# Determining the dormancy and germination requirements of two upland prairie sedges

## *Carex inops* ssp. *inops* and *Carex tumulicola*

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*A project submitted in partial fulfillment of the requirements of the Master of Environmental Horticulture*

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# TABLE OF CONTENTS

## List of Tables



## List of Figures

## A note from the author

This project was designed in response to a need expressed by Sierra Smith at the Center for Natural Lands Management's South Sound Conservation Nursery, which is that *Carex inops* ssp. *inops* and *Carex tumulicola* are important species for prairie restoration, but the seeds are difficult to germinate. Identifying how to reliably germinate seeds will help CNLM and its partners meet restoration goals by facilitating the production of sufficient quantities of plants and seeds. What follows is a description of the project and its findings. It is written for both current project partners and future researchers and propagators, with an understanding that the audience has at least a basic understanding of plant and seed biology. I hope you find it helpful.

## ABSTRACT

*Carex inops* ssp. *inops* and *Carex tumulicola* occur in upland prairies, but have had limited usage in restoration seedings due to challenges in seed propagation. Methods for breaking seed dormancy and enhancing germination were tested in germination chambers over the course of ten months. The first trial measured the effects of 0-4 months of cold wet stratification followed by simulated spring, late spring (intermediate), or summer conditions. For *C. tumulicola*, two months of winter stratification resulted in 40% germination with improved speed and uniformity, indicating that it is an obligate spring germinator. For *C. inops*, winter stratification produced a negative response; 60% germination was observed with zero months of stratification and spring or intermediate temperatures, suggesting that *C. inops* naturally germinates in the fall, an unusual strategy for a sedge.

A second experiment tested potential seed pre-treatments and their ability to enhance germination. The importance of fire was tested indirectly by evaluating germination responses to smoke water and liquid smoke diluted to 1:10,000, 1:1,000,000, and 1:100,000,000. The removal of the perigynia (a covering surrounding *Carex* seeds) was also tested on *C. inops*. *C. tumulicola* showed positive responses to both smoke water and liquid smoke, and the strongest response was observed in seeds treated with the moderate and weakest dilution. *C. inops* showed an increased germination rate with both perigynia removal and the weakest dilution of smoke water. At times, germination almost doubled that of the control.

Determining the dormancy and germination requirements for difficult to propagate species is important for efficient production of restoration plant materials. The results of this effort will allow conservation nurseries to make the best use of the seed they collect and produce.

## INTRODUCTION

Restoration is the process by which degraded and damaged landscapes are intentionally altered to improve ecological function. Restoration projects typically require the installation of native plants to meet restoration goals, and professionals are increasingly calling for genetically appropriate native plants to do the job. The definition of "genetically appropriate" can change based on the goals of the project, but one common theme is that plants are both locally adapted and genetically diverse.

The prairies of Western Washington are seated within the greater Willamette Valley-Puget Trough-Georgia Basin ecoregion (Dunwiddie & Bakker, 2011). These prairies exist due to a unique combination of glacial outwash soils, maritime climate, and a history of management by native populations who would regularly burn the prairies to encourage the growth of important food crops and discourage the encroachment of conifers (Drake, Ewing, & Dunn, 1998). Due to a more recent history of settlement, fire suppression and agriculture, and the increasing pressure of development from nearby population centers, the vast majority of these prairies are now lost. A large-scale restoration effort is now underway to protect and improve existing prairie remnants and to establish new prairies on appropriate sites.

Recovery of the Puget Sound prairies has and will continue to require the production of large quantities of plants and seed from a long list of native species. The South Sound Prairies Conservation Nursery fills this role, with a mission to "provide abundant, genetically appropriate plant material to support habitat restoration in Western Washington" (Smith & Elliott, 2015). The conservation nursery focuses on plants that will support the recovery of a number of federally listed and candidate species. Maintaining genetic diversity is a high priority and drives many aspects of the production process. Seed collection, seed cleaning and storage can all limit the diversity of seed used for direct sowing and plant propagation (Basey, Fant, & Kramer, 2015).

The nursery produces large quantities of both seed and plugs to fill the requests of project partners (Smith & Elliott, 2015). For most species, the focus is on seed production. Seeds are easier and more cost effective to store, transport, and install than containerized plants, and are the desired plant material whenever possible. For more challenging species, plugs can be produced in a greenhouse and outplanted at restoration sites, but even most plugs are usually produced from seed. Whether they are the end product or an intermediate step, seeds are a critical step in the restoration process.

Some of these species could be propagated vegetatively. Vegetative propagation, either by cuttings or divisions, is a common and desirable technique in horticulture. Plants can be produced rapidly, and with predictable and uniform traits. Because

vegetative propagation doesn't involve genetic recombination, the resulting crop will necessarily have very low genetic diversity. Because it is time consuming, costly, and does not fulfill restoration goals, vegetative propagation is only used as a last resort. Propagation by seed is the desired technique whenever possible.

Native species aren't always easy to propagate from seed. Annual food crops are often bred for fast, reliable germination, but native seeds rarely germinate right away. Survival of the individual and the population often depends on being selective about the timing of germination (Baskin & Baskin, 2014). These adaptations are beneficial to seeds, but can be challenging to propagators. The native plants of Puget Sound prairies utilize a diverse array of germination strategies. Many of these strategies have been identified by previous studies, and plants are being produced in sufficient quantities (Drake et al., 1998). For a few species, a lack of understanding about germination requirements has prevented their use in restoration plantings.

*Carex inops* ssp. *inops* and *Carex tumulicola* are two species that have proven to be challenging to propagate from seed. Both species spread readily by rhizome and could easily be produced by vegetative propagation, but seed production would allow the nursery to produce more plants with higher genetic diversity. Unfortunately, the germination requirements of these species are poorly understood, and efforts to date have always resulted in very poor germination. With a better understanding of these species' germination requirements, *Carex inops* ssp. *inops* and *Carex tumulicola* seed and plant production could be scaled up to meet the needs of the Puget Sound prairie recovery effort.

# BACKGROUND

## Species of interest

*Carex* is one of the largest genera of vascular plants, and is the largest and most globally important genus of the sedge family (Cyperaceae) (Ball & Reznicek, n.d.). Most of the 1800 species occur in northern-temperate zones, and the majority are found in wetland habitats, where they can dominate the vegetation and species diversity can be quite high (Schutz, 2000). Less common are the *Carex* of dry grasslands, forest understories, and rock outcrops. Although these systems typically have low sedge diversity, *Carex* are often locally dominant, and can be important members of these plant communities.

