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Germination of *Viola odorata*, a Genetic Resource for Fragrance in *Viola* Breeding

Shea A. Keene and Thomas A. Colquhoun

Environmental Horticulture Department, UF/IFAS, 1523 Fifield Hall, Gainesville, FL 32611, USA

keene284@ufl.edu

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Summary

The highly scented *Viola odorata* is a potential genetic resource for fragrance-focused *Viola* breeding; however, the species is thought to exhibit seed dormancy and germination of the seeds is difficult. The objective of this study was to investigate two methods to break dormancy and promote germination in *V. odorata*, including different concentrations of gibberellic acid in the culture media, and different durations of cold stratification. Ultimately, the highest germination percentage was achieved via cold stratification at 4 °C for 8 and 12 weeks.

INTRODUCTION

Viola odorata, also known as sweet violet, is a cleistogamous, temperate, perennial species in the Violaceae family that is famous for its fragrance (Marcussen, 2006). As genetic resources of aroma traits, sweet violets could be used in a fragrance-focused breeding program to develop fragrant *Viola* hybrids that can tolerate the intense heat and humidity common in the southeastern US. However, like other *Viola* species, *V. odorata* is thought to exhibit seed dormancy as its seeds are notoriously difficult

to germinate and overall germination rates are low (Banasinksa and Kuta, 1996; Barekat et al., 2013). Achieving consistent, high rates of germination is very important in a breeding program that utilizes interspecific hybridization, as poor germination would result in smaller parental and hybrid populations, and thus less genetic variance from which to select (Brown et al., 2014). Seed dormancy may be defined as a barrier that prevents the complete germination of a viable seed under otherwise favorable environmental conditions (Baskin and Baskin, 2004). Dormancy evolved across species as an adaptation to different environments—resulting in a diverse range of dormancy mechanisms—and serves to prevent germination until certain conditions have been met and the environment is favorable to the establishment of a new generation (Finch-Savage and Leubner-Metzger, 2006). The requirements to break dormancy vary just as widely and can be highly specific to different taxa (Willis et al., 2014). The most common form of dormancy exhibited by *Viola* species—non-deep physiological dormancy (PD)—may be broken by stratification or treatment with gibberellic acid (GA_3) (Franklin et al., 2017; Gehring et al., 2013). Thus, the objectives of this research were to evaluate two methods to break dormancy and achieve germination of *V. odorata* seeds, including different concentrations of GA_3 in the culture media and different durations of cold stratification.

MATERIALS AND METHODS

Experiment 1: Different GA_3 concentrations in the culture media. Seeds of *V. odorata* 'Reine de Neiges' were purchased from Jelitto Seeds (Schwarmstedt, Germany) and stored in their original packaging before use. The culture media was prepared with Murashige and Skoog (MS) basal salt mixture, sucrose, and agar. After the media was autoclaved, a filter-sterilized GA_3 stock solution was added to three of the bottles to achieve GA_3 concentrations of 2 mg/L, 6 mg/L, and 250 mg/L. Media with 0 mg/L GA_3 was also prepared. The media was then poured to solidify in petri dishes with an 8 cm diameter. Prior to sterilization, 240 seeds were mechanically scarified by gently rubbing them between two pieces of 120-grit sandpaper for 1 min. All seeds were surface sterilized in a 4.125% sodium hypochlorite solution with two drops of Tween-20 for 15 min on a gyratory shaker, then rinsed three times with sterile distilled water. Twenty seeds were transferred onto each petri dish using sterile forceps in a transfer hood. The petri dishes were sealed with a single layer of sealing film and placed on a shelf under cool white fluorescent tube lights with a 16-h light/8-h dark photoperiod and maintained at 25 °C.

