

# Exposing Seeds of *Sarcococca confusa* to Increased Concentrations and Durations of Ethyl Methanesulfonate Reduced Seed Germination, Twinning, and Plant Size

Tyler Hoskins and Ryan N. Contreras

Department of Horticulture, Oregon State University, 4017 Agricultural and Life Sciences, Corvallis, OR 97331

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**Abstract.** Sweetbox (*Sarcococca confusa*) is an evergreen shrub valued for attractive foliage, winter fragrance, black fruit, as well as shade and drought tolerance. The high degree of apomixis in *Sarcococca* limits the effectiveness of conventional breeding practices. However, mutation breeding may be a valuable tool to induce variation in seedling crops. As such, a study was conducted to expose seeds of *S. confusa* to ethyl methanesulfonate (EMS) at varying concentrations and exposure durations, and to evaluate the effects on seed germination, growth, and relative frequency of polyembryony. In 2010, seeds of *S. confusa* were treated with 0%, 0.2%, 0.4%, 0.8%, and 1.2% EMS for 24 and 48 hours. Seeds were sown and the relative germination and occurrence of polyembryony were recorded. Seedlings were later evaluated for size and phenotypic variation in the subsequent growing seasons. Percent germination was found to decrease with increasing EMS rates in the 24- and 48-hour treatments. The occurrence of polyembryony also decreased with increasing EMS rates in the 24-hour treatment but was observed to be greatest at 0.2% EMS. No significant differences in plant size index (SI) were found after four growing seasons. When mature, seeds were collected from any plants that bore fruit in each treatment and were then sown. The percentage of plants that bore fruit in each treatment decreased with increasing EMS rates, although the germination rate of these seeds did not differ. A chlorophyll mutant and several dwarf forms were identified in this population as well. They have been propagated asexually for future evaluation as new cultivars. This study demonstrates the utility of chemical mutagenesis to induce phenotypic variation in *S. confusa* while reducing the rate of polyembryony.

Sweetbox (*Sarcococca confusa*) is an evergreen shrub with attractive foliage in Buxaceae that requires shade and is relatively compact, making it an excellent choice for foundation plantings. Its white flowers are apetalous, sweetly fragrant, borne during winter, and give rise to glossy black drupes. In addition to its numerous ornamental attributes, it is regarded as highly drought tolerant (Dirr, 2009), which allows it to thrive in dry shade, which is a challenging landscape site for most plants. More compact forms of this species may be preferred for modern landscapes or container gardens. However, reports of apomixis in *Sarcococca* present challenges to breeding. Dirr (2009) reported

that seedlings of sweetbox grew true to type, such that all seedlings were identical to their seed parent. This anecdotal observation suggests either 1) self-pollination of a relatively homozygous plant resulting in a homogenous population or 2) apomixis. During early preliminary studies, seeds sown from open-pollinated plants of *S. confusa* at Oregon State University (OSU) resulted in one, two, or three seedlings (Fig. 1), indicating apo-

mixis occurs, at least facultatively, in the species.

Apomixis was first reported in *Sarcococca ruscifolia* (Orr, 1923), and a number of studies listed in the work by Nygren (1954) followed that confirmed adventive embryony (nucellary polyembryony) in several other species of *Sarcococca*. More recent studies have provided more in-depth studies to document specific mechanisms and activities during the process of apomixis in *Sarcococca* (e.g., Naumova, 1979; 1980; Naumova and Willemse, 1983).

Seedlings arising through apomixis are genetically identical to the seed parent and, in cases of polyembryony, when one seedling is sexually derived and one or more seedlings develop through apomixis, it can be difficult to identify the sexually derived seedling. Regardless of the mode of apomixis, this phenomenon presents a major obstacle to traditional breeding. Based on the presence of apomixis in the genus and our observations of polyembryony, we chose to introduce variation in this species using mutagenesis.