**Figure 1. *Carex inops* ssp. *inops* inflorescence with ripe seed. Photo take in June at Tenalquot Prairie, near Tenono, WA.**

*Carex inops* L.H. Bailey ssp. *inops*, or long-stolon sedge, is a member of the *Carex pensylvanica* complex (Crins & Ball, 1983). This complex consists of two eastern species (*Carex pensylvanica* Lam. and *Carex lucorum* Willd. ex Link) and one western species (*Carex inops*) with two subspecies (*Carex inops* ssp. *heliophila* and *Carex inops* ssp. *inops*). *Carex inops* ssp. *inops* (hereafter this subspecies will be referred to as "CAIN") is the most restricted in its range, extending from Northern California in the south to lower British Columbia in the north. CAIN occurs at high and low elevations on both sides of the Cascades. Herbarium records indicate that CAIN is locally common in the open forests and dry grasslands of the San Juan Islands and South Puget Sound prairies ("Consortium of Pacific Northwest Herbaria," n.d.).

*Carex tumulicola* Mack., or foothill sedge, occurs from California north to British Columbia, where there are roughly 10 known occurrences on the Southeast coast of Vancouver Island (Miller, Fairbarns, & Hartwell, 2008). *Carex tumulicola* (CATU) was listed as endangered in Canada in 2008 due to this limited extent north of the border. CATU inhabits grasslands and forest openings from low to mid elevation (Ball & Reznicek, n.d.). In Canada, all occurrences are from vernally moist meadows and shrub thickets in Garry oak meadows (Miller et al., 2008). In Washington, most herbarium records are from the San Juan Islands and Whidbey Island, although there are two records from the South Puget Sound region, where CAIN is more common ("Consortium of Pacific Northwest Herbaria," n.d.).

**Figure 2. *Carex tumulicola* inflorescence with ripe seed. Photo taken in September at Naas Prairie on Whidbey Island, WA.**

*Carex divulsa* Stokes (Berkeley sedge or grey sedge) is a Eurasian sedge that was mistaken for CATU and sold widely in California during the 1990s and 2000s,

advertised as a native species with horticultural virtues (Curto, 2006). *C. divulsa* is weedy, is escaping into wild areas, and is still commonly mistaken for CATU. The city of Portland just published a fact sheet warning restoration ecologists of this problem when accepting CATU plants from nurseries(City of Portland & Portland Parks and Recreation, 2016). The misidentification of *C. divulsa* may have contributed to our poor understanding of CATU germination ecology.

## Ecology of sedges

*Carex* are herbaceous perennials that are well suited to asexual reproduction, all possessing rhizomes that grow to various lengths (Wilson et al., 2008). Although many sedges rely heavily on vegetative reproduction, all sedges still produce seeds. Flowers are borne on above-ground stems that form during the previous year. The seeds are achenes, and are surrounded by a modified bract called a perigynium (plural, perigynia). The achene and perigynium are usually dispersed as one unit. Most perigynia can float, thus providing an important seed dispersal function for wetland sedges.

Sedges are an important food source for wildlife in some habitats. Small seed-eating birds consume sedge seeds, sometimes in large quantities (Wilson et al., 2008). Many mammals, from voles to beavers to bears, count *Carex* foliage as an important part of their diet. For upland sedges, including CAIN, the perigynia base is often succulent. Ants have been know to grasp the perigynia to drag seeds off to their nests for food (Wilson et al., 2008). I observed ants chewing on the bases of CAIN seeds (while they were still attached to the plant) when seed was being collected for this project.

Sedge seeds are often long-lived. Because disturbances are uncommon in most sedge habitats, these long-lived seeds tend to build up in the soil, creating soil seed banks. Seed bank studies have identified the seeds of sedges that have survived for more than 15, 45, and even 130 years buried in the soil (Schutz, 2000). For sedges that usually spread rhizomatously, this soil seed bank provides a back up source of propagules in case of a minor or major disturbance.

Lang & Halpern (2007) investigated the seed bank of a montane meadow in western Oregon that had been invaded by conifers. Soil samples revealed that a large proportion of viable seed in the soil was CAIN, and that the seeds of most other meadow species had died in both recently invaded (<75 year old forest) and older (95-200 year) forest plots. Although CAIN was still found growing in the understory, the annual seed input would be low, especially in the shade of the forest canopy. While CAIN density was only 11.4% in young forests, the soil contained 465 CAIN seeds per square meter. Old forests had only 2.4% CAIN density, but still had 160 seeds per square meter. The meadow plots, on the other hand, had 18.6% density and only 108 seeds per square meter.

Halpern, Antos, McKenzie, & Olson (2015) revisited these plots a few years later to study the effects of meadow recovery after forest removal with and without burning. Carex cover expanded in most plots, but was three times greater in burned than unburned plots. The greatest change was seen in burned plots with greater tree influence (older forest). In these plots, CAIN cover increased dramatically due to abundant recruitment from the seed bank, essentially inhibiting meadow recovery due to its dominance.

A number of upland sedges, including *Carex deflexa, C. geyeri, C. rossii,* and CAIN are known to thrive after fire. Fire intensity matters; low severity fires reduce competition and increase soil nutrients, while high severity fires can kill shallow sedge rhizomes. Both survival of the rhizomes and recruitment from the soil seed bank contribute to high sedge regeneration after low intensity fires.

## The biology and ecology of germination

Seeds are resilient reproductive units, often able to endure more extreme temperatures and certainly more extreme drought than their adult counterparts (Baskin & Baskin, 2014). Seedlings, on the other hand, are extremely vulnerable. Without a well-established root system and energy stored in hardened leaf and stem tissues, seedlings are susceptible to drought, fungal infection, extreme temperatures, and herbivory. Germination is risky business, and waiting patiently until the time is right increases the chance that a seedling will survive the establishment phase. The ability to delay germination until the appropriate season, called seed dormancy, is one adaptation that deals with this challenge, and is an important strategy for many plants.

Seed dormancy describes the physiological prevention of germination, even in the presence of ideal conditions of moisture and temperature (Baskin & Baskin, 2014). The release of dormancy is accomplished through exposure to the cold, wet conditions which are typical of winter in the temperate regions of the world. During winter exposure, or " cold stratification" (a simulated winter season we can create in a nursery environment), seeds undergo a physiological change which releases this block and gives the seed the potential to germinate. This physiological change can be permanent or temporary. If temporary, dormancy will return after a specified period of time or when the temperature rises above a certain threshold.

