EFFECTS OF COLD STRATIFICATION AND HORMONES ON SEED GERMINATION OF *SARRACENIA ALATA*

K.A. Hopkins[1] and D.A. Gravatt[2*]
[1]*Horticultural Science, Texas A&M University, College Station, TX 77843*
[2] *Department of Biology, Stephen F. Austin State University, Nacogdoches, TX 75962*
**Corresponding author; Email: dgravatt@sfasu.edu*

Abstract.–Seed germination requirements for *S. alata* were investigated using cold stratification and phytohormones. Treatments included no stratification (control), two week, three week, four week, five week, and six week cold stratification. Hormone treatments included no stratification with cytokinin, no stratification with gibberellins, no stratification with cytokinin and gibberellins, no stratification with auxin, and one with six week stratification with abscisic acid. Three weeks cold stratification was required to yield a significant increase in percent germination. Gibberellin was the only hormone which caused a significant increase in germination in *S. alata* seeds.

Keywords: abscisic acid, auxin, cytokinin, gibberellin, pitcher plant

Carnivorous plants have specialized traps to capture and digest animal prey for nutrient assimilation. *Sarracenia alata*, a pitcher plant native to regions in south eastern North America, including parts of East Texas, produces a pitfall trap. Carnivorous plants typically grow in nutrient poor soil, usually with diffuse light and water-saturated soil conditions (Givnish et al. 1984). The various forms of traps allow these plants to capture and digest animal prey to gain nutrients that would otherwise be obtained from the soil. These adaptations allow carnivorous plants to thrive in an environment not habitable for most other plants which require a high level of nutrient gain from the soil. These fascinating plants are rich with potential for plant hobbiest to begin to cultivate and grow them in their gardens and landscapes. With this interest comes a call for the propagation of stock to meet this demand. Rather than destructively collecting whole plants from natural populations, seeds can be collected and grown for use.

It is well known that many seeds require specific conditions to germinate (Angevine & Chabot 1979; Baskin & Baskin 1998; 2001).

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These conditions include, but are not limited to stratification, scarification, fire treatment and hormone presence. Understanding these requirements is important for further studies which may require seedlings or involve germination. The germination requirements for the carnivorous plant *S. alata* have not been extensively studied. This study helps to elucidate the cold stratification requirements and to explore the role hormones play in breaking dormancy in *S. alata.*

Germination initiates when the seed begins to take up water, and then a radicle emerges; the process is over when the embryonic axis elongates (Bewley & Black 1982). The emerging radicle is the first visual sign of germination, and is the indicator we used in this study to determine the point of successful germination. Germination also indicates the end of a seed's dormancy. Seed dormancy is a temporary failure or block of a viable seed to complete germination under physical conditions that would normally be favorable (Kucera et al. 2005). These dormant seeds have additional requirements that must be met before germination can occur. These requirements can include the presence or absence of light, stratification, fire, and hormones (Koornneef et al. 2002). There are two types of dormancy. One is embryonic dormancy, when the embryo itself is dormant (Bewley 1997). The other type of dormancy is coat-enhanced dormancy (a.k.a., mechanical), which happens when the embryo is constrained by its seed coat (Bewley 1997). In the case of this study with *S. alata*, we tested the requirements for release of embryotic dormancy using cold stratification and explored the effects of plant hormones.

Cold stratification is a treatment performed on seeds where they are subjected to cooler than mean temperatures and moist environments in order to overcome embryonic dormancy. This treatment simulates the environmental factors of a winter chilling requirement of the plant. In some species, germination will not occur or may be delayed if the cold requirement is not met (Gotsch & Ellison 1998). It is also possible that germination may be affected by endogenous plant hormones or those artificially introduced. There are five classical plant hormones: auxin, gibberellin, ethylene, cytokinin,

and abscisic acid. For this study, ethylene was not included; therefore only the effects of auxin, gibberellin, cytokinin, and abscisic acid on *S. alata* germination were examined. Auxin promotes root initiation, meristem development, and shoot elongation (Kucera et al. 2005). Gibberellins release dormancy, promote germination, and counteract the effects of abscisic acid (Kucera et al. 2005). Cytokinin regulates cell division, and promotes seed germination (Kucera et al. 2005). Abscisic acid plays a positive role in dormancy induction, that is, it prevents (down regulates) germination (Kucera et al. 2005).

