Ethyl Methanesulfonate and Caffeine Mutagenetic Treatment to Four Ornamental *Silene* Species[1]

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Abstract

Mutagenesis breeding for horticultural crops is valuable not only for creating new cultivars, but also expanding the genetic pool for hybridization. Four *Silene* species were subjected to ethyl methanesulfonate (EMS) and/or caffeine mutagenesis to obtain valuable ornamental traits and to test the effects of different mutagens and combinations. Species responded differently to the mutagens. Generally, caffeine plus EMS treatments had a greater effect on mutation rate than either treatment applied alone. Caffeine alone was found to enhance seed vigor of *S. flos-cuculi*. Caffeine as a plant mutagen should be further investigated to determine the most efficient concentration as well as effects on other plant species, as several desirable mutants were obtained with leaf variegation.

Index words: *Lychnis*, campion, catchfly, mutation, ornamental breeding, EMS.

Species used in this study: *Silene coronaria* (L.) Desr., *S.* ×*haageana* Lem. 'Molten Lava', *S. chalcedonica* L., and *S. flos-cuculi* L.

Chemicals used in this study: EMS (Ethyl methanesulfonate); Jet-Alert caffeine (caffeine).

Significance to the Horticulture Industry

Induced mutagenesis is utilized to potentially generate beneficial mutations for breeding and genetics study. The purpose of this ornamental mutation breeding study was to observe the M1 effects (M1 means the first generation of plants that was treated by mutagens) of a known mutagenesis chemical (ethyl methanesulfonate known as EMS) and caffeine to induce phenotypic mutations in seedlings to create new germplasm for further breeding. Mutations appeared on the current generation readily on campion (*Silene*) species, which demonstrated chemical mutation could be a rapid and accessible approach for the ornamental nursery industry with a desire to breed for ornamental traits. Also, the EMS and caffeine concentrations used in this research are good references for nurseries desiring to conduct ornamental mutation breeding. Moreover, caffeine, tried as a chemical mutagen in the current study, induced mutation in certain campion species, and should be tested further as it is less toxic to the environment and handlers than EMS.

Introduction

The ornamental value of the genus *Silene* L., common name campion or catchfly, is recognized commercially by horticulturists, yet has not been well exploited as another genus, *Dianthus* L., within the same family, the Caryophyllaceae. Induced mutagenesis, which has a high mutation frequency, has been used to induce variegation in leaves, develop new flower colors, alter plant growth habit, and ultimately create new cultivars in ornamental breeding. Mohan Jain (2006) reported that approximately one fourth of the total 2,300 mutant plant varieties developed are ornamentals. Artificial mutations could be induced by physical radiation or chemical mutagens, as well as through somatic mutations in tissue culture. The effects of different mutagens are also an important subject for related fields, since different mutagens have different effects on DNA or RNA due to their specific mutative mechanism (Snustad and Simmons 2006). Ethyl methanesulfonate (EMS), a common chemical mutagen, is known to cause point mutations by effective alkylation of guanine bases, which results in A-T base pair substitution for G-C pairs during the next round of duplication, whereas, caffeine causes chromosome aberration and sister chromatid exchange in mammalian cells (Bittueva et al. 2007). Its mechanism on plants is not yet clear, and no plant cultivar has been reportedly developed through caffeine mutation. Caffeine was therefore selected as a mutagen to test along with EMS in this experiment due to its low toxicity to humans and because of its mutation effects on animals. Preliminary trials showed that some species of *Silene* are EMS sensitive. This research was designed both for mutation breeding and to evaluate the effects of different mutagens on phenotypic traits in *Silene* seedlings.

