Viola pedatifida G. Don (prairie violet) fruits and seeds. Fruit split open with seeds ready to ballistically disperse (*center of photo*) and fruit pointed upward ready to be collected (*to the right*).

Seed collection, storage, and germination practices may affect *Viola* reintroduction outcomes

Sam Kilgore, Kayri Havens, Andrea Kramer, Ashlyn Lythgoe, Linda MacKechnie, and Marcello De Vitis

ABSTRACT

Numerous factors can influence the ability to successfully procure and use seeds to support plant reintroductions, including challenges with seed collection, storage, and germination. *Viola* species (Violaceae) are often missing from regional restoration activities because of such obstacles. Using 6 *Viola* species native to prairie and woodland habitats in the Midwestern United States, we investigated how timing of seed collection, seed storage conditions and duration, and seed germination pretreatment influences seed viability and germination. Specific germination pretreatments tested were cold stratification length, priming with polyethylene glycol, and gibberellic acid. Our results indicate that very short-term seed storage, from 1 d to 4 mo, can influence the depth of primary dormancy in violet seeds, significantly affecting subsequent germination. Cold stratification was the most effective seed pretreatment in breaking dormancy across the 6 species studied, whereas responses to other pretreatments (for example, gibberellic acid and priming) were largely species-specific. Even though cold stratification was consistently the most effective pretreatment, responses significantly differed among and within species. We provide a "best practices" checklist and recommend more detailed record-keeping when seeds are collected, stored, and pretreated for use in restoration.

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KEY WORDS

cold stratification, seed germination, seed storage, restoration, *Viola labradorica*, *Viola lanceolata*, *Viola pedatifida*, *Viola pubescens*, *Viola sagittata*, *Viola sororia*, Violaceae

NOMENCLATURE

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Seeds are an excellent propagule source for restoration, as they are portable, can often be stored for many years, and can capture substantial amounts of genetic diversity (Broadhurst and others 2008; Rajjou and Debeaujon 2008). Seed supply, however, is often unable to meet restoration demand (Merritt and Dixon 2011). Not all species are equally represented in native seed markets (Ladouceur and others 2018; White and others 2018; Hancock and others 2020), and for many large-scale projects entire plant genera are not available (Barak and others 2017). The pool of species available from commercial and institutional seed suppliers can impose an important biodiversity filter in ecological restoration projects (Ladouceur and others 2018). The species most commonly absent from the native seed market are usually those that are rare or protected (Ladouceur and others 2018; White and others 2018) and species with seeds that are difficult to collect, germinate, or grow (Ladouceur and others 2018). Understanding species biotic and abiotic requirements and matching them to the restoration site can increase restoration success (Falk and others 1996; Godefroid and others 2011). But for many species, we lack the in-depth knowledge required to maximize reintroduction success.

Species in the genus *Viola* (Violaceae) are often targets for restoration in the Midwestern US because they provide important early-spring resources for pollinators. They also act as larval hosts for at-risk Fritillary butterfly species that require suitable *Viola* populations in undisturbed tallgrass prairies to complete their life cycle (Debinski and Kelly 1998). However, they are often excluded from restoration efforts because they present many challenges at the seed collection, storage, and propagation steps (Banas 2020). Because of these challenges, many violet species are not commercially available in quantities large enough to support restoration on regional scales (White and others 2018). Even when *Viola* species are included in restoration projects, seeds or reintroduced plants often fail to establish or persist (Banas 2020; Durkin 2020; Nyberg 2020).

Wild seed collection is the first challenge presented by *Viola* species because of the small stature of these plants and explosive dehiscence of seeds from ripe fruit capsules. Successful seed collection depends on a short window of 1 to 3 d between seed maturity and when seeds are ballistically dispersed up to 5 m away from the parent plant (Beattie and Lyons 1975). Collectors have developed a strategy of using fruit orientation as a proxy for seed maturity. As the fruits of most *Viola* species mature, they progress from pointing below to above the horizon over the course of 2 to 3 d. As soon as fruits begin to point upward, capsules are collected while they are still closed. After collection, they are left to split open and release seeds in a controlled space to avoid seed loss (personal communication from nursery managers). This collection protocol harvests seeds slightly before natural dispersal, but at a developmental point when germination and seedling survival can still occur. Since seed maturity is a gradient, it is critical not to collect seeds too early as they might fail to germinate. If collected prematurely, the seeds may not be fully ripe, which may influence the depth of dormancy, dormancy-breaking requirements, and ultimately seedling survival (Priestly 1986 in Lippitt and others 1994).

Lack of knowledge on optimal seed storage may also limit successful conservation and restoration of *Viola* species. Seed storage is known to affect dormancy in seeds (reviewed in Graeber and others 2012), and suboptimal storage conditions, such as long-duration high temperature and humidity, are known to drastically decrease seed viability of many species (Harrington 1972; Baskin and Baskin 2014). Hence, understanding optimal storage conditions in wild species is important. Short-term storage has been investigated for *Viola tricolor* L. (johnny jumpup) (6 and 12 mo storage; Demir and others 2011), but to our knowledge, no other study has investigated the impact of short-term storage on wild violet seeds. Regional growers and practitioners store *Viola* seeds across a range of durations (from very short-term storage, such as less than 24 h,

Viola pedatifida fruits with seeds. From top to bottom: unripe seeds collected too early, ripe tan seeds in a naturally split-open fruit, fruit pointed upward ready for collection.

to medium- and long-term storage) and under a range of conditions (for example, in plastic bags at ambient conditions or in more controlled conditions, such as inside refrigerators or sealed containers at approximately 13 °C [55.4 °F] and 40% RH) (Banas 2020; Durkin 2020; Schultz 2020; Nyberg 2020). While the impact of commonly applied durations and conditions of storage has been investigated on many wild species, little research has examined the impact of very short- (24 h) and short-term (3–18 mo, according to Hong and others 1996) storage. Seed collectors and restoration practitioners apply storage conditions right after collection, even when seeds need to be used shortly after collection for propagation or restoration purposes. Understanding if and how these conditions affect seed viability and germination can help the restoration community maximize viability and germination.

Conservation and restoration of *Viola* species are also limited by lack of information on optimal seed germination protocols. Physiological dormancy (PD), or the physiological inhibition of radicle emergence until specific conditions have been met (Baskin and Baskin 2014), has been recorded in at least 24 *Viola* species globally (reviewed in Baskin and Baskin 2014; see also Baskin and Baskin 1972; Baskin and Baskin 1975; Grime and others 1981; Geneve 2003; Jensen 2004; Eckstein and others 2006; Gehring and others 2013; Meineri and others 2013; Elisafenko 2015; Franklin and others 2017; Godefroid and Van de Vyver 2019). In *Viola*, PD is broken by cold stratification (reviewed in Baskin and Baskin 2014). Optimal duration of cold stratification required for maximum germination for many *Viola* species is unknown. The success of other seed pretreatments to break seed dormancy is highly variable within and between *Viola* species, with germination percentages ranging from 10% to 80% (reviewed in Baskin and Baskin 2014, but see also Deno 1993, 1996, 1998; Barekat and others 2013; Gehring and others 2013). Other potentially successful dormancy-breaking approaches include the use of exogenous gibberellic acid (GA), which has shown success in breaking dormancy of both wild (Solbrig 1981; Deno 1993, 1996, 1998; Franklin and others 2017) and cultivated (Barekat and others 2013) *Viola* species, and osmo-priming seeds with polyethylene glycol (PEG), which has shown success when used on cultivated *Viola* species (Carpenter and Boucher 1991; Yoon and others 1997; Geneve 1998) but is unstudied on their wild counterparts.

