A Germination Study: Dormancy in ray achenes of *Holocarpha macradenia*, a rare coastal prairie forb

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Introduction

The extensive efforts to restore the extremely rare plant *Holocarpha macradenia* are hampered by a lack of understanding of seed germination requirements, which were elusive before this work. This plant is a rare forb inhabiting coastal prairie habitats of California's central coast. It has been listed as Endangered by the State of California, and Threatened by the Federal Government.

Holocarpha macradenia flower heads are composite inflorescences with both disc and ray flowers. Both types of flowers are fertile, so each inflorescence is capable of producing both disc and ray seeds. (Image 1)

Image 1: Disc and ray seeds of *Holocarpha macradenia*

Disc and ray seeds (aka 'achenes') differ in their readiness to germinate, which may be an evolutionary adaptation to variable interannual growing conditions. The disc achenes are known to germinate readily with the addition of water under a wide range of temperatures. However, the ray achenes do not readily germinate under normal nursery or greenhouse conditions. They sit dormant, even after months in a moistened pot of soil. Presumably, these seeds form the long term seedbank for this plant. They are the plant's "insurance policy" against years with poor environmental growing conditions, or years when seed set is low for other other reasons (seed predation, plant death, etc). As an annual plant, if all seeds were to germinate the first year after they are produced, then one bad year without a successful seed set would decimate the population. In theory, at least some of these dormant ray achenes are able to persist for

multiple years in the seed bank, waiting out the unfavorable years, and germinating when conditions are “right” for good plant performance. Past researchers have not been able to determine the precise environmental process or cue that stimulates these seeds to germinate, though some regeneration has occurred in the field after disturbance such as scraping and fire (Bainbridge, 2003).

Under a contract with the City of Santa Cruz, and funded by a grant from the United States Department of Fish and Wildlife (USFWS), the University of California at Santa Cruz (UCSC) Greenhouses have performed laboratory seed germination experiments in an attempt to understand this plant’s germination ecology and environmental triggers that initiate ray achene germination. Understanding this cue could add to our understanding of the year-to-year population dynamics and could inform future conservation activities.

We were able to determine the average baseline viability of ray achenes at 40%. The most successful experimental germination methods in this study achieved up to 43% germination of ray achenes. Thus, some of the methods discussed below appear to be sufficient in stimulating germination in the entirety of viable seeds.

An appendix to this document makes summary recommendations for those interested in germinating ray achenes, and a second appendix makes suggestions for future directions in Santa Cruz tarplant ray achene germination ecology research.

Materials and Methods:

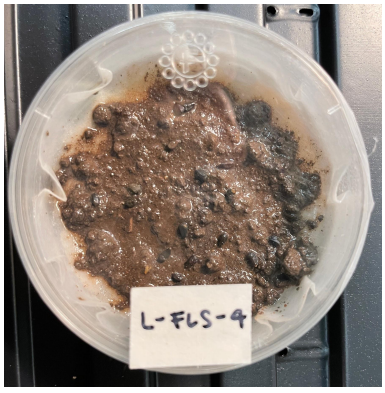
The seed used for these experiments was harvested from 40 robust plants grown ex-situ at the UCSC Greenhouses in 2019 under contract with the City of Santa Cruz. The seed germination experiments described here were conducted in Spring 2022.

Seeds were sorted to ensure that only ray achenes were present in the samples tested. These seeds are morphologically distinct: disc achenes tend to be lighter in

color, and linear in shape; ray achenes are darker, wider, wedge shaped, and proximally blunt-truncated. The location of the hilum differs slightly too: disc achenes attach to the receptacle straight underneath the proximal end, whereas ray achenes attach at the side of the blunter proximal end.

To assess baseline seed viability, a sample of disc and ray achenes was sent to the Oregon State University Seed Lab for tetrazolium testing. The sample of 208 ray achenes tested at 40% viability. For those curious readers: the disc achenes from this same seed batch were tetrazolium tested at 73% viability.