Materials and Methods

Three replications of 100 seeds of *Silene coronaria* (L.) Desr., *S.* ×*haageana* Lem. 'Molten Lava', *S. chalcedonica* L., and *S. flos-cuculi* L. were placed separately in coffee filters tied with a string to make a bag. Seeds were then soaked in different chemical mutagens for 24 hours at 15C (59F) in a growth chamber (Nor-Lake, Hudson, WI). The four species were chosen based on their different ornamental traits and different germination rates. Chemical mutagens included ethyl methanesulfonate (EMS) (Acros Organics, Morris Plains, NJ) and 200 mg tablets of Jet-Alert caffeine (Bell Pharmaceuticals, Minneapolis, MN). Mutagen treatments included 0.6% EMS (v/v), 10% caffeine (w/v), 20% caffeine (w/v), 0.6% EMS plus 10% caffeine, 0.6% EMS plus 20%

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Table 1. Mean germination time, plant height, and leaf chlorophyll content in response to ethyl methanesulfonate (EMS) and caffeine mutagen treatments applied to seed of *Silene coronaria*[z].

Mutagen	Mean germination time (days)	Plant height (cm)	Chlorophyll[y]
Control	15.94 ± 2.04a[y]	7.69 ± 0.55a	33.19 ± 1.60a
0.6% EMS	19.53 ± 2.41a	4.10 ± 0.41b	31.94 ± 3.11a
10% Caffeine	16.63 ± 1.00a	7.36 ± 0.80a	34.76 ± 2.54a
20% Caffeine	17.70 ± 0.54a	7.54 ± 0.50a	30.37 ± 2.53a
0.6% EMS + 10% Caffeine	20.04 ± 2.43a	6.01 ± 0.87a	31.96 ± 1.77a
0.6% EMS + 20% Caffeine	17.52 ± 0.45a	4.10 ± 1.92b	28.33 ± 2.32a
$LSD_{0.05}$	NS	1.75	NS

[z]Different letters indicate means significantly different within columns at $P \leq 0.05$.

[y]Readings taken from a SPAD chlorophyll meter, unitless.

caffeine, and a control with only deionized water. Deionized water was used as the solvent for all mutagens.

After soaking in the mutagen solutions, seeds were rinsed three times for two minutes under tap water, then planted in 20 cm (8 in) diameter pots (Itml, Middlefield, OH) on a heat mat. The planting medium was a bark, peat moss, vermiculite, and perlite blend (Metro-Mix 702, Sun Gro Horticulture, Bellevue, WA). All treatments and replications for each species were completely randomized in the Oklahoma State University Horticultural Research Greenhouses in Stillwater, OK. Media was watered as needed. This experiment was conducted from March to August, 2011. The temperatures in greenhouses were set at 21C (70F) daytime and 18C (64F) during night, and actual temperatures varied with weather conditions.

Seed vigor, seedling surviving, number of mutants, as well as M1 morphologic variation were recorded for the treated *Silene* M1 seedlings. Seedlings were counted daily after germination until the germination rates were stable for three consecutive days. Seed vigor was determined by mean germination time (MGT) (Matthews et al. 2011).

$$MGT = \sum (f \cdot x) / \sum x$$

x, the newly germinated seeds on the f^{th} day, f, the number of days since the planting day, $\sum x$, total number of germinated seeds. Plant heights were measured 70 days after planting. Canopy height were measured for *S. coronaria* and *S. flos-cuculi*, and stem length were recorded for *S. xhaageana* 'Molten Lava' and *S. chalcedonica*. Leaf chlorophyll content was measured using a SPAD-502 chlorophyll meter (Spectrum Technologies, Plainfield, IL). Flowering days are the days from the planting date (March 11) to first flowering. The mutation rate was calculated based on the last day of the germination number (rate) recorded for the seedling surviving dynamics. Mutants for each replication of every treatment were counted after all other data were collected. In order to trace seedling survival dynamics, seedling numbers for each treatment and replication were counted 10 days apart after the germination rates were stable for three consecutive days. Data were collected on mean germination time, and on three plants of each replication for plant height, chlorophyll content as well as flower date of *S. ×haageana* 'Molten Lava' and *S. chalcedonica*.