The common practices described above are currently used to collect and store seeds of wild violets and may have an impact on the viability and germination of seeds used in restoration efforts. To inform restoration work using violet seeds, we conducted experiments to understand how seed viability and germination are influenced by 1) seed maturity at time of collection; 2) very short- and short-term storage; and 3) dormancy-breaking pretreatments. Specifically, we looked at how collecting seeds immediately prior to dispersal (a frequently used collection protocol in *Viola*) affects the likelihood that seeds will germinate under standard conditions. We also tested the effect of storage on dormancy establishment to see if seed storage conditions contribute to the low germination frequently reported by practitioners. In addition, we investigated the impact of cold stratification, gibberellic acid application, and seed priming on dormancy breakage, because these treatments have been shown to achieve relatively high germination percentages in wild and (or) cultivated *Viola* species. We focused on 6 *Viola* species from a variety of Midwestern US habitats to provide general answers to nursery and land managers who want to include *Viola* species in conservation and restoration projects.

METHODS

Study Species

Six perennial *Viola* species native to the Chicago region were selected for this study (Figure 1). Three species (*Viola lanceolata* L. [bog white violet], *V. pedatifida* G. Don [prairie violet], and *V. sagittata* Aiton [arrow-leaved violet]) occur primarily in prairie habitat and are being actively produced for and used in restoration activities in the region (Wilhelm and Rericha 2017). Two species (*V. pubescens* Aiton [downy yellow violet] and *V. labradorica* Schrank [dog violet or alpine violet; recently synonymized with *Viola conspersa* Rchb. and listed as threatened in Illinois (USDA NRCS 2021)]) occur in woodland habitat and are less commonly produced and used in restoration efforts (Wilhelm and Rericha 2017). The sixth species (*Viola sororia* Willd. [common blue violet]) is abundant in a wide range of disturbed habitats, including lawns, and is typically not used in restoration efforts (Table 1).

Experimental Approach

We conducted 4 germination trials following the same general methodology described here to understand the impact of 1) seed maturity; 2) one-d storage conditions; 3) short-term (4 and 16 wk) storage conditions; and 4) pretreatments on seed germination. When modifications were applied, they are described in their respective sections. We sowed 4 replicates of 25 surface-sterilized seeds on a 1.5% agar medium in sterile 35 mm (1.38 in) Petri dishes for each accession and treatment. Seeds were surface sterilized in a 1% commercial bleach solution for 30 s and rinsed 3 times in sterile deionized water prior to sowing. For tests employing GA or PEG, seeds were soaked for either 24 h in GA solutions or 7 d in PEG, rinsed twice in deionized water, and blotted to remove any traces of the treatment reagent prior to surface sterilization. Following plating, seeds were placed in a climate-controlled incubator at alternating temperatures of 25/15 °C (77/59 °F) and light/dark with a 12-h photoperiod, as this temperature regime maximized germination while minimizing mold growth (authors' personal observation, unpublished data). Germination was assessed every 2 to 3 d (and germinants removed) until

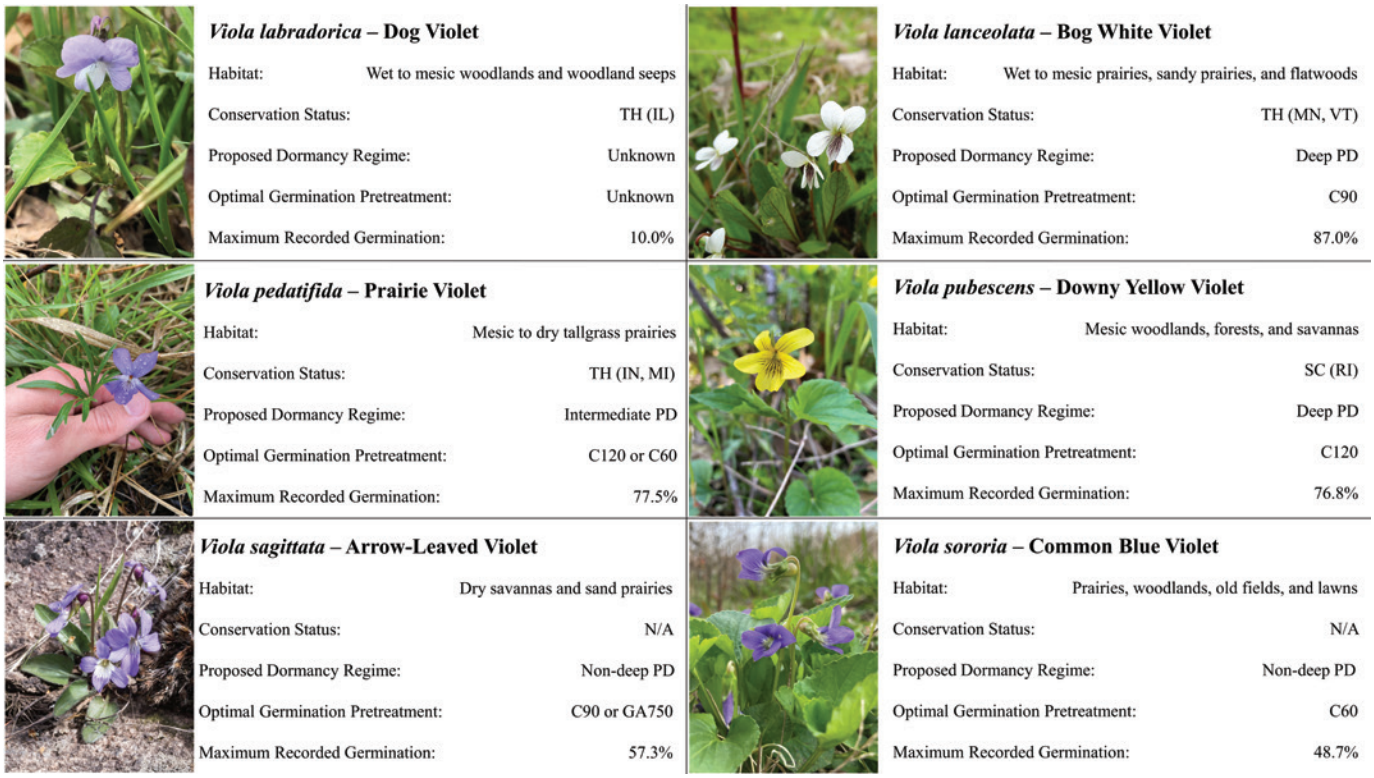


Figure 1. Viola species tested, the habitat in which they are found, conservation status, and select results.