For experimental germination trials, small petri dishes (60mm x 15mm) were prepared with filter paper. Each petri dish contained 20 ray achenes under one of 15 treatments. Seeds were either pre-treated (e.g, soaked overnight) or treatment applied in the petri dish as appropriate (see Table 1 for a list of treatments). Dishes were then sealed with a strip of Parafilm to prevent excessive desiccation. (Images 2 and 3)



Images 2 and 3: Seed treatments inside of sealed petri dishes
Image 4: Incubator with a 12 hr photoperiod containing 5 replicates of each treatment

Each of these treatments was replicated 5 times under total darkness and 5 times under a 12 hour photoperiod inside of separate incubators. (Image 4) These petri

dishes were the *cold stratified* set. Both incubators were held at 40ºF constant temperature for 2 months, then warmed to 70ºF constant temperature for an additional 2 months. At this point, a second batch of petri dishes identical to all of the treatments was sown and placed in these 70ºF incubators and monitored for 2 months. This second batch is the *warm temperature* batch, which was sown to see if there was a difference in germination under cooler temperatures. Figure 1 shows a representation of all 4 of the environmental conditions tested. All petri dishes were monitored 2x/week for germination and were randomly rotated within the incubator each time they were monitored.

Table 1: Treatments applied to seeds under all 4 environmental conditions. Treatments fit into 4 categories: Gibberellic acid (purple), scarification (blue), fire (red), and soil microbial activity (green).

Treatment name	Details of pretreatment
Control	Control - no treatment, placed on moist filter paper in petri dish
Gibberellic acid 100ppm	Soaked in 100ppm gibberellic acid overnight; filter paper moistened with this solution
Gibberellic acid 250ppm	Soaked in 250ppm gibberellic acid overnight; filter paper moistened with this solution
Gibberellic acid 500ppm	Soaked in 500ppm gibberellic acid overnight; filter paper moistened with this solution
Sandpaper scarification	Scarified by gently rubbing between two sheets of sandpaper
Sulfuric acid 2 min	Soaked in concentrated sulfuric acid for 2 minutes
Sulfuric acid 5 min	Soaked in concentrated sulfuric acid for 5 minutes
Burned grass	½ tsp of burned grass included in petri dish
Charate	½ tsp of burned charate (coyote brush) included in petri dish
Smoke	Soaked in smoke water overnight, and filter paper soaked with this solution. Smoke water prepared with 1:500 concentration of Wrights liquid smoke
Heated to 50ºC	Heated at 50ºC for 17 hours before placing in petri dish
Heated to 65ºC	Heated at 65ºC for 6 hours before placing in petri dish
Field soil	½ tsp of field soil included in petri dish
Decomposing grass	½ tsp of dried grass included in petri dish
Cowpie	½ tsp of cow pie included in petri dish

Figure 1: Graphic representation of the 4 environmental conditions under which all treatments in Table 1 were tested

Results and Discussion

While this experiment was able to find significant results from treatments, it would have been more ideal to use larger sample sizes for greater statistical power. This is difficult to do when using rare seeds. 5 replicates with 20 seeds each worked here, but it would be preferable to have 50 seeds per sample.

At first glance, it appears that germination was significantly better under the 40º cold stratification condition than under warm 70º temperatures ($t_{228.12}$=-5.37, p<.0001, Figure 2) and under the 12 hour photoperiod condition than in complete darkness ($t_{286.26}$=1.7716, p=.0388, Figure 3). However, the significance of these statistical trends may have been affected by the magnitude of individual seed treatments within these conditions. We will look at these interaction effects in more detail after we examine the individual seed treatments.