Mutation breeding is an important tool to improve plants exhibiting apomixis (Lapins, 1983). Although *S. confusa* appears to be a facultative apomict, it is inefficient and cumbersome to identify and separate sexually derived seedlings from nucellar embryos. As such, the objectives of our study were 1) to expose seeds of *S. confusa* to EMS at varying concentrations and exposure durations to evaluate the effects on seed germination, 2) to evaluate growth effects from various treatments, and 3) to determine whether the relative frequency of polyembryony is affected by EMS treatment.

## Materials and Methods

**Plant material and experiment design.** Mature fruit of *S. confusa* were collected on 10 Dec. 2009 from established plants on the campus of OSU (Corvallis, OR). Fruit were stored in plastic bags at 4 °C until mutation treatments were initiated. The study consisted of five EMS concentrations (0%, 0.2%, 0.4%, 0.8%, and 1.2%) with two treatment durations (24 and 48 h) and was arranged in a completely randomized design.

**Mutation treatments.** On 15 Feb. 2010, the pericarp was removed manually from

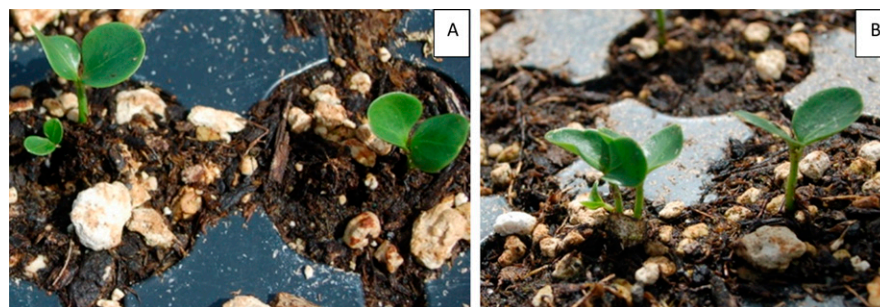


Fig. 1. Germinating seedlings of *Sarcococca confusa* sown as a single seed in each cell, showing (A) one or two seedlings and (B) one or three seedlings, demonstrating the presence of polyembryony.

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T.H. is a Faculty Research Assistant.

R.N.C. is an Associate Professor.

R.N.C. is the corresponding author. E-mail: ryan.contreras@oregonstate.edu.

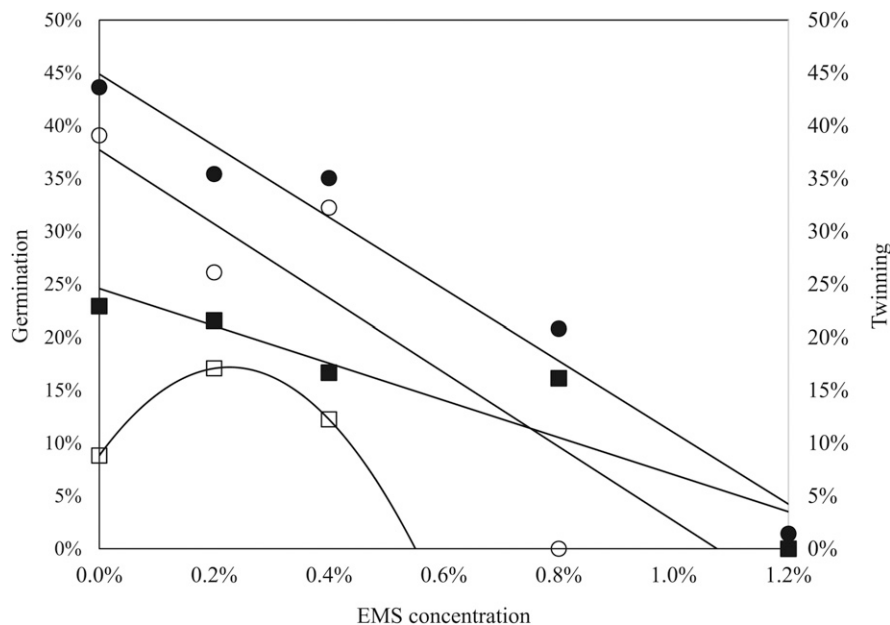


Fig. 2. Germination and twinning percentages of *Sarcococca confusa* when seeds were exposed to increasing concentrations of ethyl methanesulfonate (EMS) for 24 and 48 h. Percent germination of seeds treated for 24 h (●;  $y = -33.878x + 0.4488$ ;  $r^2 = 0.9642$ ) and 48 h (○;  $y = -35.072x + 0.3772$ ;  $r^2 = 0.8456$ ), and the percentage of twins that occurred among germinated seeds treated for 24 h (■;  $y = -17.567x + 0.2459$ ;  $r^2 = 0.8579$ ) and 48 h (□;  $y = -16.347x^2 + 73.943x + 0.0882$ ;  $r^2 = 1$ ).