The study by Ellison (2001) reviewed the germination requirements and dormancy of eight *Sarracenia* species and thirteen *S. purpurea* populations. Ellison's study included an examination of cold stratification requirements for *S. alata*. Cold stratification periods of two, three, four, and five weeks were tested. Ellison concluded that cold stratification promoted quicker germination and increased the number of seeds that germinated (Ellison 2001). Since both cold stratification and hormones have previously been used to break dormancy, our study provides a more detailed examination of the cold stratification requirements and the role of hormones in the release of dormancy of *Sarracenia alata* seeds.

The objective of this study was to observe the germination response of *Sarracenia alata* seeds to increasing cold stratification periods to find an optimum stratification length for germination of the species. A second objective was to observe the germination response to the hormones cytokinin (CK), gibberellin (GA), abscisic acid (ABA), and auxin (IAA). Perhaps a hormone application could have the same results as a lengthy stratification period, saving time for the grower.

MATERIALS & METHODS

Seed collection and preparation.–For this experiment, *Sarracenia alata* seed capsules were harvested from an herbaceous seep near Boykin Springs Lake in the Angelina National Forest located near

Zavalla, TX in late September 2015 and stored in sealed containers at room temperature until needed. Fifty seeds were randomly selected for viability and were dissected to examine the condition of the embryo. Seed embryos were stained with tetrazolium to determine seed viability (Lakon 1949).

Beginning in early October 2015, seeds were surface sterilized with a 10 % bleach solution for 60 seconds, then rinsed with reverse osmosis water three times. The seeds were then divided randomly into eleven treatments. The first six treatments included (1) no stratification (control), (2) two week stratification, (3) three week stratification, (4) four week stratification, (5) five week stratification, and (6) six week stratification. The stratification start days were staggered to allow for the end of all treatments to simultaneously occur at the end of the study. Cold stratification treatments were applied by placing seeds between moist paper towels in plastic storage bags. These storage bags were then placed in a 4 °C refrigerator for the prescribed stratification period.

The remaining five treatments were the application of plant hormones (7) no stratification with cytokinin (Kinetin, Sigma-Aldrich, USA, PubChem SID: 24896197), (8) no stratification with gibberellin (gibberellic acid, Sigma-Aldrich, USA, PubChem Substance ID: 24895317), (9) no stratification with cytokinin and gibberellin, (10) no stratification with auxin (3-Indoleacetic acid, Sigma-Aldrich, USA, PubChem Substance ID: 24896015), and (11) one with six week stratification followed by the application of abscisic acid ((+)-Abscisic acid, Sigma-Aldrich, USA, PubChem Substance ID: 329769948). The hormone treatments were applied by allowing the seeds to soak for two hours at a concentration of 5 mM for each of the treatments. After soaking, seeds were removed from the hormone solution and placed on C-FERN media in 100 mm by 15 mm plastic petri dishes.

Statistical analysis.–JMP software was used for the statistical analysis of the data. The percent germination data was transformed using arcsine (McDonald 2014). Once transformed, ANOVA was

performed to test for differences between treatments. Post ANOVA analysis included fit of least squares and Tukey's Honest Significant Difference test.

RESULTS

Seed viability.–Seed viability was performed in late November, 2015. Of the 50 dissected seeds, 96% of the embryos tested positive for viability, as indicated by the tetrazolium red staining method. Freshly collected seeds (n=50) placed directly on C-FERN media failed to germinate after 60 days of being placed in the germination chamber.

Germination success.–*Sarracenia alata* seed germination results revealed that after 30 days, there were statistically significant differences between treatment means ($P<0.001$). Germination responses to the cold stratification treatments are illustrated in Fig. 1. These treatments included the control (no stratification), as well as two, three, four, five and six week stratification treatments for a total of six treatments. Statistical differences between treatments at day 30 can be seen in Table 1. When looking at the mean germination rates for the cold stratification treatments (Fig. 1) at day 30, it is evident that increased cold stratification periods promoted greater rates of germination. For the cold stratification treatments (Fig. 1), the two week treatment (12.5%) had no statistical difference in percent germination when compared to the control (2.5%; $P<0.001$). All other treatments were statistically different from the control and two week treatment. These treatments include three week, four week, five week, and six week stratification treatments. Percent germination yields increased significantly from the control at the three week stratification treatment. Maximum percent germination was observed in the six week stratification treatment at 95%. The range of percent germination between three week and six week stratification treatments was 27.5% to 95% germination. There was a positive relationship between cold stratification length and percent germination.