TABLE 1

Details of the seed accessions used for this study: species; location of the source population; month and year of seed collection; name of the accession; relationship (to specify if there is any relationship between 2 of the accessions); origin (W = wild, N = nursery, GC = growth chamber); seed storage conditions prior to beginning the germination experiment; seed storage duration.

Accession ID	Species	Source population	Collection months/Year	Relationship	Origin	Seed storage conditions	Storage duration
LabS	*Viola labradorica*	Glencoe IL	May 2018		W	23 °C, paper bag	11 mo
LanS	*Viola lanceolata*	Morocco IN	Apr–Jun 2018		N	4 °C, sealed plastic bag	10–12 mo
PedS1	*Viola pedatifida*	Lake County IL	May–July 2018	Parent population of Ped2-Ped4	N	23 °C, sealed plastic bag	11–13 mo
PedS2		Lake County IL	Mar–May 2018	F1 of Ped1	GC	23 °C, paper bag	9–11 mo
PedS3			July 2019		GC	Variable, see methods	1 d
PedS4		Grayslake IL	July 2019	F1 of Ped1	N	Variable, see methods	1 d
PubS1	*Viola pubescens*	Lake County IL	Mar–May 2018	Parent population Pub3	GC	23 °C, paper bag	9–11 mo
PubS2		Lake Forest IL	Mar–May 2018		W	23 °C, paper bag	9–11 mo
PubS3		Lake County IL	July 2019	F1 of Pub1	GC	Variable, see methods	1 d
SagS	*Viola sagittata*	Morocco IN	Apr–Jun 2018		N	4 °C, sealed plastic bag	10–12 mo
SorS1	*Viola sororia*	West Chicago IL	May–July 2018		W	23 °C, paper bag	11–13 mo
SorS2		Highland Park IL	July 2019		W	Variable, see methods	1 d

Notes: 4 °C = 39.2 °F; 23 °C = 73.4 °F

no further germination was observed for 3 consecutive d (tests lasted approximately 3 wk). We scored germination based on visible radicle emergence (> 1 mm [0.04 in]). After final germination was recorded, the status of the ungerminated seeds was assessed using the seed press test (firm, presumed alive and empty/rotten, presumed dead; Kitchen and Monsen 2001), and the final germination proportion was adjusted with respect to the number of viable seeds.

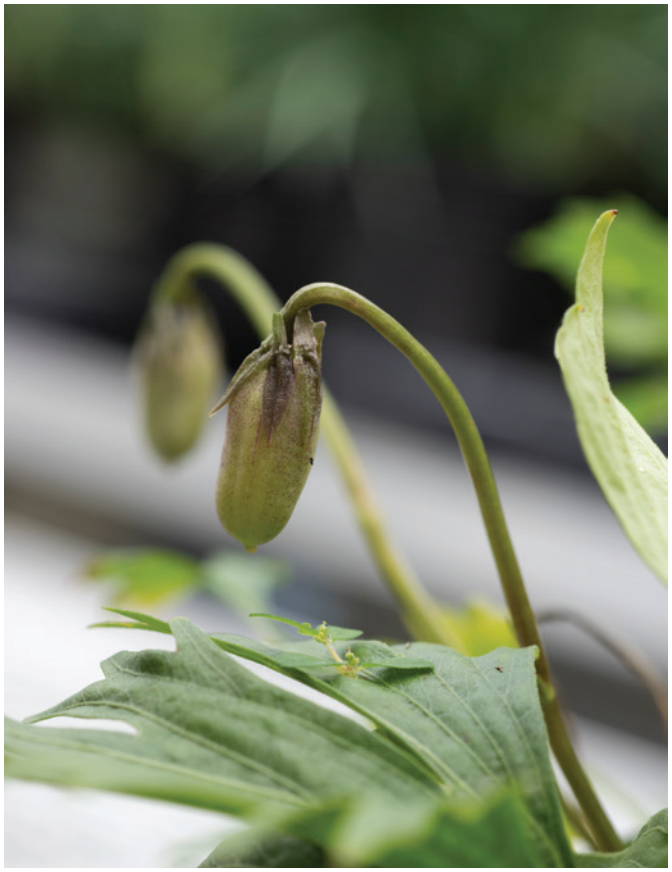
Viola pubescens seeds and elaiosomes.

Impact of Seed Maturity on Germination

Viola pedatifida (PedS3; Table 1) fruits were collected in July 2019 from 30 plants cultivated in a greenhouse at the Chicago Botanic Garden with 3 treatments representing different stages of seed maturation: 1) fruit facing downward; 2) fruit facing upward; and 3) early dehiscence (seeds are visible through open fruit but before dispersal). We collected one fruit per plant per treatment. The collection stages (1–3) were used as a proxy for seed maturation. After collection, fruits were placed into paper bags at room temperature (approximately 20 °C [68 °F] and 48% RH) for 24 h to let the fruits release the seeds. Once separated from chaff, seeds from each group were placed between 2 blotter papers, moistened with 750 ppm GA solution, and allowed to imbibe for 24 h prior to being sown into Petri dishes as described above. The 750 ppm GA pretreatment was chosen here and in the next 2 sections because this concentration had shown success in other studies looking at wild *Viola* germination, and this pretreatment involves the least amount of time between seed collection and seed germination.

Impact of One-Day Storage Conditions on Germination

We collected seeds from upward-facing capsules of *V. pedatifida* PedS2 (Table 1) in July 2019 from 30 plants (1–2 fruits per plant) cultivated in a greenhouse at the Chicago Botanic Garden and randomly divided them into 2 groups: uncleaned (seeds left within fruits) and cleaned (seeds removed from fruits). Of each group of seeds, 4 replicates were exposed for 24 h to 5 storage condition treatments routinely used by restoration practitioners: 1) Control: seeds stored in a paper bag in a climate-controlled room (20 °C [68 °F], 48% RH); 2) Cold/Dry: in a paper bag in the refrigerator (3 °C [37.4°F], 48% RH); 3) Warm/Humid: in an air-tight plastic bag in warm and humid conditions (30 °C [86 °F], 75% RH); 4) Warm/Dry: in a paper bag in warm dry conditions (30 °C [86 °F], 22% RH); and 5) Seed Bank: dehydrated at 20% RH for 2 wk and then frozen in a sealed plastic bag at –20 °C (–4 °F). All replicates



Viola pedatifida fruits in the pointed-down orientation. Not yet ready to be collected but should begin turning upward in 2 to 3 d.

were kept in the dark by placing them in light-excluding bags. At the end of each storage treatment, seeds were treated in a 750 ppm GA solution for 24 h prior to germination testing. Because of limitations in seed availability, only 19 to 21 seeds were sown per Petri dish instead of 25. Treatment #5 was excluded from the cleaned group due to lack of seeds, making a total of 9 treatments.