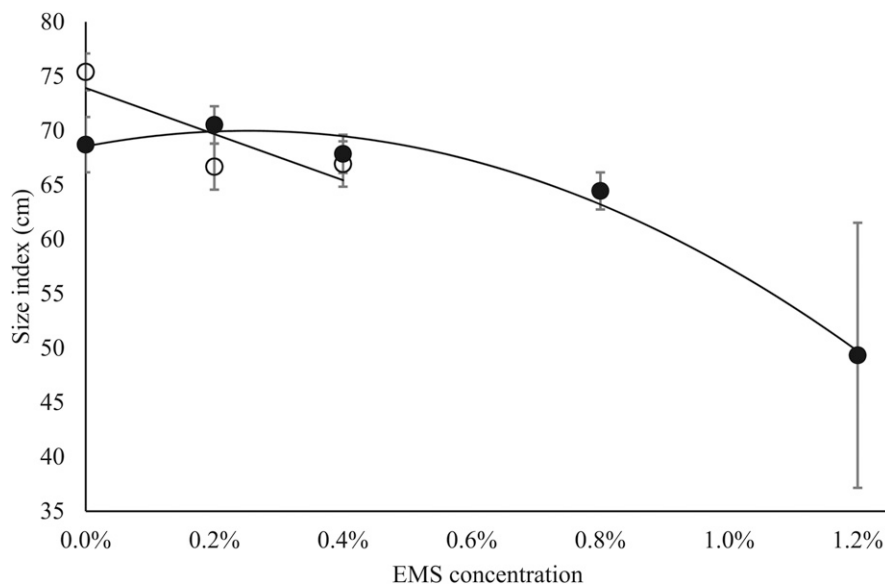


Fig. 3. Size index [ $SI = (\text{Height} \times \text{Width}_1 \times \text{Width}_2)^{1/3}$ ] of 4-year-old *Sarcococca confusa* plants derived from seed exposed to increasing concentrations of ethyl methanesulfonate (EMS) for 24 h (●;  $y = -225.318x^2 + 1135.1x + 68.551$ ;  $r^2 = 0.984$ ) and 48 h (○;  $y = -2118.2x + 73.91$ ;  $r^2 = 0.7285$ ). Plants had been grown for three seasons in containers and one in a field.

seed immediately before treatment. Seeds were separated into groups according to treatment and placed in 500-mL Erlenmeyer flasks with 1 mL/seed of a solution containing EMS at the respective concentration in a 0.1 M phosphate buffer. Flasks were sealed with parafilm and seeds were soaked while agitating at 125 rpm at 20 °C for 24 and 48 h. After treatment, seeds were rinsed under running tap water for 15 min and sown into 72-cell trays using Sunshine LA4 P potting mix (Sun Gro Horticulture, Agawam, MA)

and placed into a glasshouse with day/night set temperatures of 24 °C/17 °C, with a 14-h photoperiod.

**Plant growth.** On 20 July 2010, germinated seedlings were transplanted into 0.6-L square containers filled with a 1:1, by volume, blend of a proprietary potting mix (includes aged bark, compost, pumice, perlite, sphagnum peatmoss, coconut coir, fish bone meal, feather meal, bat guano, alfalfa meal, blood meal, shrimp meal, basalt rock dust, kelp meal, dried poultry waste and