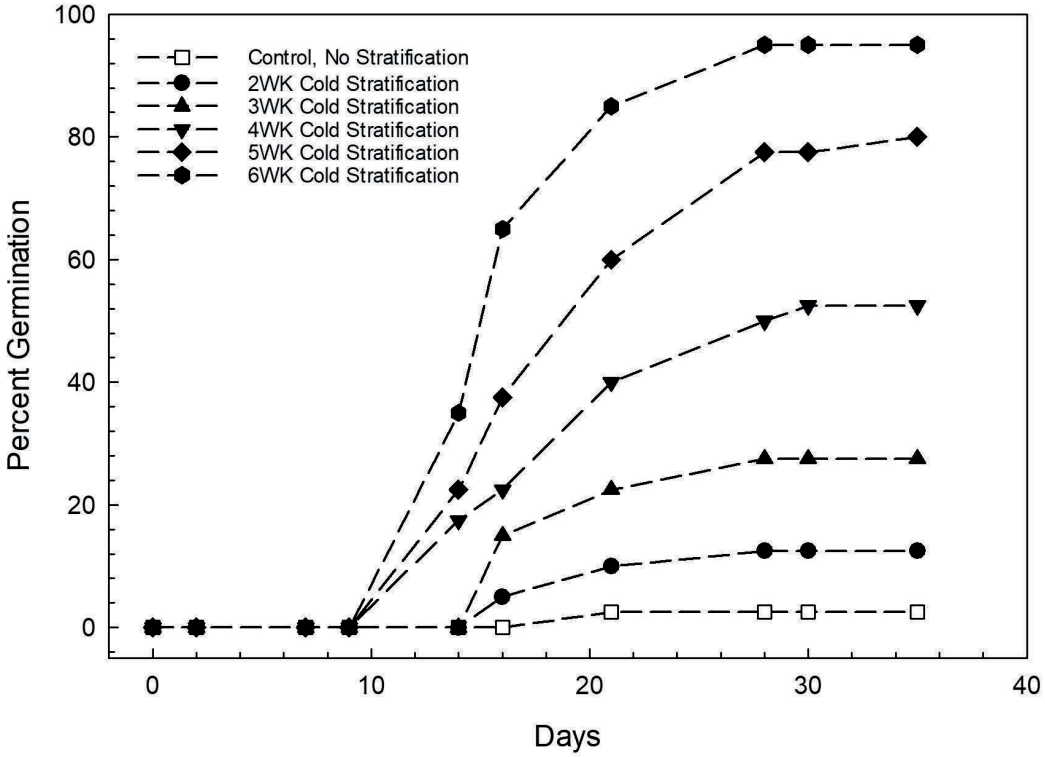

Figure 1. Percent germination observed across 35 days using cold stratification lengths of 2-, 3-, 4-, 5- and 6-weeks and a control with no stratification.

Plant hormone treatments.–For the hormone treatments (Fig. 2 and Table 1), the cytokinin and auxin treatments had no statistical difference from the control. The gibberellin, gibberellin plus cytokinin, six week stratification plus abscisic acid, and six week stratification treatments were all statistically different from the control (see Table 1, $P < 0.001$). Percent germination of the treatments that were statistically different from the control ranged from 30% to 95% germination (Table 1). Maximum percent germination was observed in the six week cold stratification treatment, 95%. Treating seeds with cytokinin plus gibberellin provided for a maximum rate for the promotion of germination at 32.5%, (see Fig. 1 and Table 1). The addition of abscisic acid (ABA) to the six week cold stratification treatment caused a significant decrease in the percent germination from 95 % (six week stratification only) to 40% (six week stratification and ABA). The auxin (IAA), control, and cytokinin treatments were not statistically different and exhibited germination