Data were analyzed by ANOVA separately by species using the PROC GLM procedure with mean separation using the Duncan test (SAS/STAT®, version 9.3, SAS Institute, Cary, NC), $P \leq 0.05$. All percentage data were transformed by arcsin χ before analysis.

Results and Discussion

S. coronaria. Ethyl methanesulfonate and EMS plus 20% caffeine applied to *S. coronaria* significantly reduced plant height compared to the control, whereas 10% caffeine, 20% caffeine, and EMS plus 10% caffeine did not affect plant height for this species (Table 1). No treatment affected seed vigor (mean germination time) and leaf chlorophyll content of *S. coronaria*. Days to first flower was not recorded, since this species does not flower without vernalization under greenhouse conditions. No differences among treatments was noted for seeding survival [Fig. 1. (a)]. None of the treatments induced mutations based on observations of leaf color and morphology done visually (Table 2).

S. ×haageana *'Molten Lava'*. Caffeine application induced mutation in *S. ×haageana* 'Molten Lava' (Table 2). Significant differences were found within the caffeine treatments or EMS plus caffeine treatments for mean germination time, plant height, and flower date (Table 3). All treatments except EMS treatment delayed mean germination time, indicating low seedling vigor. All treatments except 10% caffeine significantly reduced plant height. All EMS plus caffeine treatments and both high and low rates of caffeine delayed the flowering date and extended the vegetative growth period significantly. No treatment affected chlorophyll content. Only the EMS plus 20% caffeine treatment significantly increased the mutation rate for *S. ×haageana*. The EMS, EMS plus 10% caffeine, and EMS plus 20% caffeine treatments dramatically lowered seeding survival rate of *S. ×haageana* one month after application compared to the other three treatments after the germination rate was stable for at least three consecutive days (Fig. 1). The EMS plus 20% caffeine treatment further decreased seedling survival rate on May 22, 2011 [Fig. 1. (b)], and caused some dwarfed plants with smaller flowers. *Silene ×haageana* is rich with pigment in the calyxes, while some mutants lacked reddish color in the calyxes. As for plant pigments, the other mutant found in this species showed blended spots on the red petals (data not shown). All the mutants had abnormal stamens.