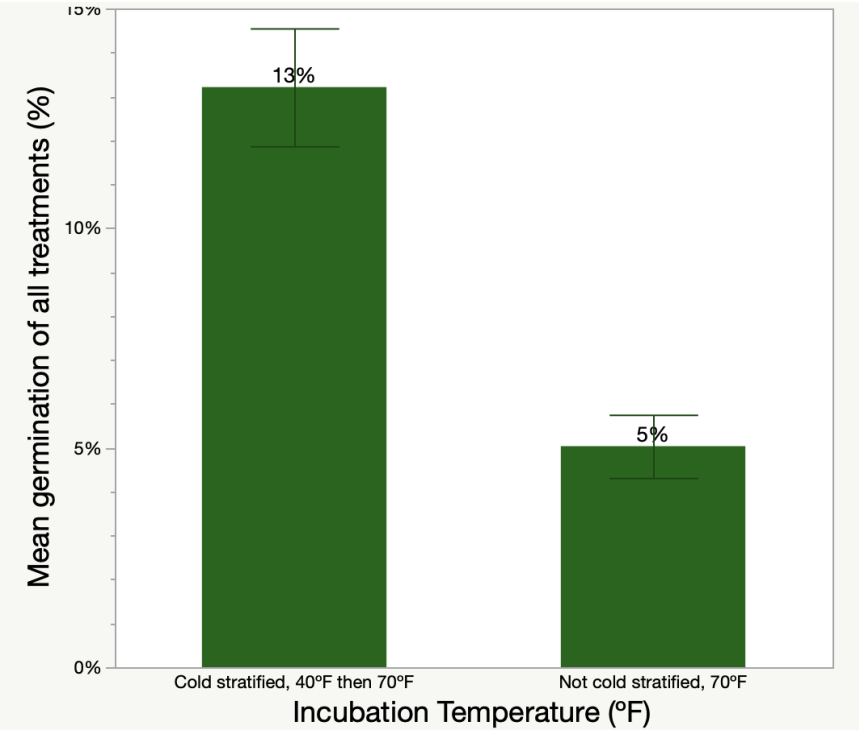


Figure 2: Total germination in cold stratified vs not cold stratified treatments.

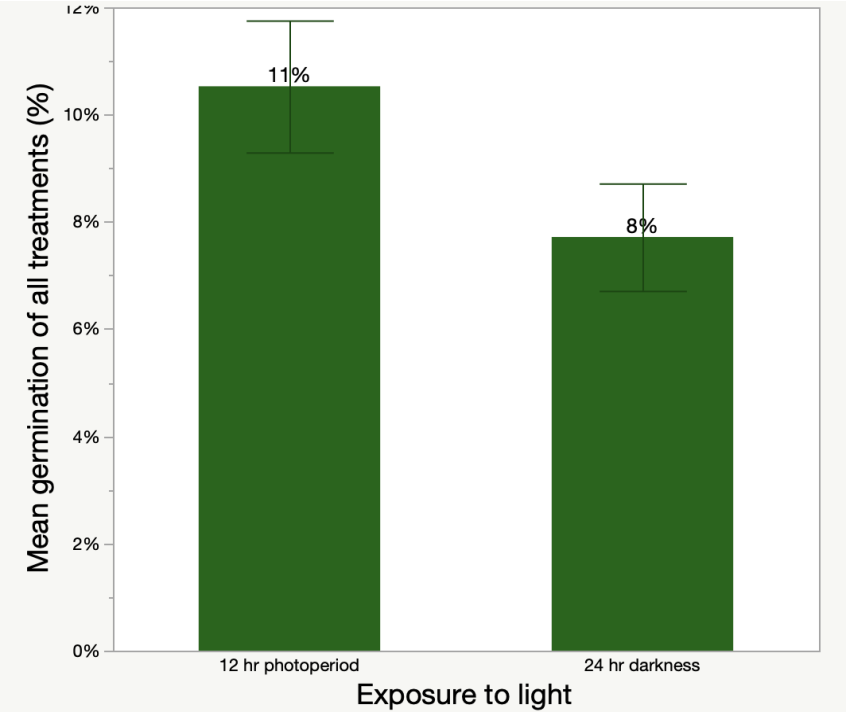


Figure 3: Total germination under 12 hour photoperiod vs total darkness

In addition to these trends in environmental conditions, the individual seed treatments had a significant effect on germination (F_{14}=8.9676, p<.0001). Specifically: compared to the control treatments, treatments of gibberellic acid at 100ppm (p<.0001), gibberellic acid at 250ppm (p<.0001), sulfuric acid for 2 minutes (p=.0084), and sulfuric acid for 5 minutes (p=.0001) all showed significant increases in germination. Those significant treatments are indicated with a gold star in Figure 4. No significant effects were found in any of the soil microbial activity treatments (field soil, cowpie, or decomposing grass) or grassland fire treatments (heat at 50ºC or 65ºC, smoke soak, burned charate, or burned grass.) Results of these treatments were similar whether under 12 hour photoperiod or complete darkness, and under cold stratification or warm temperatures.

