot emergence and mean days to shoot emergence of 'Bob Gordon' and 'Ozark' elderberry seeds. Seed soaking treatments included H_2O_2 for 15 min; GA_{4+7} for 24 h; H_2O_2 followed by GA_{4+7}; H_2SO_4 for 30 s; and tap water for 15 min before cold stratification at 4°C for 150 d. Two additional treatments included H_2O_2 or no H_2O_2 before 60 d at 21 °C followed by 90 d stratification at 4°C. For both cultivars, seed treated with H_2O_2, followed by GA_{4+7}, and cold stratified for 150 d had the highest percent shoot emergence (94%) when compared with other treatments. The same treatment without H_2O_2 had greater shoot emergence (84%) than seeds that were soaked in tap water and cold stratified for 150 d (72%). Percent shoot emergence for seeds soaked in either treatment of H_2O_2, and then maintained at for 60 d at 21 °C before 90 d cold stratification, and those soaked in tap water before 150 d cold stratification were similar. Scarified seeds (i.e., H_2SO_4 treatment) failed to produce shoots. For both cultivars, seeds treated with GA_{4+7} had the fewest mean days to shoot emergence (11 to 15 d). In contrast, 'Bob Gordon' seeds soaked in tap water and cold stratified for 150 d required fewer days for shoot emergence (21) than 'Ozark' (25). Although sexual propagation of elderberry has been considered difficult previously, all treatments used in this study, except for H_2SO_4, had over 70% shoot emergence. However, very high germination can be achieved by using a disinfectant on fresh seed followed by a GA_{4+7} soak for 24 h, and cold stratification before seeding.

Increased sales of elderberry products over the last decade have driven the demand for improved elderberry germplasm (Charlebois et al., 2010; Mohebalian et al., 2012). Traits, such as large drupe size with small seeds, uniformity of ripening within and among pendulous umbels, increased yield on strong, erect canes, and resistance to disease, mites, and insect pests, are desirable (Charlebois et al., 2010; Darrow 1975). Most widely-grown American elderberry [*Sambucus nigra* L. subsp. *canadensis* (L.) Bolli] cultivars have been selected from wild plants growing in Arkansas, Kansas, Missouri, and Oklahoma during the last decade (Byers et al., 2012). To enhance genetic diversity, new and existing programs in Europe and the United States are using conventional breeding methods to make controlled crosses and then collect seeds from the progeny for subsequent germination and evaluation of seedlings in the field (K. Kaack, personal communication). Thus, practices that enhance seed germination may be useful to enhance elderberry breeding efforts.

American elderberry plants produce small drupes, containing multiple seeds, each with an outer hard endocarp (Brinkman, 1974). Within each seed is a dormant, spatulate-shaped embryo (Hidayati et al., 2000). After fruit harvest, these orthodox-type seeds have a low germination percentage without

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stratification (Rose, 1919; Worley and Nixon, 1974). Early studies demonstrated that moist American elderberry seed required temperatures, ranging from 0 to 5 °C, when overwintered outdoors for germination to occur (Adams, 1927; Davis, 1927). Heit (1967) stated that American elderberry had the highest germination after seed scarification with H_2SO_4 (concentration not reported) for 10 to 20 min. However, Cunningham and Farmer (1982) found that scarification of American elderberry seed with concentrated H_2SO_4 for 10 or 20 min damaged most embryos and resulted in less than 1% germination. Norton (1986) later reported 55% germination when *Sambucus cerulea* seeds were incubated in 1000 $mg \cdot L^{-1}$ GA_3 for 30 d at 4 °C. More recently, H_2O_2 has been used as a surface disinfectant for pathogens (Hartmann et al., 2002; Wojtyla et al., 2016) on seeds before planting and as a soaking treatment before stratification to enhance germination (Rosner et al., 2003).