In effect, this mechanism ensures that seeds germinate in the spring. In temperate regions, there is a distinct advantage to spring germination. Winters often bring freezing temperatures and seedlings that germinate in the fall would be at high risk of exposure. Western Washington winters are rather mild (however freezing temperatures do still occur), and summers are hot and dry; neither season is really suitable for a young seedling. By being able to detect the season, seeds can time germination to minimize risk and maximize the window of time for growth.

The transition from a strictly dormant to a non-dormant state is called conditional dormancy (Baskin & Baskin, 2014). Conditional dormancy describes any state in which some amount of germination is possible. The transition through conditional dormancy is characterized by the widening (or narrowing) of the temperature range which can support germination, and an increase (or decrease) in the percentage of germination which occurs at those temperatures. For some species the strongest state of dormancy ever experienced is conditional dormancy. Others are strictly dormant at maturation, and then cycle through a state of conditional dormancy and non-dormancy on an annual basis after primary dormancy is relieved.

The release of dormancy opens up a window of opportunity for germination, but does not guarantee that germination will occur. The seeds of some species are opportunistic, willing to germinate as soon as the season is right regardless of the surrounding environment. Others, often those species with longer-lived seeds which are able to establish soil seed banks, are more discerning.

Germination of any seed requires the presence of two basic elements: water and oxygen. Imbibed seeds are metabolically active and able to detect cues in their environment. Water is also required for the expansion of the radicle, which breaks through the seed coat and initiates germination. The other basic requirement for seed germination is the presence of oxygen. Oxygen is a required input for the process of respiration, and without it seeds will eventually deplete their resources and die.

Beyond dormancy and the basic requirement for water and oxygen, the initiation of germination often depends on additional cues. Dormancy helps a seed identify the correct season, but germination cues help a seed detect whether the surrounding environment might be favorable for germination. The most basic germination cue is temperature. For example, a species which is relieved of dormancy by two months of winter stratification might be non-dormant by January, but may not begin to germinate until May or June if warmer temperatures are required for germination. Most species achieve maximum germination within a narrow (optimum) temperature range, but are able to achieve some level of germination within a wider range of temperatures (Baskin & Baskin, 2014).

One group of cues with a clear ecological role are those that help seeds detect gaps in the canopy. Whether that gap is from a fallen tree in the forest or a patch of prairie with a reduced litter layer from a recent burn, gap detection allows seeds to identify opportunities for reduced competition. Where resources are scarce, reduced competition means increased chances for survival.

Both seed dormancy and germination cues are important mechanisms for long-term survival in wild populations. Dormancy is especially common in species of temperate regions and germination cues are often attuned to the disturbances that frequent a particular habitat. But in the context of propagation, seed dormancy can pose a real challenge. Incorporating any species into a restoration program requires

the production of large quantities of plants or seeds, and non-uniform germination can limit genetic diversity in the resulting crop (Basey et al., 2015).

## Common sedge germination requirements

Sedges typically exhibit physiological dormancy at maturity (Baskin & Baskin, 2014). Schutz (1997) found that the seeds of six sedges were conditionally dormant at maturity. Kettenring & Galatowitsch (2007) found that 14 wetland *Carex* all had conditionally dormant seeds at maturity. Schutz & Milberg (1997) found that 13 populations of *Carex canescens* from different regions and climates were all conditionally dormant at maturity, although to various degrees. As with most physiologically dormant temperate species, dormancy was relieved by cold moist stratification for these sedges (Kettenring & Galatowitsch, 2007; Schutz, 1997; Schutz & Milberg, 1997; Schutz & Rave, 1999).

A reduction in dormancy after a period of after-ripening (extended warm dry conditions) is common in species of dry habitats, and has been observed in sedges of dry and wetland habitats. Germination increased in three out of six temperate wetland *Carex* after 4 and 8 weeks of warm dry storage (Schutz, 1997). Of a different group of 13 sedges, cold stratification increased germination in four species, while after-ripening increased germination in the other nine (Schutz, 2000).

Germination is typically highest for *Carex* in warm temperatures. For most *Carex* tested to date, germination is highest at 20°C or 25°C (Schutz, 2000). Kettenring & Galatowitsch (2007) found that most of the 14 North American *Carex* they studied germinated best at the alternating temperature regime of 27°C/15°C (daytime temperature/nighttime temperature). The ability to germinate in lower temperatures is often not gained until dormancy is relieved. For example, of 32 sedges studied by Schutz, none were able to germinate at a constant 10°C (2000). After six months of cold stratification, 22 of those species gained the ability to germinate at 10°C, but 18 of those species did not exceed 10% germination. Some forest sedges have a lower optimum germination temperature, but in general, temperature requirements for *Carex* germination are quite high.

Most sedges inhabit areas where disturbances are infrequent (Wilson et al., 2008). After dormancy has been relieved, gap detection methods appear to play a strong role in stimulating germination.

Large daily temperature fluctuations, which occur when soil is directly exposed to the sky rather than covered by a canopy, are a strong signal of a canopy gap and often stimulate germination (Baskin & Baskin, 2014). Wolfgang Schutz (2000) found that for 29 out of 34 temperate European *Carex*, the fluctuating temperature range 22°C (daytime temp) and 10°C (nighttime temp) resulted in more germination than the constant temperature 15°C (the daily average of 22°C/10°C).

Light is another mechanism for gap detection. The requirement for ample light can allow a seed to detect whether or not it is buried, and the requirement for a specific light quality (typically a high red:far red ratio) tells a seed whether or not it is being shaded by other plants (Baskin & Baskin, 2014). Many *Carex* either require light to germinate, or experience a boost in germination from exposure to light (Wilson et al., 2008). Schutz & Rave (1999) found that light was required for germination of 32 sedges at five constant temperatures between 10-30°C. Even after cold stratification, 10 of those sedges still had an absolute requirement for light.

Gap detection cues are often redundant. For example, in some species, fluctuating temperatures can stimulate germination in the dark (Schutz, 2000). Fluctuating temperatures can be an important depth detection mechanism for buried seeds, which may not have sufficient light to stimulate germination but may be close enough to the surface to detect temperature fluctuations (Baskin & Baskin, 2014).