S. chalcedonica. *Silene chalcedonica* is EMS sensitive. Every treatment with EMS incurred differences in comparison

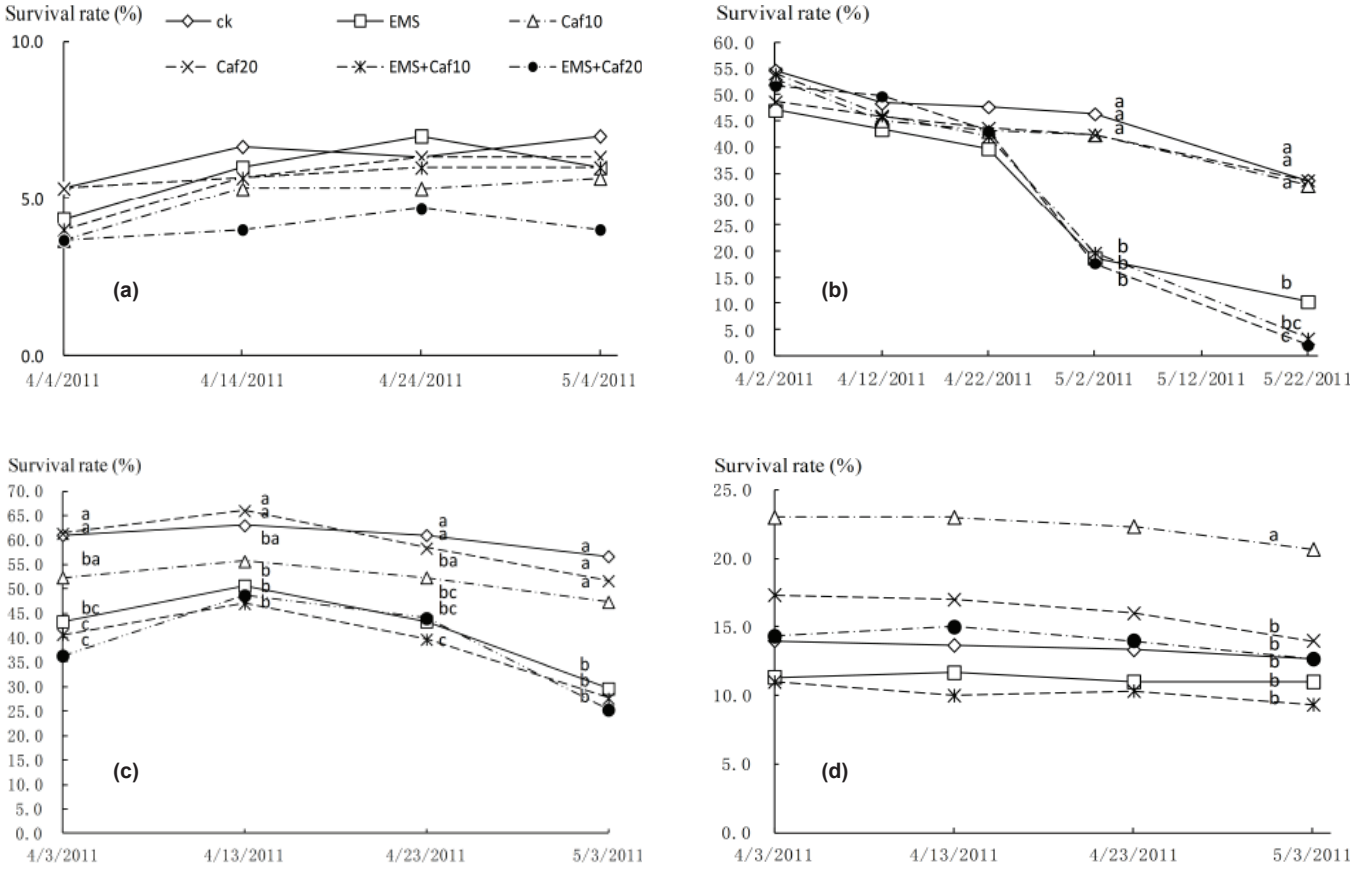

Fig. 1. Seedling survival dynamics as affected by ethyl methanesulfonate (EMS) and caffeine mutagen treatments applied to seed after the germination rate became stable for three consecutive days. Different letters on the same date denote seedling survival rate different at $P \leq 0.05$. (a) *Silene coronaria*, (b) *S.* ×*haageana*, (c) *S. chalcedonica*, and (d) *S. flos-cuculi.*

to mean germination time, plant height, chlorophyll content, and flowering data, yet caffeine only treatments had no significant effects (Table 4). The same was also observed for the mutation rate (Table 2). The EMS, EMS plus 10% caffeine, and EMS plus 20% caffeine treatments had a greater effect on seeding survival rate of *S. chalcedonica*. One month after recording began (seedling survival rate was stable for three consecutive days), these treatments killed more seedlings than other treatments that contained no EMS [Fig. 1. (c)] and survival decreased over time. Several mutants were obtained through mutagenesis for *S. chalcedonica*, which included leaf shape and color variation. Stamen abnormality due to mutagenesis was also found, as the case reported for *S.* ×*haageana* (Table 3).

S. flos-cuculi. All treatments with EMS affected plant height and leaf chlorophyll content, making it an EMS sensitive species (Table 5). However, the 10% caffeine treatment decreased mean germination time, which means caffeine caused stronger seed vigor than other treatments, including the control. EMS plus 20% caffeine application increased the mutation rate of *S. flos-cuculi* (Table 2). The flowering dates of *S. flos-cuculi* were not analyzed since the heat during summer inhibited flowering, making it difficult to determine the effects of mutation. All treatments, except 10% caffeine, had no effect on *S. flos-cuculi*'s seedling survival rates. The 10% caffeine treatment surprisingly caused a significant increase in seedling survival rate a month after germination rate was stable for three consecutive days [Fig. 1. (d)].