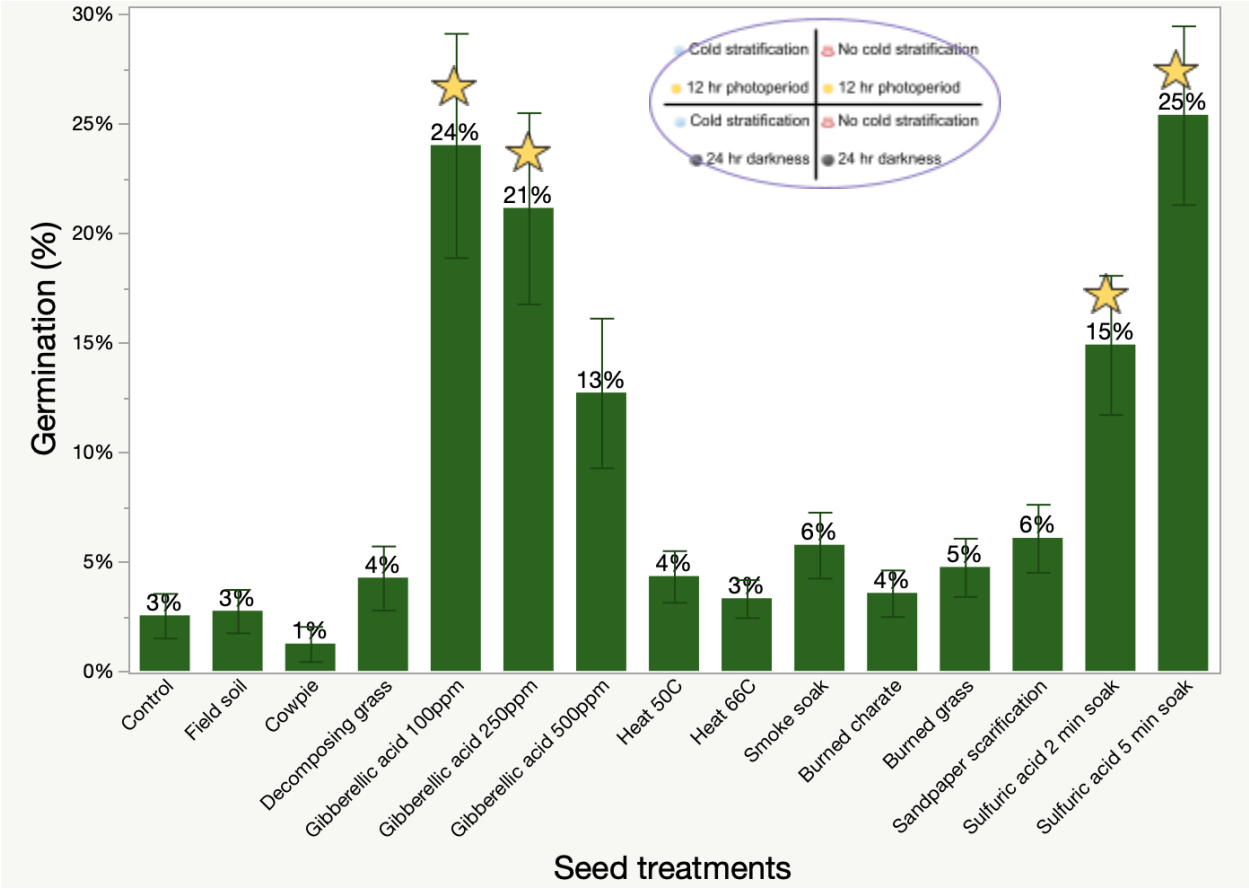


Figure 4: Mean germination of each seed treatment, averaged across all four environmental conditions

Among the three different scarification treatments, only the 2 minute and 5 minute soaks in sulfuric acid were effective; the sandpaper treatment was not sufficient to stimulate a significant amount of germination under any of the growing condition variables. We can speculate that the sandpaper treatments may not have worn away enough of the seed coat, or perhaps the right *region* of the seed coat, to allow sufficient water and gas exchange. A more precise method of mechanical scarification may provide a different result. Research shows that some species of seeds with impermeable seed coats are known to imbibe from the hilar region of the seed coat (Jaganathan et al, 2019) but this sandpaper treatment was most likely to impact the broad side of the seeds instead. The success of the 5 minute acid scarification treatment is interesting because it suggests that these seeds may be able to survive passing through the gut of an animal. If this is the case, perhaps these ray achenes aren't only the long-term seed bank of the plant, but could also have a survival advantage in years with high seed predation, or even a dispersal advantage.