Impact of Short-Term Storage Conditions on Germination

For this experiment, we collected upward-facing closed fruits from *V. pedatifida* (PedS4), *V. pubescens* (PubS3), and *V. sororia* (SorS2) in July 2019 (Table 1). Once collected, seeds were allowed to release from their fruits inside paper bags under room temperature conditions for 24 h before treatments began. Then, seeds were randomly assigned to 9 treatments, with each treatment containing 4 replicates of 75 to 100 seeds total, according to availability for each accession. The 9 treatments resulted from crossing 2 temperature (4 °C [39.2 °F] and 23 °C [73.4 °F]), 2 humidity (22 or 75% RH), and 2 duration (4 or 16 wk) conditions, plus a no-storage (immediate sow) treatment acting as the control. To ensure seeds stayed at specific humidity and temperature levels, seed replicates were suspended above saturated salt solutions in sealed,

climate-controlled boxes with either sodium chloride (75% RH) or potassium acetate (22% RH) (Young 1967), at either room temperature (approximately 23 °C [73.4 °F]) or in the refrigerator (4 °C [39.2 °F]). Following storage, seeds were surface sterilized and treated with a solution of 750 ppm GA for 24 h prior to germination testing.

Impact of Seed Pretreatments on Germination

Upward-facing fruits were collected from all 6 study species across 8 populations (LabS, LanS, PedS1, PedS2, PubS1, PubS2, SagS, and SorS1) between May and November 2018 (Table 1). Once collected, seeds were allowed to release from their fruits for between 24 and 48 h before being separated from chaff and stored (storage conditions and duration varied by accession; see Table 1).

Ten germination pretreatments were applied to each of the 8 seed accessions at the laboratories. Treatments included soaking seeds in 250 ppm, 500 ppm, or 750 ppm GA solutions for 24 h; priming seeds in –1 MPa, –1.5 MPa, or –3 MPa PEG solution for 7 d; 60-d, 90-d, or 120-d cold stratification at 4 °C (39.2 °F); and a control (no treatment). Limited GA and PEG treatments were performed on *V. sororia* and *V. labradorica* because of seed shortages.

To replicate approaches often used by nursery managers, we also surface-sowed seeds in potting soil in 10.2 × 10.2 cm (4 × 4 in) pots and watered them during the month of November 2018. Pots remained in an unheated shed with windows until spring 2019 and were watered when dry, until they were put in an incubator under conditions explained earlier in the Experimental Approach section. Seedling emergence was recorded until no new emergence was observed for 3 consecutive d.

Each accession was classified into 1 of 3 dormancy types, following Baskin and Baskin (2004): 1) deep PD: for which GA pretreatment was ineffective at promoting germination and maximum germination was observed at 120 d cold stratification; 2) intermediate PD: seeds exhibit a small response to GA, but this is significantly lower than observed after cold stratification, with maximum germination observed at 60 or 90 d cold stratification; 3) non-deep PD: when GA was effective in inducing germination at rates not significantly different from those observed in cold stratification, and maximum germination was reached with 60 d cold stratification. After-ripening is effective in species with both non-deep PD (during which it can break dormancy) and intermediate PD (when it can lessen required stratification length), but since no after-ripening treatments were included in this experiment, it was not used as a factor classifying dormancy here (reviewed in Baskin and Baskin 2014).

Statistical Analysis

Generalized linear models were used to test whether treatments explained variation in viability-adjusted percent germination. We used stepwise backward elimination of non-significant factors ($\alpha = 0.05$; $P > 0.05$) to identify a best-fit model (Crawley 2013). In some cases, we could perform data analyses because of small sample sizes and low (almost null) germination. All analyses were performed in R (R Core Team 2018). For the methods section on Impact of Short-Term Storage Conditions on Germination, no analyses were performed on *V. sororia* or *V. pubescens* given very low germination. For the Impact of Seed Pretreatments on Germination methods, Tukey's post hoc test was used to determine significant differences between individual pretreatments using the $\alpha = 0.05$, $P < 0.05$ threshold for delineating significance. Overwintering data were not analyzed but were plotted over germination figures for each accession as an estimation of germination following common nursery protocols. Analyses were run only within species because of the large degree of variation in storage conditions of seed among the 8 tested accessions.

RESULTS

Impact of Seed Maturity on Germination

Seed maturity, using fruit position and orientation as a proxy, significantly influenced germination in *V. pedatifida* ($P < 0.005$). Germination was the highest at earlier maturation stages (fruit pointed down; 71 ± 5%), intermediate when fruit pointed upward (53 ± 5%), and the lowest at maturity (immediately before dehiscence; 22 ± 4%).

Impact of One-Day Storage Conditions on Germination

Presence of fruit tissues while drying (included or excluded, $P < 0.001$), one-d storage conditions ($P < 0.001$), and their interaction ($P < 0.001$) all significantly affected percent germination of *V. pedatifida* seeds (Figure 2). Germination in the control was 17 ± 4% for both the chaff-included and -excluded treatments. None of the storage treatments significantly increased germination above this threshold. Under the Warm/Humid treatment (30 °C [86 °F], 75% RH), the highest germination was observed when chaff was excluded (19 ± 4% germination), compared to when chaff was included (2 ± 2% germination). An opposite trend was observed in the Warm/Dry treatment (30 °C [86 °F], 22% RH), in which seeds with chaff included had 20 ± 4% germination, while seed with chaff excluded had only 6 ± 2% germination. Germination in the single Seed Bank treatment was 8 ± 3%.

Impact of Short-Term Storage Conditions on Germination

Variation in seed germination percentages was explained by storage duration ($P < 0.001$), storage conditions ($P < 0.001$), and their interaction ($P = 0.038$) in *V. pedatifida* (Figure 3). Seeds in the control treatment germinated at 28 ± 5%. Seeds stored at

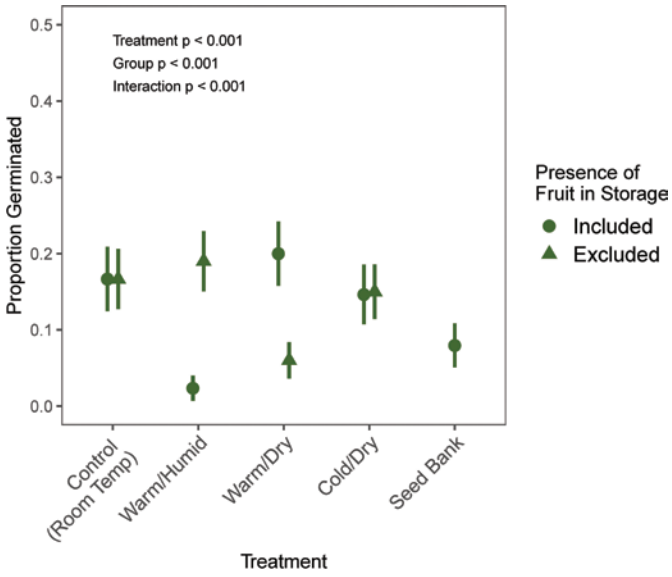


Figure 2. Germination response to one-d storage conditions in *Viola pedatifida*. Germination was significantly lower than the control when seeds were stored with fruit in the warm/humid treatment as well as when fruits were excluded and stored in the warm/dry treatment.