Table 2. Mutation rate (%) based on leaf color and morphology changes of four tested *Silene* species as affected by ethyl methanesulfonate (EMS) and caffeine mutagen treatments applied to seed[z].

Mutagen	*S. coronaria*	*S.* ×*haageana*	*S. chalcedonica*	*S. flos-cuculi*
Control	0.00	0.00d	0.00b	0.00b
0.6% EMS	0.00	26.67bc	9.92a	12.42b
10% Caffeine	0.00	0.00d	0.00b	0.00b
20% Caffeine	0.00	1.71cd	0.00b	4.32b
0.6% EMS + 10% Caffeine	0.00	42.22b	6.10a	7.04b
0.6% EMS + 20% Caffeine	0.00	88.89a	11.46a	34.17a

[z]In columns, data followed by lower case letters indicate significant difference at $P \leq 0.05$.

Table 3. Mean germination time, plant height, and leaf chlorophyll content in response to ethyl methanesulfonate (EMS) and caffeine mutagen treatments applied to seed of *Silene ×haageana 'Molten Lava'*[z].

Mutagen	Mean germination time (days)	Plant height (cm)	Chlorophyll[y]	Days to first flower
Control	11.29 ± 0.92c	12.23 ± 1.28a	27.34 ± 3.12a	74.67 ± 1.53b
0.6% EMS	12.31 ± 0.48bc	2.19 ± 0.49c	23.79 ± 2.52a	106.00 ± 3.00ab
10% Caffeine	13.48 ± 0.93ab	10.57 ± 1.25ab	26.64 ± 4.02a	77.67 ± 3.21b
20% Caffeine	14.29 ± 2.10a	9.86 ± 1.23b	28.46 ± 2.68a	77.67 ± 2.08b
0.6% EMS + 10% Caffeine	13.61 ± 0.29ab	2.03 ± 1.63c	27.98 ± 1.63a	116.33 ± 11.59a
0.6% EMS + 20% Caffeine	14.24 ± 0.64a	1.39 ± 0.52c	26.15 ± 2.15a	136.00 ± 41.39a
$LSD_{0.05}$	1.90	2.04	NS	31.43

[z]Different letters indicate means significantly different within columns at $P \leq 0.05$.

[y]Readings taken from a SPAD chlorophyll meter, unitless.

Table 4. Mean germination time, plant height, and leaf chlorophyll content in response to ethyl methanesulfonate (EMS) and caffeine mutagen treatments applied to seed of *Silene chalcedonica*[z].

Mutagen	Mean germination time (days)	Plant height (cm)	Chlorophyll[y]	Days to first flower
Control	9.54 ± 0.37b[y]	26.08 ± 1.45a	27.53 ± 0.19a	71.33 ± 2.08c
0.6% EMS	11.09 ± 0.46a	9.59 ± 2.26b	19.41 ± 1.45b	90.33 ± 1.53a
10% Caffeine	9.92 ± 0.39b	24.87 ± 2.90a	26.60 ± 1.78a	70.33 ± 2.52c
20% Caffeine	9.95 ± 0.73b	26.05 ± 7.16a	27.70 ± 2.11a	68.67 ± 1.53c
0.6% EMS + 10% Caffeine	11.01 ± 0.16a	11.06 ± 1.92b	19.85 ± 0.75b	83.00 ± 6.24b
0.6% EMS + 20% Caffeine	11.46 ± 0.82a	8.54 ± 2.46b	20.12 ± 1.79b	89.00 ± 1.73a
$LSD_{0.05}$	0.96	6.36	2.67	5.50

[z]Different letters indicate means significantly different within columns at $P \leq 0.05$.