In North America, elderberry plants of selected cultivars are clonally-propagated for commercial production of fruit. Dormant, hardwood cuttings of one-year-old canes are rooted in greenhouses in late winter or in the field in spring with or without 0.3% indole-3-butyric acid powder to promote rooting (Dirr and Heuser, 2006; M.R. Warmund, unpublished data). Less frequently, elderberry cultivars are propagated by softwood cuttings in a greenhouse. However, when large plant numbers are needed for habitat restoration, sexual propagation of elderberry may be advantageous. Sexual propagation of elderberry is also useful for reproductive biology studies and for breeding programs to increase genetic diversity for crop improvement. While scarification, stratification, and pre-treatment of seeds with various compounds have been identified to promote sexual propagation of elderberry, it is difficult to discern practices that optimize germination using commercially-available products. Thus, the objectives of this study were to evaluate percent shoot emergence and mean days to shoot emergence of 'Bob Gordon' and 'Ozark' seeds pre-treated with H_2O_2; GA_{4+7}; H_2O_2 followed by GA_{4+7}; water-diluted H_2SO_4; or tap water before cold stratification for 150 d at 4°C. Two additional treatments included H_2O_2 or no H_2O_2 before 60 d at 21 °C and then 90 d cold stratification at 4°C.

Materials and Methods

'Bob Gordon' and 'Ozark' elderberry fruit was harvested on 22 Aug. 2018 from a replicated planting of elderberry cultivars established in April 2017 at the University of Missouri Horticulture and Agroforestry Research Center near New Franklin, Missouri. Plants were spaced 1.2 m x 2.4 m apart in a Menfro silt loam (fine-silty, mixed, superactive, mesic typic hapludalfs). Five replications of three plant-plots of each cultivar were arranged in a randomized complete block design. Nitrogen fertilizer was applied in March at 56 $kg \cdot ha^{-1}$ annually and plants were grown without irrigation or pesticides. Weeds were controlled manually and the fescue ground cover was mowed. Fruit was harvested at peak ripeness (all berries in an umbel dark-purple) from 4 to 24 Aug. 2018. Immediately after harvest, berries of each cultivar were washed, de-stemmed, sealed in 3.8 L polyethylene bags (Pactiv Corp., Lake Forest, IL), and stored at 4 °C. On 28 Aug. 2018, fruit from each cultivar was bulked. Next, seed was extracted and washed with tap water to remove the pulp. Any seed that floated during washing was discarded.

The following day 'Bob Gordon' and 'Ozark' seeds received the following treatments: 3% H_2O_2 (34.014 $g \cdot mol^{-1}$; Fisher Scientific, Pittsburgh, PA); GA_{4+7} (250 $mg \cdot L^{-1}$; Provide®; Valent BioSciences, Walnut Creek, CA); 3% H_2O_2 + GA_{4+7} (250 $mg \cdot L^{-1}$); H_2SO_4 (98.072 $g \cdot mol^{-1}$; Fisher Scientific, Pittsburgh, PA): tap water (v/v); and tap water alone. The sulfuric acid solution was a 1:1 mixture with water by volume (50% solution of concentrated sulfuric acid). Soaking time for H_2O_2 and tap water was 15 min. GA_{4+7}

and H_2SO_4 soaking times were 24 h and 30 s, respectively. After the H_2O_2 or H_2SO_4 soak, seeds were rinsed with running tap water for 2 min. Seeds were not rinsed following GA_{4+7} treatments. Next, seeds were air-dried at 21 °C for 1 h. Each treatment was then placed in a 10 cm-diameter petri dish (without filter paper) and sealed in a plastic bag before 150 d stratification at 4° C. Two additional treatments were a 3% H_2O_2 soak for 15 min followed by rinsing in tap water for 2 min, or no H_2O_2 soak before 60 d at 21 °C followed by 90 d stratification at 4° C. For these treatments, H_2O_2- soaked seeds were placed in petri dishes and enclosed in 3.8 L polyethylene bags after rinsing and air-drying (21 °C) for 1 h or were placed within petri dishes after air-drying for 1 h and sealed in bags on 29 Aug. 2018.