## Existing knowledge on *Carex inops* ssp. *inops* propagation

Previous knowledge on the germination requirements of CAIN was scarce at the time when this project began. Propagation efforts from staff at the South Sound Prairies Conservation Nursery never exceeded 5% germination. Protocols were not available on the Native Plant Network Propagation Protocol Database and reaching out to area propagators turned up very few clues about how to grow CAIN.

**Figure 3. Dry *Carex inops* ssp. *inops* seeds.**

McGinnis & Meyer (2011) published a study on methods to enhance germination of closely related *Carex pensylvanica*. *C. pensylvanica* is native to Eastern U.S. forests and the seeds tested were collected in Minnesota. Perigynia removal increased germination, as did exposure to light; very little germination occurred in darkness. Germination also increased as time in storage was extended (up to 16 weeks), regardless of storage temperature (warm or cold). Cold stratification was tested as well, but results were difficult to interpret due to the 2x2x3 factorial design.

Krock, Smith, Elliott, Kennedy, & Hamman (2016) published a paper after this project was underway, in which they tested the effects of smoke-water and cold moist stratification on a number of Puget Sound prairie species. For CAIN, all combinations of a 1:100 dilution of smoke water, a deionized (DI) water control, and six stratification durations (from 0-120 days) resulted in less than 2% germination.

## Existing knowledge on *Carex tumulicola* propagation

Information on CATU propagation was also scarce. Bartow (2004) stratified seeds in a walk-in cooler for 90 days and then moved containers to a greenhouse at 70°F. Very sparse germination resulted. Containers that were left out through the winter experienced some germination the next spring.

A number of resources were identified after the beginning of this experiment which described CATU propagation methods. Guerrant & Raven (1998) found that six weeks of cold stratification resulted in 40% germination. Two other methods were also tested; seeds that experienced six weeks of warm stratification followed by six weeks of cold stratification germinated at 19%, and only 2% of fresh seeds germinated.

**Figure 4. Dry *Carex tumulicola* seeds.**

The Corvallis Plant Materials Center reported that CATU is dormant at maturity and usually germinates in late spring (Bartow, 2016). They advised sowing seeds in containers in late fall and allowing them to cold stratify outside for 4-6 weeks. Seeds should then be moved into a greenhouse to encourage germination, which requires warm conditions (75-100°F) and light.

## Summary

The overarching goal of this project was to identify how to reliably germinate CAIN and CATU seed, focusing on methods that could be used operationally by conservation nurseries and restoration practitioners.

Because of the lack of basic knowledge on dormancy and germination temperature requirements, these were the foci of the first experiment. Cold, wet stratification from 0-4 months was tested for its effects on seed dormancy. Following stratification, three temperature ranges were tested for their suitability for germination. The first experiment aimed to answer the following questions:

1. Are CAIN and CATU seeds dormant?
2. If so, does cold stratification relieve that dormancy? What is the ideal length of time in cold moist stratification?
3. What is the ideal germination temperature for CAIN and CATU?

The second experiment built on the first, and focused on smoke and perigynia removal as pre-treatments to improve germination. More background information

on the significance of these methods for germination is provided at the beginning of that chapter.

Despite the likely importance of light, after-ripening, and fluctuating temperatures on CAIN and CATU germination, these factors were not tested in these experiments. Experiments were conducted with light, fluctuating temperatures that correspond with natural conditions. After-ripening times matched natural patterns for the first experiment.

The germination response of CAIN and CATU was analyzed by plotting the distribution of germination events over time, and by comparing the percentage, speed, and uniformity of germination between treatments.

# STRATIFICATION AND GERMINATION EXPERIMENT

## MATERIALS & METHODS

### Seed Lots

Two lots of CAIN seed and one lot of CATU were evaluated in this trial. The South Sound Prairie Conservation Nursery provided 20 g of CAIN seed (362 seeds/g, 96.1% pure), which was cultivated and harvested from the Violet Prairie Seed Farm (Tenino, WA) on May 15th, 2015. This seed lot will be referred to as VP-CAIN, and is the primary CAIN seed lot tested throughout this project. 9.7 g of CAIN seed (318 seeds/g, 97.8% pure) was wild-collected on June 15th, 2015 from Tenalquot Prairie (Rainier, WA). This seed lot, referred to as TQ-CAIN, was only used in this first experiment due to the limited quantity of seed available. 31.2 g of CATU seed (681 seeds/g, 97.7% pure) was wild-collected from Naas Prairie on Whidbey Island (Coupeville, WA) on September 13th, 2015. All three seed lots were stored at room temperature (22°C) before and after this experiment. A summary of these three seed lots, and the experimental codes which will be used to refer to them from this point forward, is presented below for reference (Table 1).

**Table 1. Summary of seed lots used in this project. VP-CAIN seed was harvested at Violet Prairie Seed Farm from plants which were originally cultivated from wild-collected seed.**

| Species | Collection site | Source | Code |
|---|---|---|---|
| ***Carex inops* ssp. *inops*** | Violet Prairie Seed Farm (Tenino, WA) | Cultivated | VP-CAIN |
| ***Carex inops* ssp. *inops*** | Tenalquot Prairie (Rainier, WA) | Wild | TQ-CAIN |
| ***Carex tumulicola*** | Naas Prairie (Coupeville, WA) | Wild | CATU |

## Experimental Design

Work began in September 2015, 19 weeks after collection for VP-CAIN, 20 weeks after collection for TQ-CAIN, and 4 weeks after collection for CATU. First, seeds were assessed for water-permeability by weighing before and after soaking for 24 hours; all seeds imbibed water readily. 200 seeds from each seed lot were then assessed using the Tetrazolium (TZ) test according to the protocol described in the Tetrazolium Testing Handbook (Peters, 2000) to establish a baseline for seed viability.

Roughly 3200 seeds from each lot were prepared for testing by imbibing in aerated DI water for 24 hours. All seeds were then rinsed in a 10% concentrated bleach solution (1:10 bleach: DI water) for one minute, followed with a DI water rinse, and then blotted dry. Bleaching seeds reduces the impact of surface molds, which can thrive in humid incubation chambers, on seed viability and germination rates (J. Bakker, personal communication, August 2, 2015).