limestone; Patio Potting Soil; Rexius Inc., Eugene, OR) and unaged douglas fir bark mulch (Rexius Inc.). Containers were top-dressed with the label rate of 18N-2.6P-10K controlled-release fertilizer (CRF; APEX, 10-11 month, J.R. Simplot, Boise, ID). On 29 Dec. 2010, plants were transplanted into 3.8-L containers using the same 1:1 substrate blend just described and then top-dressed with the label rate of the same CRF. On 21 Apr. 2011, plants were transplanted into 11.4-L containers filled with the same 1:1 substrate blend described earlier and then top-dressed with the same CRF at the label rate. Plants were moved to a 47% shade house at the OSU Lewis-Brown Farm (Corvallis, OR) and irrigated for 20 min daily using 12 L·h<sup>-1</sup> spray stakes (Netafim USA Inc., Fresno, CA). Plants were grown there for two seasons, overwintered in a poly-covered greenhouse, and field-planted under a 47% shade structure on 16 Apr. 2013 at the OSU Lewis-Brown Farm.

**M2 seed population.** Honey bee hives were placed in the poly-covered greenhouse from 7 to 12 Feb. 2012 (peak flowering) to facilitate pollination. Up to 14 mature fruit per plant were collected on 14 Jan. 2013 from all plants that bore fruit. This included six plants from the 0% EMS 24-h treatment, five plants from the 0% EMS 48-h treatment, 12 plants from the 0.2% EMS 24-h treatment, four plants from the 0.2% 48-h treatment, three plants from the 0.4% EMS 24-h treatment, and three plants from the 0.4% 48-h treatment. On 16 Jan. 2013, pericarps were removed and seeds were sown in 1.3-L containers filled with Metro Mix 840PC (Sun Gro Horticulture), which were then placed in a glasshouse with day/night set temperatures of 24 °C/17 °C, with a 14-h photoperiod until germination. Final germination data were collected the first week of July 2013.

**Data collection and analysis.** The number of germinated seedlings and the occurrence of twinning (i.e., two seedlings derived from one seed) were recorded on 20 July 2010 before they were transplanted to 0.6-L containers. Germination percentage was calculated relative to the number of seedlings sown in each treatment. Twinning percentages were calculated relative to the number of germinated seedlings per treatment. SI [ $SI = (\text{Height} \times \text{Width}_1 \times \text{Width}_2)^{1/3}$ ] measurements were collected on 5 Nov. 2012 (while in 11.4-L containers) and 14 Oct. 2013 (while field-planted).

The relationship between EMS concentration and percent germination, twinning percentage, and mean SI were subject to either linear or quadratic regression for both 24- and 48-h data sets separately. Percent germination of M2 seed was calculated relative to the quantity sown and was subject to one-way analysis of variance (ANOVA) ( $\alpha = 0.05$ ). The relationship between the percentage of M1 plants that bore fruit after four seasons and the EMS rate was subject to linear regression. All data were analyzed using JMP® Pro Version 13.0.0 (SAS Institute Inc., Cary, NC).