The applications of three strengths of gibberellic acid (which all resulted in a significant increase in germination compared to control) are not to be construed as scarification treatments or mimicking natural processes. The success of these methods is exciting for those who may grow this plant in the future, however does not pinpoint an ecological trigger behind germination of these seeds in the field. Gibberellic acid is a plant hormone that naturally occurs (endogenously) in seeds, and plays a role in germination. The mechanism by which exogenous applications of this hormone stimulate germination are not very well understood. It seems as though gibberellic acid plays some role in softening seed coats and initiating enzymatic activity (Gupta and Chakrabarty, 2013). Thus, the *ex situ* success of this treatment in stimulating germination in disc achenes does not point towards a particular environmental process whereby germination is stimulated *in situ*.

Now that we have discussed the overall trends and successes with treatments overall, we can examine the significant *interaction effects* between the seed treatments and the growing condition variables. Two interactions stand out:

1. All three strengths of gibberellic acid produced comparable germination whether exposed to light or not, but performed significantly better when sown under cold stratification temperatures. (Figures 4 and 5) Perhaps a germination inhibitor related to these seeds' need for light exposure was overridden by the signal from the gibberellic acid.

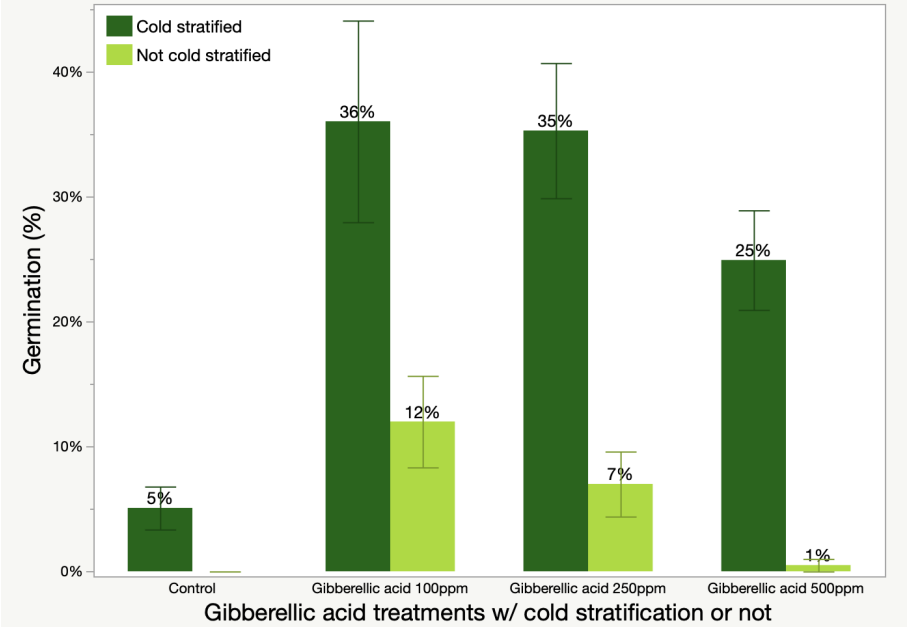


Figure 5: Gibberellic acid treatments germinated better under cold stratification than warm temperature