Experiment 2: Different durations of cold stratification. Seeds of *V. odorata* 'Rubra' were purchased from Jelitto Seeds (Schwarmstedt, Germany) and stored in their original packaging before use. Media for stratification was prepared as described in Experiment 1, with the following changes. Media for a given treatment group was prepared on the day the stratification treatment was initiated, and no GA_3 was added. No seeds were scarified before sterilization. Seeds were sterilized as described in Experiment 1. Once seeds were sown on the media (20 seeds per dish), the sealed petri dishes were stored in a laboratory refrigerator at 4 °C for the appropriate duration. Stratification was staggered so that all seeds were moved to germination conditions at

the same time. Seeds in the 0-week stratification treatments were sterilized before they were sown on the germination media. For germination, each petri dish of seeds was transferred to a 20-cell insert containing high-porosity peat-based media (PRO-MIX HP; Premier Horticulture Ltd., Rivière-du-Loup, Quebec, CA), with one seed per cell. These inserts were placed in solid-bottom plastic trays without holes and covered with clear plastic humidity domes. The covered trays were placed on a shelf under cool white fluorescent tube lights with a 16-h light/8-h dark photoperiod and maintained at 25 °C. Deionized (DI) water was added as needed to keep the seeds moist.

Experimental design, data collection, and analysis. The experimental design of Experiment 1 was a single factor (GA_3 concentration), completely randomized design (CRD) with five treatments: no scarification and 0 mg/L GA_3 (control); 0 mg/L GA_3; 2 mg/L GA_3; 6 mg/L GA_3; and 250 mg/L GA_3. All seeds were scarified, except the seeds in the control group. Each treatment was replicated three times and the experimental unit was an individual petri dish. Seeds were considered to have germinated when the radicle emerged. The experimental design of Experiment 2 was a single factor (stratification duration at 4 °C) CRD with four treatments: 0 weeks, 4 weeks, 8 weeks, and 12 weeks. Each treatment was replicated four times, and the experimental unit was an individual petri dish/20-cell insert. Seeds were considered to have germinated when the shoot/cotyledons emerged. For both experiments, a CRD was chosen because of the homogenous nature of the experimental units, and because the experiments were conducted in a laboratory with stable environmental conditions. For both experiments, the final germination percentage (FGP) was calculated for each experimental unit as the number of seeds germinated divided by the total number of seeds in each unit, then multiplied by 100. The FGP of each experiment was analyzed using analysis of variance (ANOVA) based on the model y = overall mean + treatment effect + residual. The ANOVA assumptions of homogeneity of variance and normally distributed residuals were checked with Levene's test and the Shapiro-Wilk test, respectively. If the F-statistic of the ANOVA indicated significant treatment effects ($P < 0.05$), the data were further analyzed by Tukey's range test with a *P*-value of 0.05.

RESULTS

Experiment 1. The FGP of 'Reine de Neiges' seeds was not significantly different among the GA_3 treatments (**Table 1**). Overall, the FGP was very low, reaching a maximum of only 10% to 13.33%.

Experiment 2. The FGP of 'Rubra' seeds was significantly different among the stratification duration treatments, with the highest FGP (approximately 70%) achieved for seeds stratified for 8 weeks and 12 weeks (**Table 2**). The FGP of seeds stratified for 8 weeks was not significantly different than the FGP of seeds stratified for 12 weeks.

Table 1. Effect of GA_3 concentration in the media on the germination of *V. odorata* 'Reine de Neiges' seeds.

Treatment	Cumulative number of seeds germinated[1]	Final germination percentage (%)[2]
Control	0	0.00 ns
0 mg/L GA_3	4	6.7 ns
2 mg/L GA_3	6	10.0 ns
6 mg/L GA_3	6	10.0 ns
250 mg/L GA_3	8	13.3 ns

[1] For a given treatment, the combined total number of seeds that germinated in replicates 1-3. [2] For a given treatment, the final germination percentage (FGP) is the average of the FGP of replicates 1-3. Within this column, ns indicates the ANOVA was not significant with $\alpha = 0.05$.

Table 2. Effect of cold stratification duration on the germination of *V. odorata* 'Rubra' seeds.