After cold stratification, five replications of 25 seeds of each treatment were planted in 2.5 x 2.5 x 5-cm (depth) 200-cell plug trays (Hummert International, Earth City, MO), using potting medium (ProMix; Premier Tech Horticulture, Québec, Canada) on 4 Feb. 2018. Trays were arranged in randomized complete block design in the greenhouse maintained at 21 °C under natural light and uniformly irrigated as needed. Shoot emergence (i.e., seed germination) was recorded every other day for 40 d. For germinants in each treatment, the mean number of days for shoot emergence during the 40-day greenhouse period was calculated.

Because no sulfuric acid-soaked seeds germinated with 40 d of stratification, this treatment was omitted from statistical analyses. For all others, the odds (i.e., probability) of shoot emergence of each treatment were calculated, using the GLMMIX procedure of the SAS statistical analysis software (SAS Institute, Cary, NC) with a link = logit function for a binomial distribution due to the non-normal distribution of data. Odds were calculated from the antilog of the logit value and back-transformed [% shoot emergence = odds / (1 + odds)] for reporting shoot emergence percentage for each growth regulator treatment. Mean differences among odds were determined using the LSMEANS statement ($P \leq 0.05$). Data for days to shoot emergence were

Table 1. Percent shoot emergence of 'Bob Gordon' and 'Ozark' elderberry seed treated with hydrogen peroxide, gibberellic acid, or tap water for selected periods of cold stratification at 4 °C.[z]

Seed treatment	Shoot emergence (%)[y]
H_2O_2 – 150 d stratification	76.8 bc
GA_{4+7} – 150 d stratification	83.6 b
H_2O_2 – GA_{4+7} – 150 d stratification	94.0 a
H_2O_2 – 60 d at 21 °C – 90 d stratification	78.6 bc
60 d at 21 °C – 90 d stratification	76.8 bc
tap water – 150 d stratification	72.0 c

[z] Elderberry seeds were soaked in 3% H_2SO_4 or tap water for 15 min. Seed treatments with GA_{4+7} (250 mg·L^{-1} gibberellic acid) were soaked for 24 h before cold stratification. Seeds were subsequently grown in a greenhouse for 40 d.

[y] Values represent 5 replications per treatment with 25 seeds sown per replication. PROC GLIMMIX using a logit link for binomial distributions was used to analyze shoot emergence data. Back transformed data [% shoot emergence = odds/(1+odds)] are presented. Mean differences among odds were determined using the LSMEANS statement. Means followed by the same letters are not significantly different, according to Fisher's protected LSD test ($P \leq 0.05$).

Table 2. Mean number of days to shoot emergence of 'Bob Gordon' and 'Ozark' elderberry seed treated with hydrogen peroxide, gibberellic acid, or tap water for selected periods of cold stratification at 4 °C.[z]

Cultivar	Seed treatment emergence[y]	Days to shoot
Bob Gordon	H_2O_2 – 150 d stratification	19 d
	GA_{4+7} – 150 d stratification	11 g
	H_2O_2 – GA_{4+7} – 150 d stratification	14 f
	H_2O_2 – 60 d at 21 °C – 90 d stratification	17 e
	60 d at 21 °C – 90 d stratification	18 de
	tap water – 150 d stratification	21 c
Ozark	H_2O_2 – 150 d stratification	23 b
	GA_{4+7} – 150 d stratification	11 g
	H_2O_2 – GA_{4+7} – 150 d stratification	15 f
	H_2O_2 – 60 d at 21 °C – 90 d stratification	19 d
	60 d at 21 °C – 90 d stratification	21 c
	tap water – 150 d stratification	25 a

[z] Elderberry seeds were soaked in 3% H_2SO_2 or tap water for 15 min. Seed treatments with GA_{4+7} (250 mg·L^{-1} gibberellic acid) were soaked for 24 h before cold stratification. Seeds were subsequently grown in a greenhouse for 40 d. Values represent 5 replications per treatment with 25 seeds sown per replication.

[y] Means within a column followed by the same letter are not significantly different, according to Fisher's protected LSD test ($P \leq 0.05$).

subjected to analysis of variance (ANOVA) using the PROC GLMMIX procedure of SAS and means were separated by Fisher's protected LSD test ($P \leq 0.05$).