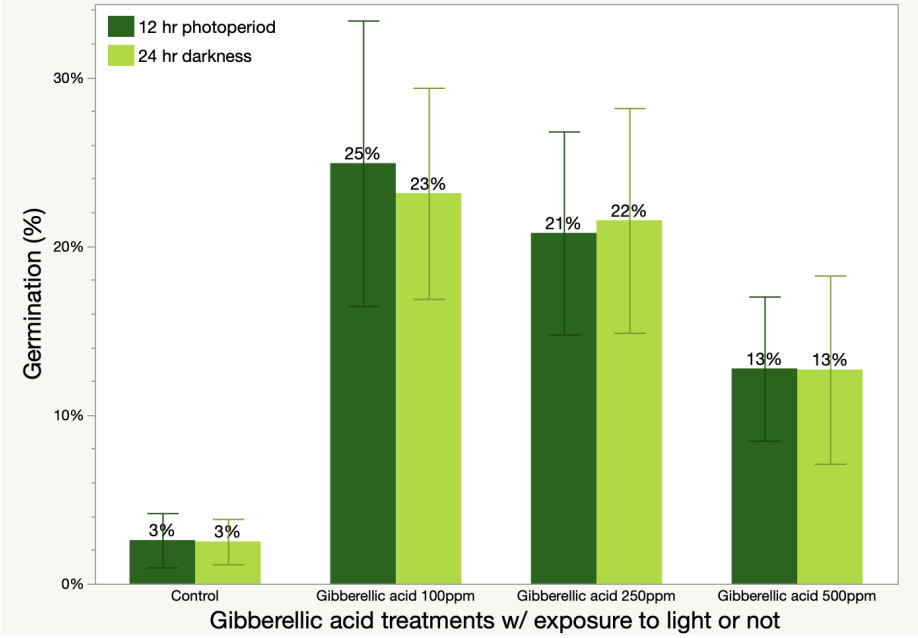


Figure 6: Gibberellic acid treatments showed similar germination rates with 12 hr exposure to light or in 24 hr darkness

2. Treatment with sulfuric acid produced significantly more germination in the 12 hour photoperiod than in 24 hour darkness, but did not require cold stratification temperatures. (Figures 6 and 7) This result is particularly interesting because it indicates that even once seeds have been scarified, they still may not germinate unless they have access to light. Perhaps this indicates that if seeds fall to the ground after scarification or pass through the gut of an animal but land under high amounts of thatch or duff, they may "know" they are in an unsuitable location for growth.

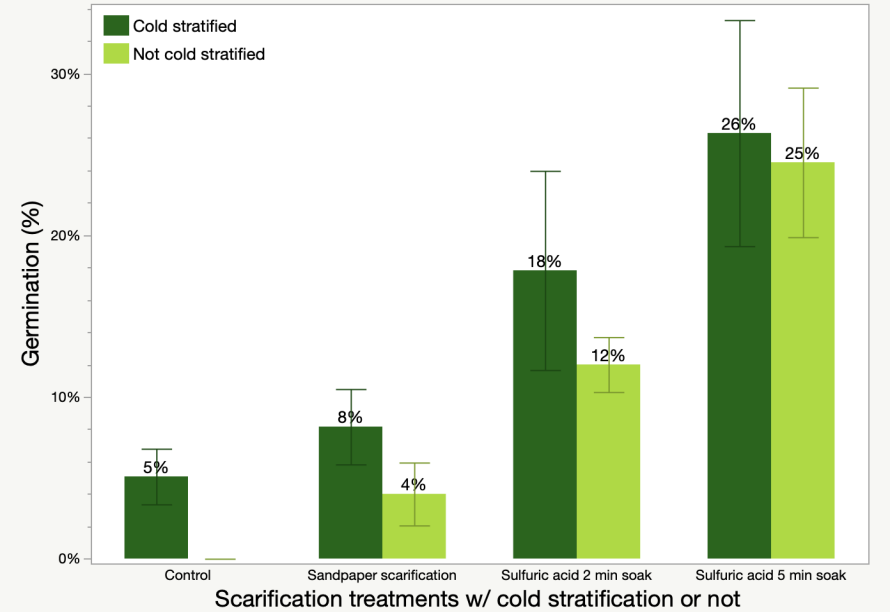


Figure 7: Scarification treatments germinated similarly with cold stratification or warm temperature