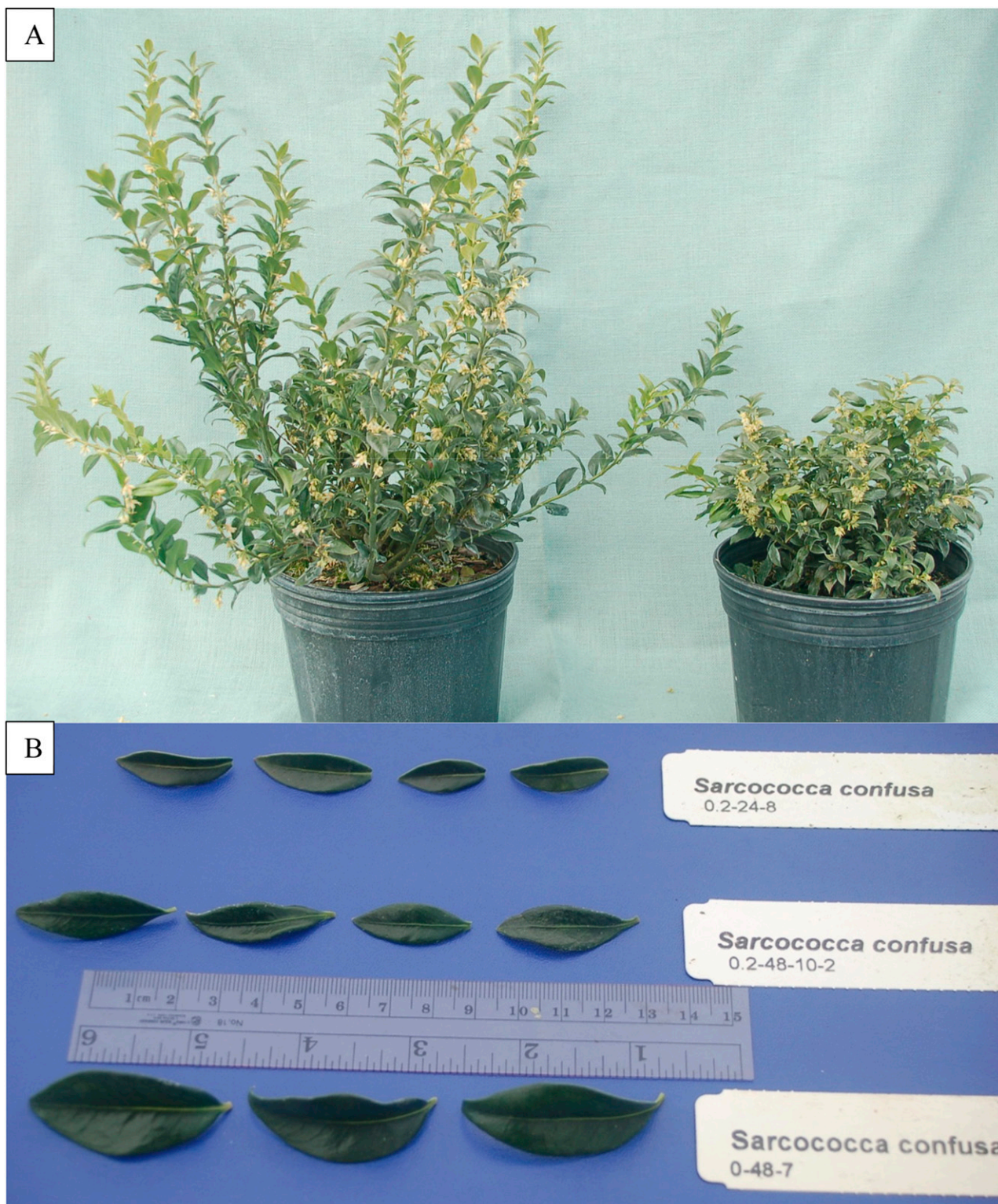


Fig. 4. Exposing seeds of *Sarcococca confusa* resulted in dwarf mutants and variation in leaf size and shape. (A) Example of size variation between wild-type (left) and dwarf mutant (right). (B) Variation in leaf size and shape between plants exposed to 0.2% EMS (top and middle) and control (bottom).

### Results and Discussion

**Percent germination.** A negative linear relationship was observed between percent seed germination and EMS concentration for both the 24- and 48-h treatments ( $r^2 = 0.9642$  and  $0.8456$ , respectively). Germination percentage was greatest in the control (0% EMS)

and decreased as EMS concentration increased (Fig. 2). No germination was observed in the 48-h treatment with 0.8% or 1.2% EMS. Median lethal dose ( $LD_{50}$ ) for the 24- and 48-h treatment groups was 0.66% and 0.54% EMS, respectively. Greer and Rinehart (2009) observed a reduction in seed germination percentage of *Hydrangea mac-*

*rophylla* seed when exposed to 2.5% or 5% EMS for 3 h in the absence of a prior cold treatment; however, cold treatment of both *H. macrophylla* and *H. paniculata* increased tolerance of both species to increasing EMS concentrations. Alcantara et al. (1996) treated *Capsicum annuum* seed with up to 1.5% EMS at 20 °C for 9 h with no significant





Fig. 5. A chlorophyll-deficient mutant of *Sarcococca confusa*. (A) Original plant identified with unstable chlorophyll mutation, (B) rooted cuttings collected from the mutant branch and stabilized to a uniform chartreuse color, and (C) seeds collected from branches exhibiting the chartreuse phenotype resulted in seedlings that were uniformly chartreuse and, in this example, also exhibited polyembryony.