Treatment	Cumulative number of seeds germinated[1]	Final germination percentage (%)[2]
12 weeks	55	68.8 A
8 weeks	56	70.0 A
4 weeks	24	30.0 B
0 weeks	9	11.3 C

[1] For a given treatment, the combined total number of seeds that germinated in replicates 1-4. [2] For a given treatment, the final germination percentage (FGP) is the average of the FGP of replicates 1-4. Within this column, values followed by different letters were significantly different as determined by Tukey's HSD ($P < 0.05$).

DISCUSSION

In Experiment 1, the FGP of 'Reine de Neiges' seeds was very low, and it was lower than the FGP achieved under similar conditions in other studies. For example, in the 2 mg/L GA_3 treatment, only 10% of 'Reine de Neiges' seeds germinated. In contrast, Banasinska and Kuta (1996) achieved approximately 60% germination for *V. odorata* seeds that were mechanically scarified and sown on MS media with 2 mg/L GA_3. Similarly, only 10% of 'Reine de Neiges' seeds germinated on MS media containing 6 mg/L GA_3, compared to 29.99% of *V. odorata* seeds sown on media with the same GA_3 concentration (Barekat et al., 2013). The failure to find significant differences ($P < 0.05$) may be due in part to inadequate replication of the treatments, as the residual degrees of freedom fell short of the rule-of-thumb value, 12 (Clewer and Scarisbrick, 2001). To a certain point, increasing the number of replicates can increase the precision of treatment comparisons and allow for the detection of smaller differences among those treatments (Casler et al.,

2015). The amount of available seed material constrained the number of replicates in this experiment. In subsequent experiments, additional seed was acquired so that more replicates could be done. Interestingly though, a percentage of violet seeds germinated at all treatment levels, except one (**Table 1**). In the control condition, non-scarified seeds on GA_3-free media did not germinate; however, scarified seeds on GA_3-free media did germinate. These results suggest that sweet violet seeds exhibit non-deep PD, as scarification can help promote germination in water-permeable seeds with non-deep PD (Baskin and Baskin, 2014). The results may also suggest that more GA_3 is not necessarily better at promoting germination. From a practical standpoint, using less (or no) GA_3 would be better, as it would save time and money since GA_3 is an expensive and heat-labile compound that must be filter-sterilized and added to the media after the media has been autoclaved. Ultimately, the results of this experiment demonstrated that seeds of a *V. odorata* cultivar can be successfully germinated under *in vitro* conditions; however, high rates of germination were not achieved.

In Experiment 2, both the 8-week and 12-week stratification durations resulted in high germination success for 'Rubra' seeds, with FGPs of approximately 70%. The germination rates achieved here are comparably high when compared to the germination rates achieved in other studies of *Viola*. For *V. odorata*, Barekat et al. (2013) achieved a maximum FGP of 74.58% and 54.31% with endosperm culture and embryo culture, respectively. However, these percentages were based on the germination of four treatment replicates with only five seeds per replicate. Moreover, such methods are expensive, as well as time- and labor-intensive, as they require aseptic conditions and *in vitro* culturing. Banasinska and Kuta (1996) achieved germination for 37 out of 61 seeds of *V. odorata*, a germination rate of approximately 60%, with mechanical scarification and *in vitro* culture on MS media supplemented with 2 mg/L GA_3. In research on other *Viola* species, Gehring et al. (2013) achieved maximum germination of approximately 50-60% for seeds of *V. pedata* that were exposed to a 12-week warm-dry treatment, followed by cold stratification for 12 weeks. Franklin et al. (2017) achieved maximum germination of 90-100% for seeds of *V. pedunculata* and *V. purpurea* that were exposed to several months of dry storage and several weeks of cold stratification at 4 °C. Like Franklin et al. (2017), the FGPs achieved here suggest that experimenting with dormancy-breaking conditions that mimic natural conditions—in this case, extended cool-wet conditions—can increase germination success.

CONCLUSION

Of the two methods evaluated in this study, the highest FGPs (approximately 70%) of seeds of a *V. odorata* cultivar were achieved via cold stratification at 4 °C for 8 and 12 weeks. Thus, based on these results, it is recommended that seeds of *V. odorata* and its cultivars be cold stratified at 4 °C for at least 8 weeks to break dormancy and promote germination.

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