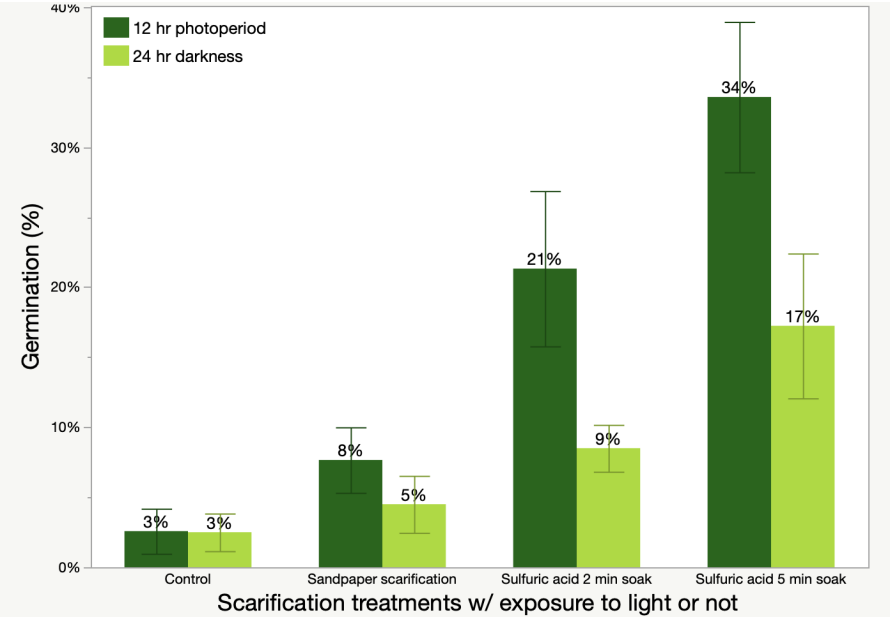


Figure 8: Scarification treatments germinated better with 12 hr exposure to light than they did in 24 hr darkness

Finally, we can examine which treatments and environmental factors *combined* worked the best. If we look only at the data collected from plants in both 40º cold stratification and 12 hour photoperiod conditions, we see that our highest mean germination rates occurred in this set. Figure 9 shows the mean germination of these treatments under only these combined environmental conditions, and excludes the other ¾ of the data collected in this study. Under these conditions, the 5 minute sulfuric acid treatment produced a mean germination of 36%, and the seeds treated with gibberellic acid at 100ppm germinated at 43%.