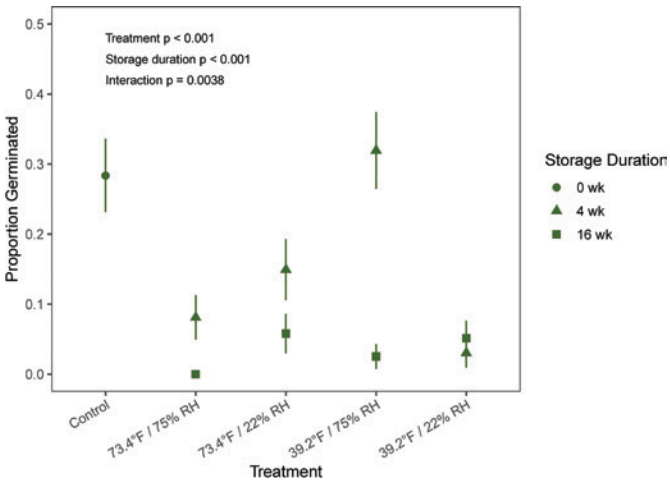


Figure 3. Viola pedatifida seeds were stored for 0, 4, and 16 wk at the above temperature/humidity conditions and pretreated with 750 ppm GA to see how storage factors affected germination. Storage had a negative impact on germination in all treatments except seeds stored for 4 wk at high temperature, high humidity.

23 °C (73.4 °F) at both 22% RH and 75% RH and for both 4 and 16 wk of storage germinated at lower percentages than the control. Germination after 16 wk of storage was consistently lower than the control. Storage for 16 wk also resulted in lower germination percentages than did 4-wk storage in all treatments except the 4 °C (39.2 °F), 22% RH storage treatment, in which there was no difference between 4- and 16-wk storage. The only storage treatment that resulted in germination not significantly lower than the control was the 4 °C (39.2 °F), 75% RH stored for 4 wk (32 ± 5%). However, when seeds were stored for 16 wk under these conditions, germination fell to 3 ± 2%. We did not run statistical analyses on the SorS2 or PubS3 accessions because of extremely low (below 10%) germination in all treatments (below 10% in SorS2 and zero germinants in PubS3). For SorS2, no germination occurred in the control treatment and the greatest germination percentage observed was 7 ± 1% from the 23 °C (73.4 °F), 22% RH, 4-wk storage treatment.

Impact of Seed Pretreatments on Germination

Germination response to the pretreatments was predominantly species-specific (Figure 4); however, we observed a few noteworthy trends. In all accessions (except for PubS2, which saw zero germination and was removed from further analyses), at least one of the cold stratification treatments enhanced germination. PEG treatments were ineffective or even damaging, GA was beneficial, but not uniformly so.

Viola labradorica

Statistical analyses were not run on this accession (LabS) because of extremely low germination (around 10%) in all treatments (Figure 4). No germination occurred in the control

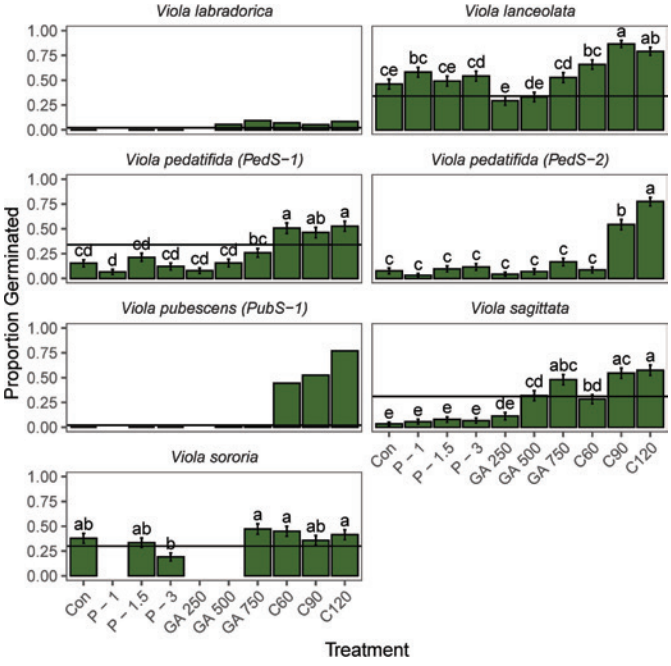


Figure 4. Viability adjusted germination of *Viola* species as a function of seed pretreatments: "Con" = control; "P - X" = Priming with PEG at –1, –1.5, and –3 MPa for 7 d; "GA X" = gibberellic acid application at 250, 500, and 750 ppm for 1 d; "CX" = cold stratification for 60, 90, and 120 d. Seeds in this experiment were stored for ~10 mo under room temperature conditions, with the exception of *V. sagittata* and *V. lanceolata*, which were stored for the same duration but at 4 °C (39.2 °F). Tukey post-hoc test results indicate significance only within a single accession, and treatments that do not share a letter notate a significant difference of $P < 0.05$. Horizontal lines indicate germination from the overwintering treatment and are not compared statistically to other treatments. Abovementioned statistical analyses were not run for *V. labradorica* because of low germination in many of the treatments, so mean values are reported without SE or Tukey values. For *V. pubescens,* statistical analyses were run for only the 3 cold stratification durations, and C120 resulted in significantly greater germination than C60 or C90 ($P < 0.05$).

treatment or the PEG treatments, while cold stratification treatments resulted in only 9% germination at most (C120). Overwintering germination was 2%.

Viola lanceolata

Seed pretreatments resulted in germination at proportions significantly different from that of the control (46 ± 5%; $P < 0.001$) in *V. lanceolata*. Except for the C60 treatment, cold stratification (C90 and C120) significantly improved the germination percentage to 87% ($P < 0.001$) and 79% ($P < 0.001$), respectively, when compared to the control (Figure 4). Neither the GA nor the PEG treatments significantly affected germination compared to the control. Overwintering resulted in 34% germination, which was similar to the control but less effective (by more than 50%) than the highest germination pretreatment (C90).