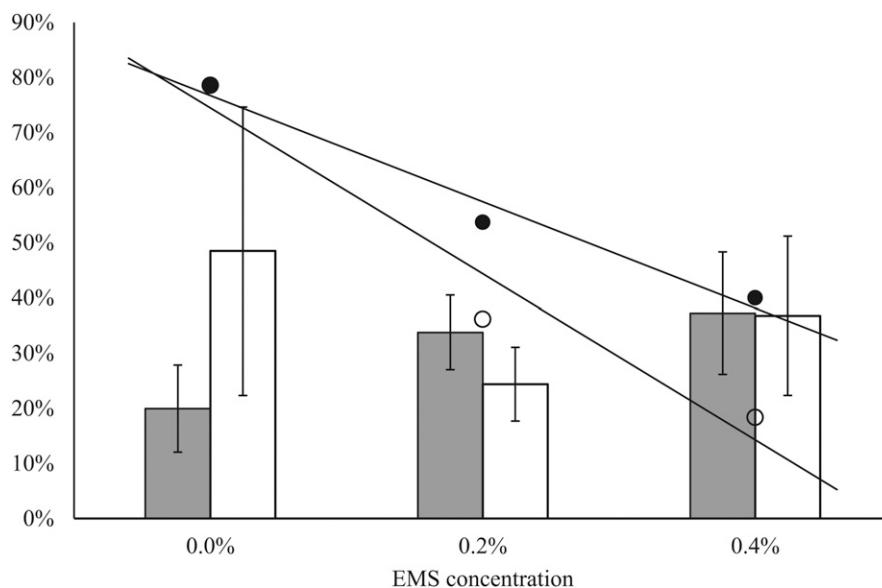


Fig. 6. Percentage of M1 *Sarcococca confusa* plants that bore fruit four seasons after seeds were treated with increasing concentrations of ethyl methanesulfonate (EMS) for 24 h (●;  $y = -0.1929x + 0.96$ ;  $r^2 = 0.9728$ ) and 48 h (○;  $y = -0.301x + 1.0457$ ;  $r^2 = 0.9473$ ), and subsequent average percent germination of M2 seeds collected from fruit-bearing M1 plants from the 24-h (gray bars) and 48-h (white bars) treatments.

reduction in germination percentage. Increasing concentration of EMS reduced seed germination of *Jatropha curcas* from 58% in the control to 25% when treated with 4% EMS (Dhakshanamoorthy et al., 2010).

**Twinning.** A negative linear relationship was observed between twinning rate and EMS concentration for the 24-h treatment ( $r^2 = 0.8579$ ), where the greatest number of twins was observed in the control, and decreased with greater EMS concentrations (Fig. 2). Twinning percentage in the 48-h treatment group was best explained by a quadratic relationship ( $r^2 = 1$ ), where the greatest number of twins occurred after exposure to 0.2% EMS.

**Plant size.** Plant SI after four growing seasons was explained by a quadratic relationship in the 24-h treatment group ( $r^2 = 0.984$ ), where the greatest SI was observed at 0.2% EMS and decreased at greater EMS concentrations, although no significant differences were found when comparing treatments using ANOVA ( $P = 0.153$ ) (Fig. 3). Plant SI in the 48-h treatment group was explained by a negative linear relationship. This data set only consisted of three data points, as germination only occurred at 0%, 0.2%, and 0.4% EMS, and is statistically fit better by a quadratic regression. However, this model would predict a substantial increase in SI if projected to greater EMS concentrations and was therefore deemed biologically irrational. Similar to the 24-h treatment group, no significant differences were found when comparing treatments using ANOVA ( $P = 0.110$ ). Several dwarf plants were identified from treated seeds and show promise as compact plants for home foundation plantings or for use in containers (Fig. 4). Treated plants exhibited altered leaf shape with reduced undulation, narrower leaf blades, and shorter leaf blades (Fig. 4). Dwarfing is a common result of mutagenesis (Lapins, 1983), and a similar reduction in plant size as well as altered leaf morphology has been observed in other taxa. *Ribes sanguineum* 'Oregon Snowflake' was developed using EMS treatment, which resulted in increased branching, reduced plant height, and altered leaf morphology (Contreras and Friddle, 2015). Greer and Rinehart (2009) recovered dwarf mutants of *Hydrangea* after treating seeds with EMS, but the frequency and degree of dwarfing was not reported.