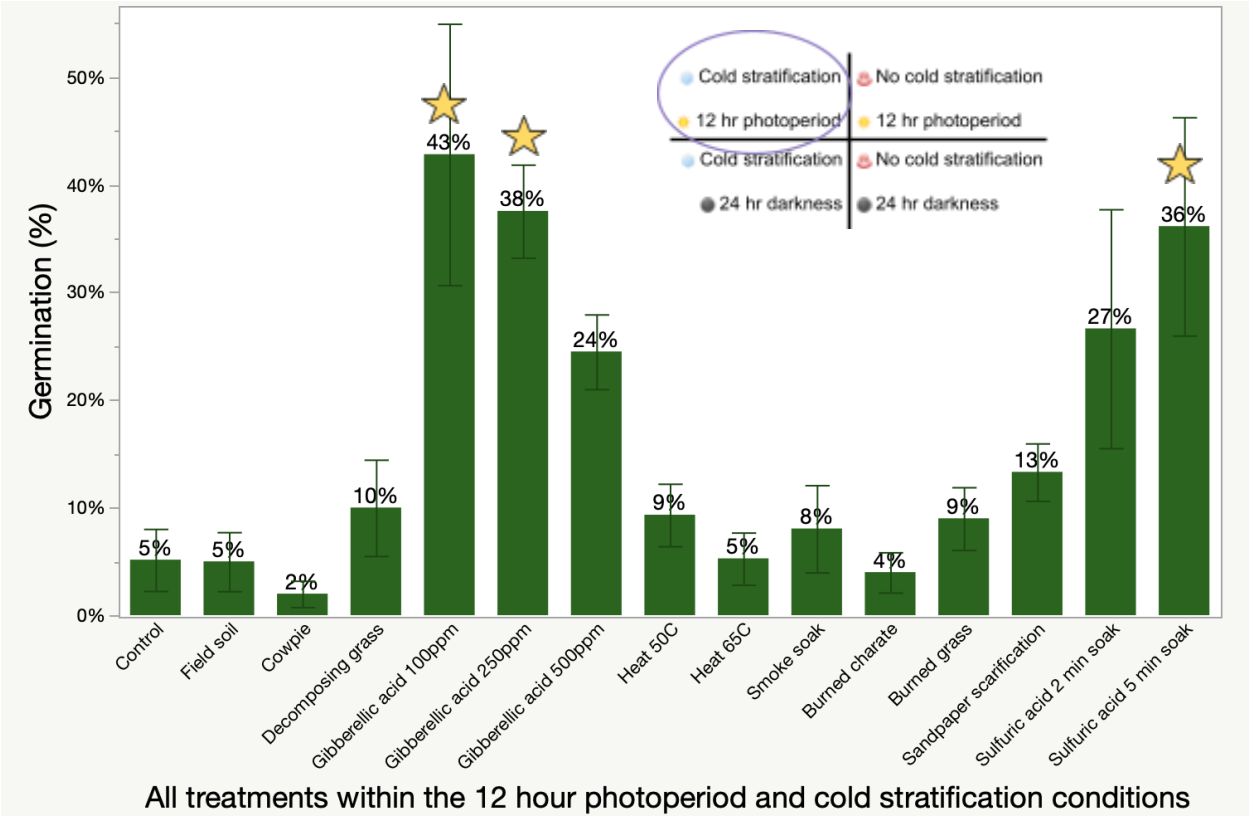


Figure 9: Some treatments in the 12 hr photoperiod and 40ºF cold stratification environmental cohort achieved significant mean germination rates between 36-43%. This represents the highest mean germination achieved by this experiment.

Conclusion

In summary, the best germination rates occurred under cold stratification temperatures of 40ºF with 12 hr diurnal exposure to light when seeds were either

scarified with sulfuric acid for 5 minutes or pretreated with gibberellic acid at 100ppm. Under these conditions, mean germination rates ranged between 36% (sulfuric acid 5 minute soak) and 43% (gibberellic acid 100ppm), which are both reasonably close to their tested batch viability rate of 40%.

Under field conditions, scarification of the ray achene seed coats is the most likely trigger for germination. However, seeds treated with sulfuric acid germinated better when exposed to light, indicating that seeds have both a physically impermeable seed coat, *as well as* some sensitivity to light to stimulate embryonic development. The gibberellic acid treatments did not require exposure to light, but this finding is consistent with other research where gibberellic acid overcame photosensitive species light requirement (Deno, 1993). Thus, it tracks that *Holocarpha macradenia* ray achenes are photosensitive and require light exposure for germination.

This work underscores the need for more research to understand the unique germination ecology of this plant, and especially how its seeds interact with its field environment and ecosystem.

Acknowledgements

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Appendix A:

Notes for practitioners who wish to germinate ray achenes:

- You'll first need to sort ray achenes from disc achenes. The best method I have found for sorting disc achenes from ray achenes is to first do a rough sorting using an Oregon Seed Blower to remove the majority of the disc achenes from your sample. It won't be perfect, and you will need to follow up with a careful manual sorting. Accidental treatment of *disc* achenes with either gibberellic acid or concentrated sulfuric acid is likely to produce an undesirable result: Unnatural elongation of seedlings may occur in disc achenes treated with gibberellic acid; concentrated sulfuric acid is likely to damage the embryo of disc achenes, which do not have as robust of a seed coat.
- Each seed batch may have a different baseline viability. I recommend tetrazolium testing to establish baseline viability of your seed lot prior to further research into effectiveness of seed treatments.
- For growers looking to produce germination in ray achenes in the simplest and most accessible way, I recommend treating ray achenes with 100ppm gibberellic acid and placing seeds in cold stratification temperatures. Germination may take multiple months under these conditions. Scarification in concentrated sulfuric acid may produce results more rapidly, however I do not recommend that most growers attempt scarification with concentrated sulfuric acid. This chemical can be dangerous to handle and should be done by trained laboratory professionals with adequate protective equipment. Since each seed batch may have variable viability, the expected result may vary widely.

Appendix B:

Suggestions and questions for future seed germination research

- Pinpointing the optimum rate of gibberellic acid may be useful for growers, especially those who may be working with a limited supply of seeds and wish to maximize their germination. Do concentrations under 100ppm provide significant benefit?
- Did the sulfuric acid treatment destroy a germination inhibitor in the seeds that mechanical scarification did not?
- Once seeds are mechanically scarified, do other treatments impact their germination? For example, does additional treatment with smoke, field soil, or cowpie affect germination compared to scarification on its own?
- Can these seeds pass through a rodent, bird, or cow's digestive tract and remain intact? How does this compare to disc achenes survivability? Does digestion break ray achene dormancy?
- How long do scarified (opened) seeds maintain viability? Does this differ between acid scarification, which may wear away much of the testa, and mechanical scarification, which may be targeted to one area?

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