[y]Readings taken from a SPAD chlorophyll meter, unitless.

Table 5. Mean germination time, plant height, and leaf chlorophyll content in response to ethyl methanesulfonate (EMS) and caffeine mutagen treatments applied to seed of *Silene flos-cuculi*[z].

Mutagen	Mean germination time (days)	Plant height (cm)	Chlorophyll[y]
Control	14.56 ± 0.69ab[y]	10.21 ± 0.34a	36.88 ± 2.25a
0.6% EMS	15.83 ± 0.53a	6.65 ± 0.04b	32.37 ± 2.86b
10% Caffeine	12.47 ± 0.75c	9.27 ± 0.85a	36.66 ± 2.09a
20% Caffeine	13.59 ± 0.86bc	9.90 ± 0.78a	37.77 ± 1.05a
0.6% EMS + 10% Caffeine	15.85 ± 1.18a	6.70 ± 0.83b	32.01 ± 1.10b
0.6% EMS + 20% Caffeine	14.88 ± 1.13ab	6.78 ± 1.09b	34.94 ± 2.70ab
$LSD_{0.05}$	1.58	1.32	3.79

[z]Different letters indicate means significantly different within columns at $P \leq 0.05$.

[y]Readings taken from a SPAD chlorophyll meter, unitless.

Mutagenesis caused leaf texture, shape, and color variation in *S. flos-cuculi*.

Mean germination time is an index showing how fast the seed lot germinates, and reflects seed vigor. The longer the mean germination time lasts, the lower the observed seed vigor. Using this index, each species had a different reaction with the mutagen treatments. *Silene coronaria* showed no difference to any treatment, *S. ×haageana* had decreased seed vigor by both the caffeine only and the EMS plus caffeine treatments, *S. chalcedonica* incurred lower seed vigor only with treatments with EMS, while in the caffeine only treatments, *S. flos-cuculi* had higher seed vigor than the control and any other treatments that contained EMS. In fact, any mutagen treatment that contained EMS did not affect the mean germination time of *S. flos-cuculi*.

Mutagenesis causes DNA or nucleotide changes and may induce more DNA repair or other mechanisms that prolong the DNA replication process, hence generally increasing mean germination time as shown in the species *S. ×haageana* and *S. chalcedonica*. Zhu et al. (1995) reported that caffeine as a post treatment agent lowered the mutation frequency of soybean (*Glycine max* (L.) Merr.) treated with EMS. Caffeine application may have increased seed vigor in S. *flos-cuculi* by facilitating DNA repair in the seeds. The effect and efficiency of certain kinds of mutagens might relate to both a difference in species seed morphology and physiological status.

Mean germination time (MGT) and seedling survival rate reflect different aspects of seed quality. A higher survival rate of 70 to 80% was targeted to yield desirable mutations versus the 50% survival rate that was often thought beneficial for producing good mutations in the past (van Harten 1998). In a preliminary trial, though, we preferred a low survival rate by setting the treatment conditions for the studied species (data not shown). A lower rate of caffeine not only shortened the mean germination time (MGT) of *S. flos-cuculi*, but also increased the seedling survival rate of this species. The results theoretically coincide with Zhu et al. (1995) report about caffeine's post-treatment impact on mutagenesis induced by EMS.

Mutant phenotypes were obtained more from the EMS plus caffeine treatments than the EMS only treatments. Caffeine applied alone mutated seedlings, with half of each leaf becoming notably lighter green than the other half, observed when *S. flos-cuculi* plants grew to the four leaf stage, but then this trait disappeared afterward. A mericlinal chimeric often occurs in mutagenic treatment for seeds, and this unstable type of mutation tends to be lost as plants grow or they develop into a stable periclinal chimeric (Geier 2012). Other leaf color changes involved border color variation in *S. coronaria*, *S. chalcedonica*, and *S. flos-cuculi*. Other crops where mutants with altered chlorophyll level have been reported with the use of EMS include peas (*Pisum sativum* L.), carrots (*Daucus carota* ssp. *sativus* L.), soybeans, lentils (*Lens culinaris* Medik.), radishes (*Raphanus sativus* L.), and barley (*Hordeum vulgare* L.) (Miller et al. 1984, van Harten 1998). A mutant *S. flos-cuculi* plant was identified with increased wax development on the leaf, changing the leaf color and potentially having enhancing drought tolerance. The effectiveness of mutation breeding for improving drought tolerance has been manifested on wheat (*Triticum aestivum* L.) (Njau et al. 2005) and barley (*Hordeum vulgare*) (Cagirgan et al. 2002).