Prepared seeds from each seed lot were randomly divided into 16 treatment groups (accessions), with 200 seeds per group. Each group was then divided evenly between 4 petri dishes (100 x 10mm, polystyrene, Carolina Biological Supply), with 50 seeds per dish. Seeds were placed on two sheets of filter paper (9cm, qualitative, Carolina Biological Supply) moistened with DI water, covered with a lid, and placed in the winter, spring, intermediate, or summer incubator (SPR: Model # GR36VLC8, WIN, INT, SUM: Model#130BLL, Percival Scientific, Inc., Perry, IA 50220).

Table 2 describes the incubation sequence and duration for each accession. This 16 accession setup was repeated for each of the three seed lots. Incubators were set to simulate the day and nighttime temperatures and light duration typical of Western Washington seasons. The details of these settings are described in Table 2. The "intermediate" growth chamber represents the shoulder season between spring and summer (late spring/early summer), and summer and fall (late summer/early fall). It is referred to as 'intermediate" for simplicity.

Accessions will occasionally be referred to by their accession code. This code has two parts. The first, signified by a W#, refers to the number of months of winter stratification that the accession received. This will be followed by a three letter code (either SPR, INT, or SUM), describing the temperature of the incubator the accession was moved to after winter stratification. The winter control, which spent all seven months in winter, is identified simply as "WIN".

**Table 2 Duration and conditions experienced by each treatment group as they moved through germination incubators. Incubator conditions: Winter: 5°C (41°F) and light for 10 hours; 2°C (36°F) and dark for 14 hours; Spring: 15°C (59°F) and light for 12 hours; 8°C (46°F) and dark for 12 hours; Intermediate: 19°C (66°F) and light for 12 hours; 11°C (52°F) and dark for 12 hours; Summer: 24°C (74°F) and light for 14 hours; 14°C (57°F) and dark for 10 hours.**

| Accession Codes | 0-4 weeks | 5-8 weeks | 9-12 weeks | 13-16 weeks | 17-20 weeks | 21-24 weeks | 25-28 weeks |
|---|---|---|---|---|---|---|---|
| WIN | Winter | | | | | | |
| W0-SPR | Spring | | | | | | |
| W0-INT | Intermediate | | | | | | |
| W0-SUM | Summer | | | | | | |
| W1-SPR | winter | spring | | | | | |
| W1-INT | winter | intermediate | | | | | |
| W1-SUM | winter | summer | | | | | |
| W2-SPR | winter | | spring | | | | |
| W2-INT | winter | | intermediate | | | | |
| W2-SUM | winter | | summer | | | | |
| W3-SPR | winter | | | Spring | | | |
| W3-INT | winter | | | Intermediate | | | |
| W3-SUM | winter | | | Summer | | | |
| W4-SPR | winter | | | | Spring | | |
| W4-INT | winter | | | | intermediate | | |
| W4-SUM | winter | | | | Summer | | |

Seeds were checked every 2-3 days for the duration of the experiment. Germinants were tallied and removed, and DI water was added when necessary. We did our best to keep filter paper from being overly wet (Baskin & Baskin, 2014), and to keep dishes at a similar moisture level, but some were occasionally drier than others at inspection time. Germination was defined as the emergence of both a root and a leaf. Although no minimum length was specified, both root and leaf had to be visible and identifiable, which usually occurred when they were at least 1 mm long. Seeds that had only a root or a leaf, or for which root and leaf were too small to be distinguished, remained in the dish until they fully met the germination criteria or died (in which case they were removed and recorded as dead).

All dishes experienced some degree of fungal growth. Fungi type and quantity varied by stratification duration, germination temperature, species, and dish. Some CAIN dishes became infested with white fungi that made the filter paper hydrophobic. Some CATU dishes experienced an infestation of small brown mushrooms, which we described as “alien antennae”. The filter paper was changed whenever the degree of fungal infestation seemed to warrant it, and notes were taken occasionally on the type and degree of infestation. Future testing might benefit from a more thorough system of tracking the timing, type, and quantity of fungal infestation in each dish, to determine whether this affected the germination response.

## TZ testing

Each treatment group was TZ tested at the end of its incubation sequence to determine the quantity of viable seeds remaining. Seeds were cut longitudinally through the embryo and incubated cut-face-down in 1% TZ at 30-35°C for 48 hours (see Appendix 6 for the full protocol). The longer than recommended staining period was chosen after poor staining was observed in a 12-24 hour staining period, and based on conversations with Sabry Elias, a TZ testing expert at Oregon State University (personal communication, January 14th, 2016). Only one half of each seed was evaluated.

Evaluation was conducted using a dissecting microscope. Seeds were placed in one of three categories: viable, 50/50, or not viable. Embryo staining, endosperm firmness, embryo size, and seed size were all taken into account in the assessment of viability. These traits were evaluated collectively, and if the balance tipped towards "viable", the seed was classified as such. Staining in the aleurone layer (a layer of cells surrounding the endosperm) was ignored.

Clearly viable seeds were large in size, had vibrant (often dark) red staining throughout the large and normally formed embryo, and a firm white endosperm. The embryo often had to be excised from the seed to confidently determine the staining pattern, as artifacts from the cutting process or leaching of TZ from the cut edge often obscured staining.

Not all seeds that were classified as viable had all of these traits. For example, some viable seeds had strong red staining and firm endosperms, but a small embryo. Other seeds were large with firm endosperms, but the embryo had light red or sometimes dark pink (but not dark red) staining- these were also called viable. Some seeds appeared to be covered in pink oil, and had slightly yellowed endosperms. Pink staining in the endosperm region could indicate fungal infestation, but oily seeds often had healthy looking embryos, so this was usually attributed to conversion of the endosperm from starch to oil as the embryo prepared for germination. These seeds were also classified as viable.

Non-viable seeds included empty seeds or seeds with only a shriveled piece of tissue within the seed coat, and filled seeds with either no staining (a white, grey, or pale yellow embryo) or at most faint pink staining. The endosperm was commonly soft and yellowed. These seeds were often crushed during the cutting process. Occasionally seeds were filled and missing an embryo entirely.

50/50 seeds were those seeds that were somewhere in the middle of the spectrum of traits. For CAIN, this usually meant the endosperm was firm and healthy looking, but the embryo staining pattern was ambiguous- usually either vibrant but patchy red, or less vibrant (bright pink) but evenly stained. Sabry Elias recommended that ambiguous seeds be split evenly between "viable" and "not viable", to be fair to the

sample (personal communication, January 14th, 2016). The 50/50 category was used to keep track of these seeds until they were later reassigned.