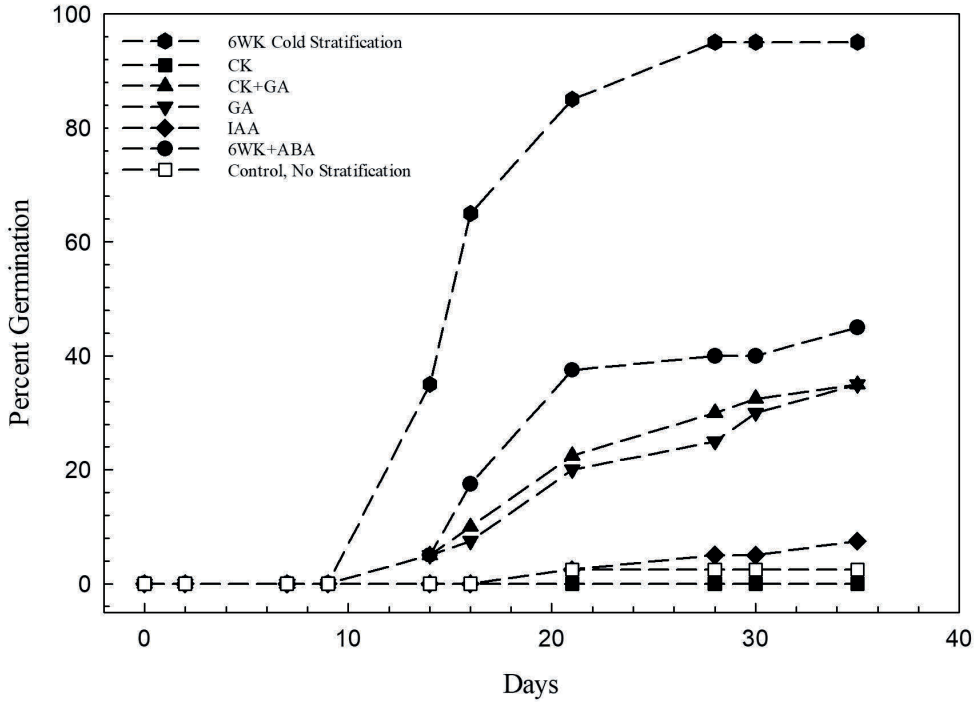

Figure 2. Percent germination observed for 35 days using plant hormones. Treatments are control (no stratification or hormone), cytokinin (CK) treatment, cytokinin plus gibberellin (GA) treatment, gibberellin treatment alone, auxin (IAA), six week cold stratification followed by abscisic acid (ABA), and six week stratification only.

rates of less than 5%. The cytokinin treatment had no germination at day 30.

DISCUSSION

This study demonstrates a significant increase in percent germination of *Sarracenia alata* seeds when an extended cold stratification is provided. This confirms the findings by Ellison (2001) where *S. alata* germination was found to be around 80% for two, four, and five week stratification. In an earlier study, six weeks of cold stratification yielded higher percent germination than four week stratification, ~85% and ~52%, respectively (Gotsch & Ellison 1998). In our study, germination rates for 6 weeks of cold stratification were higher at 95%. Moreover, the three week, four week, five week, and

Table 1. Percent germination at 30 days and fit of least squares levels for 11 stratification and hormone treatments (ABA = abscisic acid). Fit of least squares significance levels for the stratification and hormone treatments. Treatments with different letters are significantly different ($p < 0.001$).

Treatment	Mean % germination at 30 d	Fit of least squares significant difference grouping
Six week stratification	95.0	A
Five week stratification	77.5	A B
Four week stratification	52.5	B C
Six week stratification + ABA	40.0	B C D
Cytokinin + Gibberellin	32.5	C D
Gibberellin	30.0	C D
Three week stratification	27.5	C D E
Two week stratification	12.5	C D E
Auxin (IAA)	5.0	E F
Control	2.5	F
Cytokinin	0.0	F

six week cold stratification treatments were statistically different (higher percent germination) than no stratification. This indicates that any stratification period, of at least three weeks or longer, will statistically increase percent germination when compared to no cold stratification. Overall, cold stratification is necessary to release dormancy in *S. alata* as is commonly reported in the genus, *Sarracenia* (Gotsch & Ellison 1998; Ellison 2001).

The application of hormones was performed to explore the role each plays in releasing *S. alata* seeds from dormancy in the absence of cold stratification. The hormone treatments provided some fascinating results. Application of plant hormones did result in seed breaking dormancy. However, none of the hormone treatments were able to provide germination rates higher than about 30.5%, which is quite low when compared to the six week stratification treatment (95% germination). Applying cytokinin or auxin had no effect on germination of *S. alata* seeds.