Results

Percent shoot emergence varied among treatments ($P = 0.006$), but not cultivars or the interaction of these two variables (Table 1). Shoot emergence for all treatments was ≥ 72%, but shoots were most likely to emerge when treated with H_2O_2 followed by GA_{4+7} and cold stratified for 150 d (94% emergence). Seeds soaked in GA_{4+7} followed by 150 d cold stratification were more likely to emerge than those that were soaked in tap water and stratified for 150 d. Other treatments, including H_2O_2 before 150 d cold stratification, and H_2O_2 or no H_2O_2 before 60 d at 21 °C and then 90 d stratification at 4 °C had similar odds for shoot emergence.

There was a significant interaction of cultivar and treatment for mean days to shoot emergence ($P = 0.001$). Seeds of both cultivars soaked in only GA_{4+7} and cold stratified for 150 d had the most rapid shoot

emergence (11 d), but when treated with H_2O_2 before GA_{4+7} and cold stratification, emergence was delayed by 3 to 4 d (Table 2). 'Bob Gordon' seeds treated with or without H_2O_2 and placed at 21 °C for 60 d before cold stratification for 90 d did not differ in the number of days to emergence, whereas the use of H_2O_2 on 'Ozark' in these treatments decreased the time to emergence. 'Ozark' seeds that were soaked in tap water before 150 d cold stratification had the longest time to shoot emergence (25 d).

Discussion

An elderberry seed treatment of 3% H_2O_2 for 15 min before a GA_{4+7} soak for 24 h and then 150 d stratification at 4°C resulted the highest percent shoot emergence (i.e., germination), whereas the lowest percent shoot emergence occurred when seeds received only 150 d stratification (Table 1). Because germination was relatively high (≥ 72%) when seeds were soaked briefly and stratified for 150 d, propagators may simply plant more seeds to achieve sufficient plant numbers without the added expense of GA_{4+7} when the seed supply is plentiful. However, when the quantity of elderberry seed is limited, a GA_{4+7} soak before stratification may be useful to enhance the germination percentage and increase seedling numbers.

For both elderberry cultivars, treatments including the GA_{4+7} soak had faster shoot emergence than all other treatments (Table 2). However, 'Bob Gordon' shoots emerged 2 to 4 d faster than 'Ozark' compared with similar treatments that did not include the GA_{4+7}. Although the moisture content of seeds was not measured after treatment, the reason for the more rapid rate of shoot emergence following the 24 h GA_{4+7} soak may be attributed to the longer period for imbibition in this treatment compared with the shorter 15 min tap water or H_20_2 soaking periods. For treatments that did not include the GA_{4+7} soak, differences in time required for shoot emergence may be related to anatomical characteristics of cultivars, such as endocarp thickness, seed and embryo size, or other unknown factors. Although detailed studies comparing drupe tissues among cultivars have not been conducted, 'Ozark' has a larger mean endocarp size (6.9 mm^2) than 'Bob Gordon' (5.1 mm^2) (M.R. Warmund, unpublished data).

Benefits of exogenous applications of gibberellin, including overcoming seed dormancy and promoting seed germination, have been documented for several plant species (Krishnamoorthy, 1975; Weaver, 1972). *Sambucus cerulea* seeds stratified in a 10^{-3} M solution of GA_3 for 100 d had 75% germination, whereas seeds stratified in water had only 30% germination (Clancy and Maguire, 1979). Norton (1986) also reported enhanced germination (55%) when *Sambucus cerulea* seeds were treated with GA_3 at 1000 $mg \cdot L^{-1}$ for 30 d at 4 °C, but only 28% germination was achieved by Hidayati et al. (2000) when *S. canadensis* seeds were incubated in the same GA_3 concentration during for 6 weeks at 5 °C. The formulation of gibberellin also influences the percent germination when used as a pre-germination treatment. GA_{4+7} soaking resulted greater germination than a GA_3 treatment for *Galeopsis pyrenaica*, *Lycopus europaeus* (Thompson, 1969), *Juglans nigra* (Warmund and Van Sambeek, 2018), and cereals (Mayer and Poljakoff-Mayber, 1989). Although GA_3 was not tested in the present study, shoot emergence with GA_{4+7} with or without a pre-treatment of H_2O_2 was 83.6 or 94.0%, respectively.