Occasionally seeds had to be recut if the original cut was off-center. The second cut (after TZ testing was complete), often revealed a much clearer picture of embryo staining. Future TZ testing of CAIN and CATU might benefit from a revised protocol, whereby seeds are cut laterally above the embryo, and then recut longitudinally through the embryo after staining.

### Analysis

Germination counts and TZ data were compiled in a relational database (Microsoft Access, 2013). Data were then processed further in R (version 3.3.1). Three metrics were calculated to describe the response of VP-CAIN, TQ-CAIN, and CATU to winter stratification and germination temperature: germination percentage, uniformity, and germination rate. These three metrics were calculated based on the duration of time spent in the germination incubator, which was seven months for all four controls, and three months for the reminder of the accessions. The timing of germination was explored visually, by plotting both the distribution of germination events over time and the cumulative percentage of germination for each accession over time. The percentage of viable seed was also calculated for each seed lot (at the beginning of the experiment) and accession (at the end of the experiment).

**Germination percentage** is the most common metric used to describe a seed lot. It is often referred to as the "germination rate", but this terminology is incorrect. Rate necessarily involves an element of time, and germination percentage is unitless. Here, germination percentage is expressed as the mean of the number of germinants per dish, divided by the total number of seeds tested per dish, for each accession. Many studies report germination percentage relative to the number of viable seeds, but because of the difficulty involved in identifying total viable seed when seeds have strong dormancy, germination percentage is expressed relative to the total number of seeds.

Final germination percentage (% Germ), for each accession:

$$\%\ \text{Germ} = \text{mean}\left(\frac{\text{final germination count per dish}}{\text{total number of seeds tested per dish}}\right)$$

Germination rate and uniformity describe the timing of germination. Because stratification is considered a pre-treatment and seeds are not expected to germinate in these conditions, time was adjusted by subtracting the move date (the day seeds were moved out of winter stratification and into the germination incubator) from the start date, so that day 0 is the first day in SPR, INT, or SUM for each accession.

For all of the controls, which never moved incubators, day 0 is the day the experiment began.

For each accession:

Day 0 = Start Date – Move Date

**Germination rate** describes the *speed* of germination. Germination rate does not have a standardized meaning, but it is used here to identify the day on which the midpoint (50%) of germination was reached. Because this is a *relative* measure of germination, it allows us to compare accessions regardless of their final germination percentage and gives us a sense of the effects of treatment conditions on the speed of germination. Germination rate was calculated as the mean number of days it took to reach 50% of the final germination percentage for each accession. Again, because winter stratification is considered a pre-treatment, the date was adjusted as described above.

Germination rate ($T_{50}$), for each accession:

$T_{50}$ = mean(The day on which cumulative germination ≥ 0.5 * % Germ)

**Uniformity** describes the *spread* of germination, or the amount of time (in days) from the beginning to the end of active germination. Here, uniformity was calculated by, for each dish, identifying the day on which 90% of final germination occurred, and subtracting that by the day on which 10% of germination occurred. Uniformity was calculated in this way, rather than calculating the date range from the first to the last germinant, because of its decreased sensitivity to outlier germination events. Each dish contained only 50 seeds and very few accessions reached 50% germination, meaning that each seed represented at least 4% of final germination. Measuring the date range for 80% of active germination reduced the influence of outlier germination events in lots with larger germination percentages.

By this method, outlier events still influenced calculations of uniformity in accessions with smaller germination percentages. Because this could not be avoided, uniformity was only calculated for accessions which achieved a germination percentage greater than or equal to 20%. With a final germination percent of 20%, calculations of uniformity excluded the first and last germinant, calculating the date range for the middle eight germinants.

Uniformity (Uni), for each accession:

Uni = mean(Day on which cumulative germ per dish ≥ 0.9 * % Germ –
Day on which cumulative germ per dish ≥ 0.1 * % Germ)

Seed **viability** is important to include in any seed germination study. Initial seed lot viability tells us the maximum potential for germination in a seed lot. An assessment of final viability identifies viable seeds that did not germinate in that

treatment. Comparing final viability to initial seed lot viability enables us to identify treatments which reduced viability. Here, the percentage of viable seeds was calculated by splitting "50/50" seeds equally between "viable" and "not viable" categories. The number of viable seeds per dish was then added to the final number of germinants per dish, and this was divided by total seeds per dish. Values represent the mean percentage of viable seeds per accession.

Viable seeds (V), for each accession:

V = mean((0.5*(number of 50/50 seeds per dish)
+ number of viable seeds per dish
+ number of germinated seeds per dish)/
total number of seeds per dish)

Counting germinants every 2-3 days allowed us to achieve relatively high-resolution information on the timing of germination over the 7 months course of this experiment. Two types of plots were produced to allow these data to be visually explored.

First, the **distribution of germination events over time** was plotted for each accession by adding up the total number of germinants in all four dishes for each test date. To be clear, these plots represent the total germination of each accession, rather than the mean response of the four dishes within each accession.

**Cumulative germination** was also plotted to explore the timing of germination. For these plots, each point represents the mean percentage of cumulative germination (all germinants up to and including that date) for each accession. These curves allow us to analyze the *absolute* percentage of germinated seeds in each accession over time. For accessions with indeterminate germination these measures of germination timing are more meaningful than germination rate ($T_{50}$) because measures of absolute germination percentage are unaffected by the final germination percentage. Rather than choosing arbitrary points in time or germination thresholds for CAIN and CATU analysis, germination curves were analyzed for meaningful differences in these characteristics for each species.

Some accessions, especially many CAIN accessions, did not appear to have completed germination by the end of the experiment. Measures of germination timing and uniformity were influenced by the duration of the experiment for these accessions, and values would have been different had the experiment been carried out for a longer period of time. Limiting the duration of the experiment was both a practical and an ecological choice. Future restoration efforts will likely not use germination chambers to germinate seeds, and the conditions in the field or greenhouse will be influenced by the seasons. A twelve week germination test represents the maximum duration that could be expected of these seasons under natural conditions. Table 3 makes it clear that these conditions would often only

last one to two months, highlighting the importance of comparing absolute germination percentage at points in time prior to the end of the experiment.