Viola pedatifida

Both accessions PedS1 and PedS2 showed significantly different germination proportions compared to the control in response to seed pretreatments ($P < 0.001$; Figure 4). Stratification improved germination significantly for all accessions excluding PedS2 C60. For PedS1, the C60, C90, and C120 treatments all increased germination (52 ± 3%; $P < 0.001$, 46 ± 4%; $P < 0.001$, and 52 ± 3%; $P < 0.001$, respectively) compared to the control (15 ± 4%). Accession PedS1 showed no difference in response to stratification based on duration, while germination in the C90 and C120 treatments of PedS2 showed a strong positive response to increased stratification duration compared to the control (control germination 15%; C90 55%, $P < 0.001$; C120 78%, $P < 0.001$). Only the PedS1 accession was overwintered, which resulted in germination similar to all cold stratification treatments (34%).

Viola pubescens

Statistical analyses were not run on PubS1 because of extremely low germination (around 2%) in all treatments excluding the cold stratification treatments, in which the C60, C90, and C120 treatments resulted in 44%, 52%, and 77% germination, respectively (Figure 4). Similarly, it was not possible to run the statistical analyses on PubS2 given extremely low germination (0%) in all treatments. Overwintering germination was 2% for PubS1 and no germination was recorded for PedS2.

Viola sagittata

Seed pretreatments resulted in germination at proportions significantly different from the control ($P < 0.001$; Figure 4). Germination increased significantly ($P = 0.010$) to 28 ± 5% at 60 d cold stratification, 55 ± 5% at 90 d, and 57 ± 5% at 120 d, compared to the control (3 ± 2%). Two GA treatments (500 ppm and 750 ppm) significantly increased germination compared to the control (32%, $P = 0.001$; 48%, $P < 0.001$). Overwintering germination was 31%, which was greater than the control and similar to the 2 highest potency cold stratification treatments.

Viola sororia

Seeds from *V. sororia* saw no significant differences in germination proportions compared to the control (38%) in response to seed pretreatments (Figure 4). Overwintering germination was 30%, which is very similar to the control and cold stratification treatments.

DISCUSSION

Impact of Maturity on Germination

Timing of seed collection based on fruit orientation had a significant impact on the germination percentages of *V. pedatifida* seeds. Immature seeds have been shown to germinate at higher percentages or more quickly than mature ones (Baskin and Baskin 1994, but see also Kidd and West 1920; Purwantoro 2017), and this can be attributed to developmental timing within the seed (acquisition of PD). The 75% germination observed in unripe seeds (those collected from capsules oriented downward) suggests these seeds had not yet developed dormancy to the full extent observed in ripe seeds (those collected from capsules oriented upward). The observation of germination percentage gradually decreasing as seed maturation stage increases is consistent with the understanding that dormancy is acquired later in seed development (reviewed in Finkelstein and others 2008). Although collecting immature seed may maximize germination percentages with minimal pretreatment, note that immature seed typically has lower viability in storage, poorer seedling establishment, and lower future survival (Priestly 1986 in Lippitt and others 1994; Basso and others 2018).

Impact of One-Day Storage Conditions on Germination

The proportion of *V. pedatifida* seeds that germinated after being exposed to 750 ppm GA differed significantly depending on seed storage conditions experienced in a single day of storage (Figure 2). This finding indicates the importance of considering conditions experienced by seeds immediately after collection if the purpose is to germinate as many genotypes as possible for propagation and (or) restoration right after collection. We know seeds exhibit some degree of dormancy because seeds in this experiment (and all additional experiments in this study) were collected from capsules pointed above the horizon or just beginning to split open, and since primary dormancy is acquired by seeds in later maturation stages (reviewed in Finkelstein and others 2008), we understand primary dormancy is at least partially established in these *V. pedatifida* seeds. Environmental conditions during seed maturation affect the depth of dormancy (reviewed and summarized in Baskin and Baskin

2014). Since germination results differed between storage treatments, despite receiving the same 750 ppm GA pretreatment, we can infer that dormancy depth was influenced by these specific storage conditions.

High storage temperatures (30 °C [86 °F]) result in deeper primary dormancy in *V. pedatifida*, but the 2 treatments used in this study (chaff exclusion versus chaff inclusion during seed storage) also indicate that the humidity at which seeds are stored plays an influential role in dormancy establishment. The mechanism behind these dormancy differences is unclear, but the still-green chaff may have been modifying humidity within the sealed storage containers. Future studies should investigate whether a similar dormancy response is observed in seeds naturally dispersed from *V. pedatifida* plants. Additionally, while differences in depth of dormancy likely led to differences measured in our treatments, it is possible that seeds were instead killed by these higher storage temperatures. Future studies should employ the tetrazolium test to further investigate the effects of one-d seed storage conditions on dormancy and viability, and to determine whether seeds are dormant or nonviable following storage and germination trials.

Since *V. pedatifida* (Schultz 2020) and many other Midwestern *Viola* species produce seeds throughout most of the growing season (Evans 1956), the observed plastic dormancy response may be a bet-hedging strategy that allows some seeds dispersed early in the spring to germinate immediately (something that is seen in many Siberian grassland *Viola* species; Elisafenko 2015), while preventing seeds dispersed in midsummer from germinating when temperatures can be in excess of 30 °C (86 °F). These results suggest that *V. pedatifida* seeds can respond to weather fluctuations immediately prior to seed dispersal and that these conditions may affect germination patterns observed throughout the year.

Impact of Short-Term Storage Conditions on Germination

Short-term storage conditions (4–16 wk) significantly influence the dormancy of *V. pedatifida*, which is seen through changes to germination. High concentrations of GA within 24 h of seed collection successfully triggered germination of 28.2% of the seeds in our control treatment. Germination percentages similar to the control were observed in seeds stored for 4 wk at 4 °C (39.2 °F), 75% RH conditions, but germination percentage significantly decreased when seeds were stored under other combinations of temperature, humidity, and duration (Figure 3). Internal seed moisture content has been shown to influence dormancy; specifically, dormancy becomes deeper as moisture content decreases and vice versa (Baskin and Baskin 2014). *Viola* seeds stored for 4 wk at 4 °C (39.2 °F), 75% RH may have stayed in a state of relatively shallow dormancy (with depth similar to freshly collected seeds) because seeds were unable to dry down to the internal moisture contents common for recently shed seed. Seeds stored for 4 to 16 wk at 75% RH and various temperatures may have experienced some of the deleterious effects seen in seed-aging studies, such as higher mortality or increased imbibition stress. Germination of seeds stored under low humidity conditions (22% RH; 4 or 16 wk at either 4 °C [39.2 °F] or 23 °C [73.4 °F]) all saw significantly lower germination than the control, suggesting that as internal seed moisture content decreased, PD increased.