Delayed time to first flower was recorded for both *S. chalcedonica* and *S. ×haageana*. Muthusamy and Jayabalan (2011) reported that a lower mutagenic treatment rate induced early flowering in cotton (*Gossypium hirsutum* L.); however, a higher rate resulted in delayed flowering. Based on our experiment, *S. flos-cuculi* is a species that can flower the first year under greenhouse conditions; however, mutants were observed that had not flowered in the greenhouses for two years. Büttner et al. (2010) revealed EMS mutated an unknown loci located on chromosome IV in sugar beet (*Beta vulgaris* L.) beside the bolting gene *B* located on chromosome II, and the two genes both have an effect on flower time. Hohmann et al. (2005) reported an efficient EMS protocol for getting non-bolting mutated sugar beets from an early bolting sugar beet which lacked a vernalization requirement, and obtained some mutant lines that required vernalization for flowering. The non-flowering *S. flos-cuculi* mutants may need vernalization to flower. Research on the flower time change function could benefit plant production by extending vegetative growth, or shorten the breeding cycle by shortening the time to first flower.

Mutation effects on flower traits were observed from 0.6% EMS plus 20% caffeine treatment on both *S. chalcedonica* and *S. ×haageana*. Bigger flowers were observed on *S. chalcedonica* mutants, and smaller flowers were seen along with stunted plants in mutant *S. ×haageana* plants (data not shown). There were no flower color change or variegation on these selected species except there were some bleached spots on *S. ×haageana* 'Molten Lava' petals, which did not appear to be a beneficial trait. In plant mutation breeding history, deleterious mutations were observed remarkably more than beneficial ones (Boyle 2006). Another obvious change observed on flowers was that mutagenesis caused stronger pistils and weaker stamens in *S. chalcedonica* and *S. ×haageana*. Ethyl methanesulfonate has also been reported to cause mutations in the reproduction system in animals (Sega 1974, Aaron and Lee 1978). Taking advantage of *S. chalcedonica* mutants which have less pollen or no pollen as females in breeding might facilitate hybridization since based on our observation, this species has a high self-pollination rate.

The potential repair by caffeine for the mutagenesis effects of EMS (Zhu et al. 1995) was supported in this research in that *S. flos-cuculi* had higher seed vigor in the 10% caffeine treatments than the nontreated control or any treatment containing EMS (Table 5). Mutated seedlings caused by the 20% caffeine treatment, with half of the leaf becoming notable lighter green than the other half, were observed when *S. flos-cuculi* plants grew to the four leaf stage, then this trait disappeared, suggesting it was not a genetically stable change. There were no mature mutated plants produced by caffeine-only treatments in this study, so mutagenesis might require higher caffeine concentration or longer treatment duration than which was used in this experiment. The rate of 20% caffeine is the maximum value which could be made using the Jet-Alert caffeine tablets, which has a much higher amount of caffeine (20 mM or approximately 3.88 $g \cdot L^{-1}$) than was used as a post treatment on soybean (Zhu et al. 1995). Therefore, it is a reasonable deduction that caffeine has a repair function for naturally or mutagen-deteriorated seeds in low concentration, yet has a mutagenesis effect when used in high doses. The use of caffeine as a mutagen should be tested further on other species as well as evaluating the effects on the next generation of the mutagenized plants (M2).

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