**Table 3 Average monthly high and low temperatures and monthly precipitation for Olympia, WA. Measured at Olympia Regional Airport (Longitude: -122.903, Latitude: 46.9733) from 1981-2010 ("Climate Olympia - Washington," n.d.). Germination temperature identifies the incubator that is the closest match to the average temperature range for each month.**

| Month | Jan | Feb | Mar | Apr | May | Jun | Jul | Aug | Sept | Oct | Nov | Dec |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Germ temp | WIN | WIN | SPR | SPR | INT | INT | SUM | SUM | INT | SPR | SPR | WIN |
| High (°F) | 46 | 49 | 54 | 59 | 65 | 71 | 77 | 78 | 72 | 60 | 50 | 44 |
| High (°C) | 7.8 | 9.4 | 12.2 | 15 | 18.3 | 21.7 | 25 | 25.6 | 22.2 | 15.6 | 10 | 6.7 |
| Low (°F) | 34 | 33 | 35 | 38 | 43 | 48 | 51 | 51 | 46 | 40 | 36 | 33 |
| Low (°C) | 1.1 | .6 | 1.7 | 3.3 | 6.1 | 8.9 | 10.6 | 10.6 | 7.8 | 4.4 | 2.2 | .6 |
| Precip. (in) | 7.83 | 5.28 | 5.28 | 3.54 | 2.32 | 1.77 | 0.63 | 0.94 | 1.69 | 4.61 | 8.62 | 7.44 |

The winter incubator malfunctioned on December 26th, 2016 when the interior temperature reached 28°C (the temperature was likely above 5°C for up to 24 hours). For the 21 accessions that were in the winter incubator at that time, this unplanned heat shock may have influenced the germination response. Table 4 lists the affected accessions and the percentage of germination that occurred in winter for each.

For the affected accessions (especially winter controls), it is difficult to be confident whether germination was caused by the heat shock from the winter incubator malfunction or the loss of dormancy from extended stratification. For all winter accessions, 100% of germination occurred after the incubator failure.

The winter germination described in Table 4 likely increased the germination percentage, lengthened the date range used to calculate uniformity (decreasing uniformity), and decreased the germination rate for affected accessions. It is also possible that these accessions were affected beyond the additional germination documented above, but there is no way to distinguish these effects from the prescribed treatments. Measures of germination percentage, uniformity and germination rate are assumed to be near the expected value for these metrics; exceptions are identified below.

**Table 4. Germination in the winter incubator after the winter incubator failure. Lists all accessions which experienced the winter incubator malfunction. "Days between failure and move date" describes the duration of time spent in the winter incubator after failure, and before being moved to the germination incubator. "Percentage of germination that occurred in winter" lists the contribution of winter germination to the total germination percentage for each accession. *For WIN controls, this column lists the percentage of germinants that occurred after the winter incubator failure- 100% of germination occurred after this date for all three accessions.**

| Seed lot | Months of strat. | Germ. temp. | Days between failure and move date | Germinants in winter | Total germinants | Percentage of germination that occurred in winter |
|---|---|---|---|---|---|---|
| VP-CAIN | 4 | SPR | 23 | 0 | 57 | 0% |
| VP-CAIN | 4 | INT | 23 | 3 | 12 | 25% |
| VP-CAIN | 4 | SUM | 23 | 3 | 13 | 23% |
| VP-CAIN | 7 | WIN | NA | 20 | 20 | *100% |
| TQ-CAIN | 2 | SPR | 3 | 0 | 57 | 0% |
| TQ-CAIN | 2 | INT | 3 | 0 | 21 | 0% |
| TQ-CAIN | 2 | SUM | 3 | 0 | 9 | 33% |
| TQ-CAIN | 3 | SPR | 31 | 0 | 47 | 0% |
| TQ-CAIN | 3 | INT | 31 | 0 | 13 | 0% |
| TQ-CAIN | 3 | SUM | 31 | 0 | 17 | 0% |
| TQ-CAIN | 4 | SPR | 59 | 1 | 44 | 2% |
| TQ-CAIN | 4 | INT | 59 | 1 | 19 | 5% |
| TQ-CAIN | 4 | SUM | 59 | 0 | 21 | 0% |
| TQ-CAIN | 7 | WIN | NA | 13 | 13 | *100% |
| CATU | 3 | SPR | 11 | 0 | 61 | 0% |
| CATU | 3 | INT | 11 | 4 | 54 | 7% |
| CATU | 3 | SUM | 11 | 3 | 31 | 10% |
| CATU | 4 | SPR | 39 | 14 | 64 | 22% |
| CATU | 4 | INT | 39 | 8 | 42 | 19% |
| CATU | 4 | SUM | 39 | 16 | 40 | 40% |
| CATU | 7 | WIN | NA | 30 | 30 | *100% |

## RESULTS

### Germination Percentage

VP-CAIN and TQ-CAIN showed similar responses to germination temperature and winter stratification (Figure 5). For VP-CAIN, germination was highest in the accessions that were immediately placed in the spring and intermediate growth chambers (W0-SPR and W0-INT), and declined as seeds were exposed to increasing amounts of winter stratification. Maximum germination was between 48-60%. Although germination percentages declined with increasing winter stratification,

germination remained higher in spring (20-29%) than intermediate (6-10%) temperatures in the VP-CAIN accessions exposed to three and four months of winter and TQ- CAIN accessions exposed to two to four months of winter stratification. Germination was low in summer temperatures regardless of the duration of stratification. Cumulative germination in the winter control was also low.

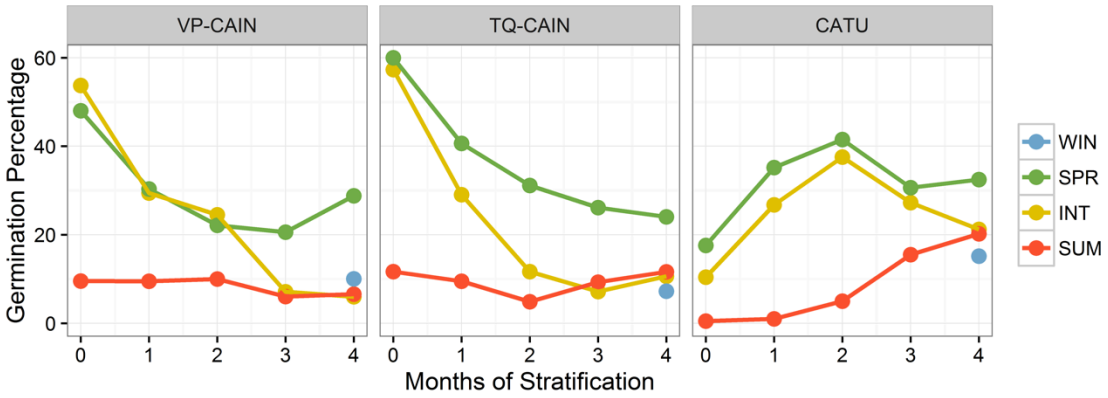


**Figure 5. Final germination percentage for each accession after the stratification and germination trial. Each point represents an accession, where the position on the x-axis represents the number of months in stratification and the color represents the germination temperature. Note that points for the control accessions (W0-SPR, W0-INT, W0-SUM, and WIN) represent germination percentages for the full seven months of incubation.**