Germination responses observed for *V. sororia* were different from those observed in *V. pedatifida*, with very little germination observed in this collection of *V. sororia* (Figure 3) when pretreating seeds with 750 ppm GA. Although GA has been shown to be effective in breaking dormancy in *V. sororia* (Solbrig 1981), this experiment yielded next to no germination. Solbrig (1981) treated seeds in 500 ppm GA for 10 d at 3 °C (37.4 °F) and was able to achieve a maximum 76.8% germination. However, the most common protocol for pretreating seeds using GA is to allow seeds to imbibe the solution for between 30 min and 72 h at concentrations not exceeding 1000 ppm, which is shown to be effective in many *Viola* species (Deno 1993, 1996, 1998; Barekat and others 2013; Franklin and others 2017). The treatments reported by Solbrig (1981) may have had a slight stratifying effect on the seeds, which may have been, even if very short, enough to trigger higher germination in this species. Additionally, seeds from this experiment were collected directly from the soil seedbank, so the extent to which dormancy may have already been broken in this collection is unknown. This experiment suggests the use of GA as a pretreatment may be ineffective at breaking dormancy in freshly collected *V. sororia* seeds.

The results discussed here are consistent with the earlier suggestion in the results section (Impact of One-Day Storage Conditions on Germination) that the prolonged non-deep dormancy seen in the 4 °C (39.2 °F), 75% RH treatment may act as a bet-hedging strategy to allow seeds dispersed early in the spring, under cool, humid conditions, to germinate immediately during conditions ideal for seedling establishment. For long-term seed storage, however, high humidity would likely be detrimental. In a similar experiment, freshly collected *V. pubescens* seeds were stored at 30 °C (86 °F), 75% RH for 16 wk and resulted in near 100% seed mortality (Kilgore, unpublished data). High temperature and high humidity conditions are contrary to the standardized best practices for orthodox seed storage (Maschinski and Haskins 2012; Baskin and Baskin 2014; De Vitis and others 2020). Although increased seed mortality was not observed in *V. pedatifida* or *V. sororia* seeds stored under these high temperature and high humidity conditions, it is well documented that long-term storage under these conditions will result in higher seed mortality than occurs in lower temperature and humidity storage conditions (summarized in Justice and Bass 1978; Hong and others 1996).

These results offer insight for both germination studies and *ex situ* conservation efforts of challenging species similar to *Viola.* Uncontrolled storage conditions prior to seed pretreatments could lead to decreased germination potential for a seedlot, especially when dormancy-breaking pretreatments are suboptimal (or unknown). Additionally, increased seed mortality may be occurring because of improper storage, but we were not able to distinguish this in our trials. This mortality is especially problematic for species that are extremely valuable for use in large-scale restoration projects, which are almost always limited by shortages of high-quality seeds (Wijdeven and Kuzee 2000; Pywell and others 2002).

Impact of Seed Pretreatments on Germination

Germination responses differed between the tested accessions, but when observed across all species, pretreating seeds with cold stratification was the most reliable method to maximize germination percentages (Figure 1; Figure 4). These results, as well as other similar studies (Pegtel 1998; Barekat and others 2013; Gehring and others 2013; Elisafenko 2015), support the use of cold stratification as the pretreatment of choice for *Viola* species by local land managers and practitioners (Banas 2020; Durkin 2020; Nyberg 2020; Schultz 2020). Few studies investigated the optimal duration of stratification required to break the dormancy (tested only in Gehring and others 2013 and Pegtel 1998 on one species per study).

Overwintering data show a species-specific response, for which the treatment was either beneficial (*V. pedatifida, V. sagittata*) or equally effective (*V. lanceolata, V. sororia*) compared to the control. The most striking difference was observed in *V. pubescens*—overwintering resulted in nearly no germination, yet the species responded strongly to long-duration cold stratification. This response likely indicates that the overwintering conditions were either not cold enough or not long enough to result in germination in this species, since seeds from the same collection germinated when put in cold stratification a few months later.

Chemical seed treatment, such as osmo-priming, has been shown to be effective in increasing germination percentages in pansies (Carpenter and Boucher 1991; Yoon and others 1997; Rajabalipour and others 2013), but its effect on breaking dormancy of wild *Viola* species is understudied. Pansy seeds show non-deep PD, broken during dry storage at a warm temperature (that is, after-ripening) (Tiwari and others 2016). Horticultural and crop species such as pansy are selected for greatly reduced dormancy regimes compared to their wild counterparts (Batalla and others 2020); however, many ornamental plant species can still exhibit some form of non-deep dormancy that can be problematic for their germination, assessment of viability, and large-scale production (Geneve 1998). Our osmo-priming of wild *Viola* species seed was unsuccessful in breaking dormancy (Figure 4). Outside of its effectiveness in breaking dormancy in morphophysiologically dormant species (see Baskin and Baskin 2014), priming has been shown to be effective in breaking dormancy in physiologically dormant species when that dormancy is non-deep (see Geneve 1998). Interestingly, the 2 *Viola* species that exhibited non-deep PD (*V. sagittata* and *V. sororia*) showed no increases in germination when primed with any of 3 PEG solutions for 7 d. These results indicate that priming seeds with PEG at rates tested here was ineffective at increasing germination percentage in these 6 species.

Pretreating seeds with GA had also been effective in breaking dormancy and triggering germination in *Viola* species (Solbrig 1981; Deno 1993, 1996, 1998; Barekat and others 2013; Franklin and others 2017). *Viola sagittata* was the only species in this study for which the use of GA-induced germination was equal to that of the maximum potency cold stratification treatment. GA was most effective at triggering germination in species that exhibit non-deep PD (reviewed in Baskin and Baskin 2014), so likely several of the species tested here exhibit dormancy deeper than non-deep PD.

Germination Response and Proposed Dormancy Regimes

PD has been recorded in at least 24 *Viola* species globally (reviewed in Baskin and Baskin 2014; see also Baskin and Baskin 1972, 1975; Grime and others 1981; Geneve 2003; Jensen 2004; Eckstein and others 2006; Gehring and others 2013; Meineri and others 2013; Elisafenko 2015; Franklin and others 2017; Godefroid and Van de Vyver 2019) while nondormancy has been recorded less frequently (15 species stored for several years; Elisafenko 2015). This report of nondormancy in Elisafenko (2015) occurred in seeds stored for months to years, and since long-term storage is known to lessen dormancy break requirements (reviewed in Baskin and Baskin 2014), it is possible that the long storage durations reported here influenced dormancy classification. Ideally, studies classifying dormancy type should be performed on freshly collected seeds. Dormancy depth is rarely determined in the few publications that report dormancy type, and this information often needs to be inferred from species for which germination behavior is reported (Baskin and Baskin 2014). Dormancy classifications for each of our studied species can be found in Figure 1 based on the guidelines described in Baskin and Baskin (2014).

Viola labradorica exhibited very low germination across all tested treatments and was the only accession that had no treatment achieve germination above 10%. These results are consistent with Deno (1993) who found similar germination percentages for the control treatment (16%) and 1000 ppm GA (10%). Future work on *V. labradorica* should investigate seed viability at the time of collection and throughout storage.