Auxin is a plant hormone which plays a role in regulating cell enlargement, cell division in the cambium and root initiation (Davies

2010). Auxin by itself may not be necessary for seed germination as discussed by Miransari & Smith (2014). Germination percentage of auxin treated seeds, 5%, in this study support previous findings with germination rates not different than the control treatment, 2.5%.

Cytokinins are a group of adenine derived compounds characterized by the ability to induce cell division when in the presence of auxin, in tissue culture (Davies 2010). The most common cytokinin in plants is zeatin. Bewley & Fountain (1972) reported cytokinin alone promoted germination in lettuce seed embryos. On the other hand, studies report that cytokinins alone have little or no effect on germination (Ikuma & Thimann 1963; Khan 1971). Our seed germination rates, using cytokinin alone, did not promote seed germination (see Fig. 2 and Table 1) and support these findings.

Gibberellins are synthesized from glycerol-3-phosphate, via isopentenyl diphosphate, in young tissues of the developing shoots and developing seeds (Davies 2010). Gibberellin is necessary for seed germination (Khan 1971; Groot & Karssen1987; Bewley 1997; Koornneef et al. 2002; Miransari & Smith 2014). A study by Unhak (2003) noted a positive effect on percent germination of gibberellin on the pitcher plant *Darlingtonia californica,* when compared to a treatment with no added gibberellin. Unhak (2003) found that the highest percentage of germination of 80% was with cold treated seeds at 4°C. Results for *S. alata* gibberellin treatment are consistent with the gibberellin treatment results of Unhak (2003). In this study, gibberellin and gibberellin with cytokinin were the only treatments that significantly increased the release of dormancy of *S. alata* (Fig. 2 and Table 1). Results from this study support those reported previously (Khan 1971; Groot & Karssen1987; Bewley 1997; Koornneef et al. 2002; Miransari & Smith 2014). The gibberellin plus cytokinin, and gibberellin alone, were not statistically different from each other (Table 1), indicating that the addition of cytokinin had little effect on dormancy release.

Abscisic acid is formed from glycerol-3-phosphate, via isopentenyl diphosphate and carotenoids in roots and leaves. Seeds are high in

ABA which may be either imported from leaves or produced *in situ* (Davies 2010). ABA and gibberellin can act antagonistically, where gibberellin is required to overcome an ABA-induced dormant state (Debeaujon & Koornneef 2000). Studies report that ABA affects the induction and maintenance of some aspects of seed dormancy (Bewley 1997; Finch-Savage & Leubner-Metzger 2006; Davies 2010; Miransari & Smith 2014). ABA concentrations of less than 10 µM have been found to have antagonistic affects on the process of seed germination in *Arabidopsis thaliana* (Kucera et al. 2005; Muller et al. 2006). The addition of abscisic acid (ABA) to the six week cold stratification treatment caused a significant decrease in the percent germination from 95% (six week stratification) to only 40%. The significant reduction of germination by ABA supports the body of evidence that this hormone is an important positive regulator of the maintenance of the dormant state. ABA appeared to reverse the effects of cold stratification, and presumably endogenous gibberellin, and is consistent with results obtained for *Fagus sylvatica* seeds reported by Nicolás et al. (1996).

It is clear from results presented in this study that *S. alata* requires and benefits from cold stratification to break dormancy. At least three weeks or longer stratification periods will statistically increase percent germination. If a six week cold stratification period cannot be supplied, then a treatment with 5 mM gibberellin can be substituted and provide an approximate germination rate of 30%. This study does suggest that gibberellin can increase germination. Further research is necessary to determine the optimum concentration of gibberellin and application time to obtain maximum germination rates. Another conceivable study would be to investigate optimum percent germination by including gibberellin in the agar medium. The results from this study indicated that the highest percent germination occurs with six weeks of stratification to yield 95% germination. While not tested in this study, our data suggest a combination of a shorter colder stratification period, of at least three weeks, and gibberellin treatment could yield a similar percent germination as the six week treatment but requiring less stratification time.

Seeds of *Sarracenia alata* expressed a significant increase in germination with cold stratification periods of three weeks or more, with 95% at six weeks. A significant increase in germination rates of seeds was observed in the gibberellin treatment. Future studies could focus on determining which concentrations of gibberellin and different application techniques determine optimum germination rates.

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