**Chlorophyll mutant.** A portion of one plant exhibited an irregular chlorophyll mutation. This branch was propagated from stem cuttings and later stabilized to a uniform chartreuse color (Fig. 5). Subsequent clonal propagation from stem cuttings has shown this mutation to be stable after three rounds of serial propagation. In addition, seeds were collected from a branch showing the chlorophyll mutation, and seedlings exhibited uniform chartreuse leaf color from the cotyledon stage and at least some seeds were polyembryonic (Fig. 5). Smith and Brand (2012) developed and introduced *Buddleja davidii* 'Summer Skies', a variegated cultivar of

butterfly bush that resulted from exposing seeds to EMS. In contrast to the uniform chartreuse chlorophyll mutation we observed, 'Summer Skies' exhibited a marginal variegation with dark-green centers, which the authors presume was a periclinal chimera as a result of its appearance and stability. Greer and Rinehart (2009) reported variegated and chlorotic mutants resulting from seed treatment of *Hydrangea* with EMS, but no details were included on pattern or prevalence.

**M2 seed germination.** A negative linear relationship was observed between the percentage of fruit-bearing plants after three growing seasons and EMS rate for both the 24- and 48-h treatment groups, where the greatest percentage of fruit-bearing plants occurred in the absence of EMS; the least percentage was seen in the 0.4% group (Fig. 6). No fruit was found on M1 plants grown from seeds treated with 0.8% or 1.2% EMS. There were no differences in germination percentage of M2 seeds among treatments in the 24- or 48-h treatment groups ( $P = 0.506$  and  $0.526$ , respectively).

Apomixis is often associated with polyploidy, as in *Cotoneaster*, which has a natural ploidy series wherein tetraploids are mostly obligate apomicts and diploids produce sexual progeny (Sax, 1954). Previous reports of apomixis in *Sarcococca* include both diploids (*S. humilis*, *S. hookeriana*) and the tetraploid *S. ruscifolia* (Nygren, 1954). The only prior report in *S. confusa*, which is a tetraploid ( $2n = 4x = 56$ ) (Denaeghel et al., 2017), is the anecdotal report from Dirr (2009) and our own findings of polyembryony. *Sarcococca confusa* was used as a pollen parent in crosses with *S. ruscifolia* 'Dragon's Gate', but Denaeghel

et al. (2017) did not attempt to use it as a seed parent. They also did not report any evidence of apomixis, and all seedlings in their crossing study had 10% or greater amplified fragment length polymorphism markers from the male parent, which indicates their progeny were derived sexually (Denaeghel et al., 2017). Although there are conflicting reports of apomixis among various species of *Sarcococca*, we have repeatedly observed polyembryony among seeds sown, making traditional breeding through sexual recombination of parents challenging and inefficient. Our study demonstrates the utility of chemical mutagenesis to induce useful variation among sweetbox seedlings and to reduce the rate of polyembryony or twinning.

Other studies have investigated the impact of various pretreatments before mutagenesis, such as imbibition or cold treatment. We did not compare any such treatments, but held all seeds at 4 °C within the pericarp, which presumably provided some degree of cold stratification as a result of the presence of moisture within the fruit. This approach aligns with recommendations made by Lapins (1983). Greer and Rinehart (2009) found that *Hydrangea macrophylla* and *H. paniculata* were both more tolerant of greater concentrations of EMS after cold treatment. On the other hand, Alcantara et al. (1996) observed no greater tolerance in *Capsicum annuum* after imbibition before EMS treatment of seeds.

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