Germination responses for *Viola lanceolata* suggested the occurrence of deep PD (germination percentages increasing

with increasing length of cold stratification). It is also possible that some dormancy was lost in the long storage duration since substantial germination was observed in the control and various GA treatments.

Germination responses to cold stratification differed between the 2 *V. pedatifida* accessions (Figure 4). Differences may be attributable to the environmental conditions experienced by the 2 populations (that is, maternal environment) since they are genetically related. Maternal effects have been shown to affect a variety of seed characteristics including mass, dormancy depth, and germination response (Galloway 2005; Donohue 2009). These results show that classifying dormancy in plants should be carefully done and should always take into consideration the conditions experienced by the source plant population (maternal environmental conditions), especially when the seeds are collected from cultivated plants, as cultivation, already after one generation, may influence seed dormancy. When considering germination response of these 2 accessions together, *V. pedatifida* exhibited an intermediate PD. Future research could investigate the effect of providing a 4- to 6-wk period of after-ripening prior to long-duration cold stratification to more clearly determine if *V. pedatifida* exhibits intermediate PD, as an after-ripening period has been shown to reduce required cold stratification durations in species with this dormancy type (reviewed in Baskin and Baskin 2014).

Viola pubescens germinated only when pretreated with cold stratification. The observed germination response recorded here was a classic example of a species with deep PD; long periods of cold stratification are required to induce maximum germination, and GA concentration had no impact on germination (Geneve 2003; Baskin and Baskin 2014). Seeds in accession PubS2 may have been immature, as the outlined collection protocol could not be followed given the sessile capsules produced by cleistogamous flowers of this species. Future studies should investigate the impact of even longer duration cold stratification on germination since sequential increases in germination were observed in response to that treatment.

Viola sagittata seeds (SagS) were the only seeds to show germination in response to GA equal to that of cold stratification. Since germination did not significantly increase between the C90 and C120 treatments, we could conclude that this 55% germination mark achieved in both treatments was probably the maximum germination achievable with a single round of stratification for *V. sagittata*. Since the other half of the ungerminated seeds were still viable, the stratification method applied here was not sufficient to break dormancy in all seeds in this accession. It is possible that the ungerminated yet viable seeds might have required different conditions to break dormancy. *Viola sagittata* exhibited non-deep PD. Future research should investigate the effect of after-ripening on *Viola* seed germination, as this treatment has proven successful in lessening the germination requirements of other *Viola* species as well as species with non-deep PD in general (Baskin and Baskin 1972; Gehring and others 2013) and may have increased germination here. Future work should also investigate the impact of an alternating stratification temperature regime to investigate the potential for increased germination after multiple rounds of stratification.

Viola sororia showed consistent germination across all treatments with no treatment achieving a higher germination percentage than the control (38%). Observed germination responses could be consistent with a non-deep PD dormancy regime (Baskin and Baskin 2014), and specifically one in which the long storage duration (9–11 mo) lessened the dormancy break requirements. These germination results are contrary to those that were earlier observed and discussed in the Impact of Short-Term Storage Conditions on Germination sections, when almost no germination was observed, which support the suggestion that storage affected dormancy depth. Solbrig (1981) observed highly variable germination percentages (78% to 26%) and average days to germination (14–26 d) from seed collected from a variety of *V. sororia* ramets within a larger population when using a 750 ppm GA pretreatment. The effectiveness of pretreatments to break dormancy need to be interpreted in light of potential seed after-ripening (as seen in other *Viola*; Baskin and Baskin 1972; Gehring and others 2013).

CONCLUSIONS

In this study we have investigated common best practices when working with wild violet seeds. We suggest in this section which practices could be the most effective to obtain high germination percentages in order to maximize the number of genotypes propagated and, eventually, represented in the reintroduced population. These suggestions are based on the specific results we obtained for the tested populations. As a general rule, when drawing these types of conclusions, practitioners and scientists should take into consideration the seed source, as cultivation may have an impact on seed development, dormancy, and germination.

Seed Collection

- As suggested by many recognized and evidence-based guidelines, seeds should be collected at the time of peak ripeness (at the time of natural dispersal). If this is not possible because of the species biology and (or) logistical issues, try to collect seeds immediately prior to dispersal when fruits are still closed but pointing above the horizon. This position of the fruit indicates that seeds inside the fruit are likely mature.

Seed Drying and Storage

- After collection of closed fruits, store them in contained spaces (for example, paper bags to avoid seed loss due to the ballistic dispersal) and under constant room conditions, such as 20 °C (68 °F) and 50% RH (<24 h), to allow for natural release and dry-down of seeds.
- After dispersal, separate seed from chaff. If necessary, extract seed from fruits using forceps.
- If using seeds collected from upward-facing closed fruits, the following storage conditions are recommended:
  Very short-term storage (1 d): 4 °C (39.2 °F) and ~75% RH
  Short-term storage (3–18 mo): 4 °C (39.2 °F) and ~22% RH

Seed Germination

Viola lanceolata, V. sagittata, and *V. sororia* should be cold stratified for 90 d, and *V. pedatifida* and *V. pubescens* for 120 d, to maximize germination with the shortest possible pretreatment. Also note that our research finds no evidence of deleterious effects (decreased germination) when stratifying any of these 6 species for 120 d. Long duration stratification may be the safest option to maximize germination across numerous populations with different pretreatment requirements (see observed trends in *V. pedatifida* accessions). Typically, germination will occur over a period of 21 d. Observed dormancy regime should be reported if data are to be reported in a publication.

The genus *Viola* occupies an often-unfilled niche in Midwestern habitat restorations, and this research indicates that limitations to utilizing these plants begin well before seed germination. It is imperative that practitioners who work with these species understand both their life history and seed biology to maximize germination and therefore their availability in restoration efforts. Numerous factors were shown to significantly affect germination of these species, so understanding how environmental conditions and pretreatments are having an impact on seed between collection and germination is crucial. After maximizing germination, plant establishment into suitable habitat is the next step critical to the successful reintroduction of these species.

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AUTHOR INFORMATION

Sam Kilgore
Graduate Student
Chicago Botanic Garden
Glencoe, IL 60022
Northwestern University
Evanston, IL 60208
samkilgore94@gmail.com

Kayri Havens
Senior Director, Ecology and Conservation
Chicago Botanic Garden
Glencoe, IL 60022
khavens@chicagobotanic.org

Andrea Kramer
Director of Restoration Ecology
Chicago Botanic Garden
Glencoe, IL 60022
akramer@chicagobotanic.org

Ashlyn Lythgoe
REU Student
Chicago Botanic Garden
Glencoe, IL 60022
Purdue University
West Lafayette, IN 47907
ashlythgoe@gmail.com

Linda MacKechnie
Seed Biologist
Ball Horticultural Company
West Chicago, IL 60185
LMackechnie@ballhort.com

Marcello De Vitis
Postdoctoral Research Associate
Chicago Botanic Garden
Glencoe, IL 60022
mdevitis@chicagobotanic.org