While the use of H_2O_2 before a GA_{4+7} soak and 150 d stratification enhanced shoot emergence, it did not produce similar results when used with 150 d stratification or before 60 d at 21 °C followed by 90 d stratification (Table 1). Hydrogen peroxide has been used as a surface disinfectant on seeds to control fungal and bacterial pathogens before planting (Hartmann et al., 2002; Trappe, 1961). Rosner et al. (2003) reported that *Ribes cereum* seeds soaked in 3% H_2O_2 for 4 h before 120 d cold stratification had greater percent germination when compared with 0, 8, or 16

h soaks. The decreased germination percentages after the 8 or 16 h H_2O_2 soaking periods in their study were attributed to degradation of the seed coat and increased percentages of seed rot during stratification. In a preliminary experiment with 'Bob Gordon' elderberry, percent shoot emergence of seeds soaked in 3% H_2O_2 for 15 min or 4 h followed by 150 d stratification had similar percent shoot emergence (66 and 68%, respectively) (M.R. Warmund, unpublished data).

Seed germination occurred after exogenous applications of either H_2O_2 or gibberellic acid, but their roles in seed germination are unknown (Wojtyla et al., 2016). However, evidence of crosstalk between H_2O_2 and signaling molecules (i.e., gibberellins, abscisic acid, ethylene, etc.) that regulate germination has been published (Wojtyla et al., 2016).

Storage of elderberry seed for 60 d at room temperature (21 °C) before 90 d cold stratification at 4 °C did not adversely affect percent shoot emergence when compared with 150 d cold stratification (Table 1). Heit (1967) also reported "fair" germination when American elderberry seeds were exposed to 20 to 30 °C for 2 months followed by stratification at 1 to 4 °C. American elderberry seeds incubated at 25 °C day/ 15 °C night for 12 weeks under lighted conditions (~40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) before stratification at 5 °C for 12 weeks had ≥ 73 % germination (Hidayati et al., 2000). While percent shoot emergence (i.e., germination) was not maximized with only 90 d stratification in our study, this method may be useful when large numbers of plants are needed by nurserymen to fulfill late plant orders for spring sales or when researchers require rapid production of sexually-propagated elderberry plants. Shorter cold stratification times also could also reduce energy costs associated with refrigeration.

Although seed coats appeared intact following the H_2SO_4 treatment for 30 s and signs of rot were not apparent after stratification, these seeds did not produce shoots (or roots) when visually inspected after 40 d in the greenhouse. In a preliminary experiment, 'Bob Gordon' and 'Ozark' elderberry seeds treated with 1 H_2SO_4: 1 tap water for 5 min had damaged seed coats and most seeds rotted during cold stratification (M.R. Warmund, unpublished data). Cunningham and Farmer (1982) also reported scarification of American elderberry seed for 10 or 20 min in concentrated H_2SO_4 caused physical damage to the embryo. In contrast, Heit (1967) reported that optimal American elderberry seed germination occurred after a 10 to 20 min H_2SO_4 treatment followed by cold stratification for 2 months, but the concentration of the H_2SO_4 was not reported.

Several methods have been tested to enhance elderberry seed germination with varying degrees of success during the last century (Brinkman, 1974). However, results from this study demonstrated that germination of high-yielding cultivars, such as 'Bob Gordon' and 'Ozark' elderberry used for commercial production, can be maximized using 3% H_2SO_2 for 15 min as a disinfectant, rinsing seeds with tap water, followed by a GA_{4+7} soak (250 $mg \cdot L^{-1}$) for 24 h and cold stratification at 4 °C for 150 d. This method provides propagators with a reliable protocol for rapid plant production after stratification, especially when seeds are grown in a greenhouse.

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