CATU showed a similar response to germination temperature. Low germination was observed in summer temperatures and the winter control, while higher germination was recorded in spring and intermediate temperatures. Winter stratification, however, increased rather than decreased germination percentages. Germination doubled after one month of winter (from 10-18% to 27-35%) in spring and intermediate accessions, and was highest overall after two months of winter stratification. Maximum germination was recorded at 41.5% (W2-SPR).

CATU germination decreased after three and four months of winter stratification in spring and intermediate temperatures. This decline is likely due to the effects of increased fungal growth observed in the W3 and W4-SPR and INT accessions rather than an increase in dormancy. Also of note was the substantial increase in germination in summer temperatures after three and four months of winter stratification. W4-SUM reached 20.2% germination, roughly half of the maximum germination percentage (41.5%) recorded for this species. This suggests that, for CATU, additional stratification widens the temperature range that is suitable for germination by continuing to reduce dormancy. Although germination decreased in the W3 and W4-SPR and INT accessions, increased germination in W3-SUM and W4-SUM suggests that four months of stratification achieved the lowest state of dormancy for CATU.

## Germination Timing

The amount of time it takes for germination to occur is a characteristic of the germination response that can be important for nursery personnel and restoration professionals to be aware of. Observing germination curves can help us identify patterns which might be worth investigating. Figures 6, 7, and 8 show the number of germinants recorded on each day during the three months spent in the germination incubators. Figure 9 displays cumulative germination for each seed lot, again over the three months spent in the germination incubators.

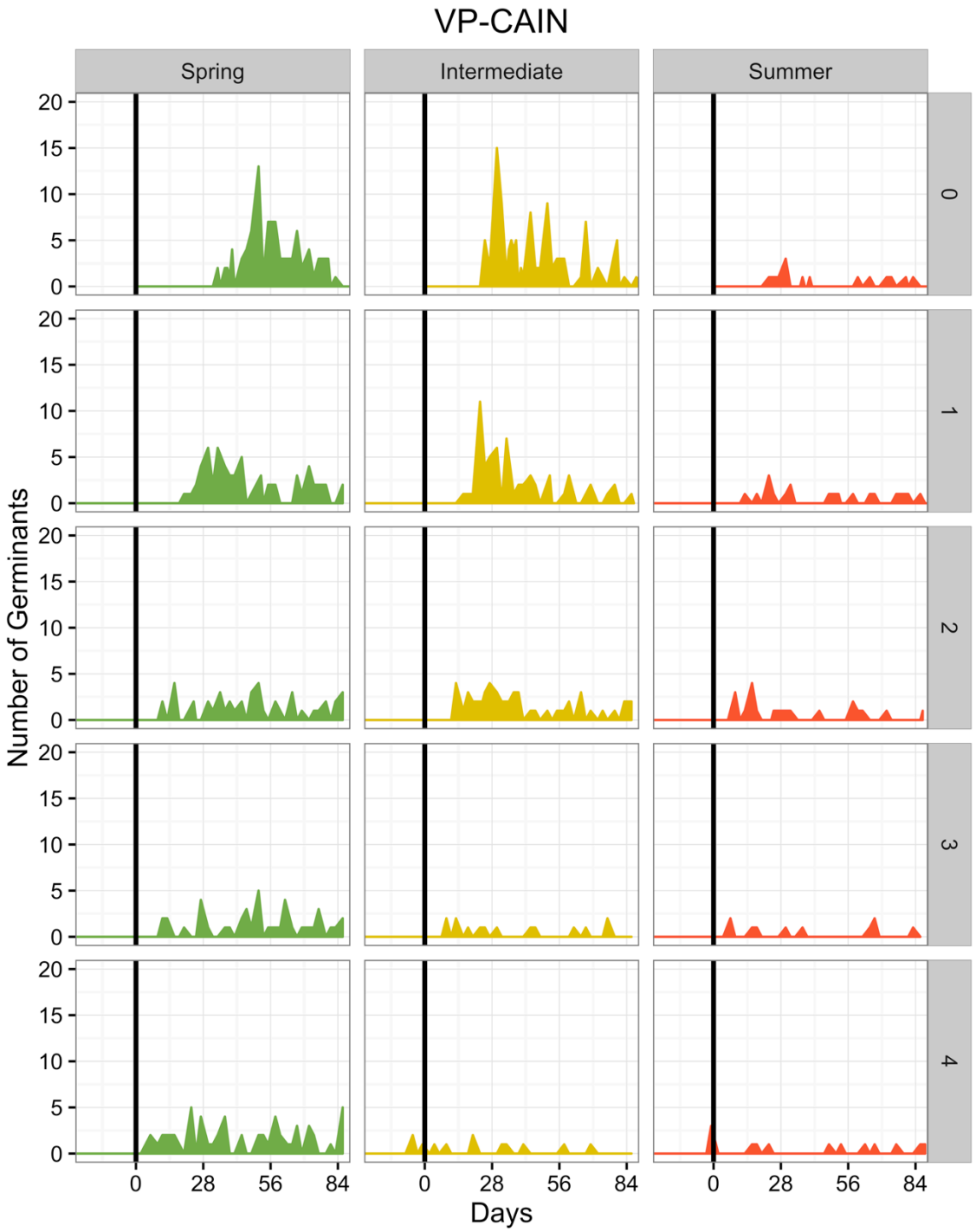


**Figure 6. Distribution of VP-CAIN germination events over time during the three months spent in the germination incubators. Rows represent the number of months spent in winter stratification. The vertical black line represents the day that each accession was moved to the germination incubator.**

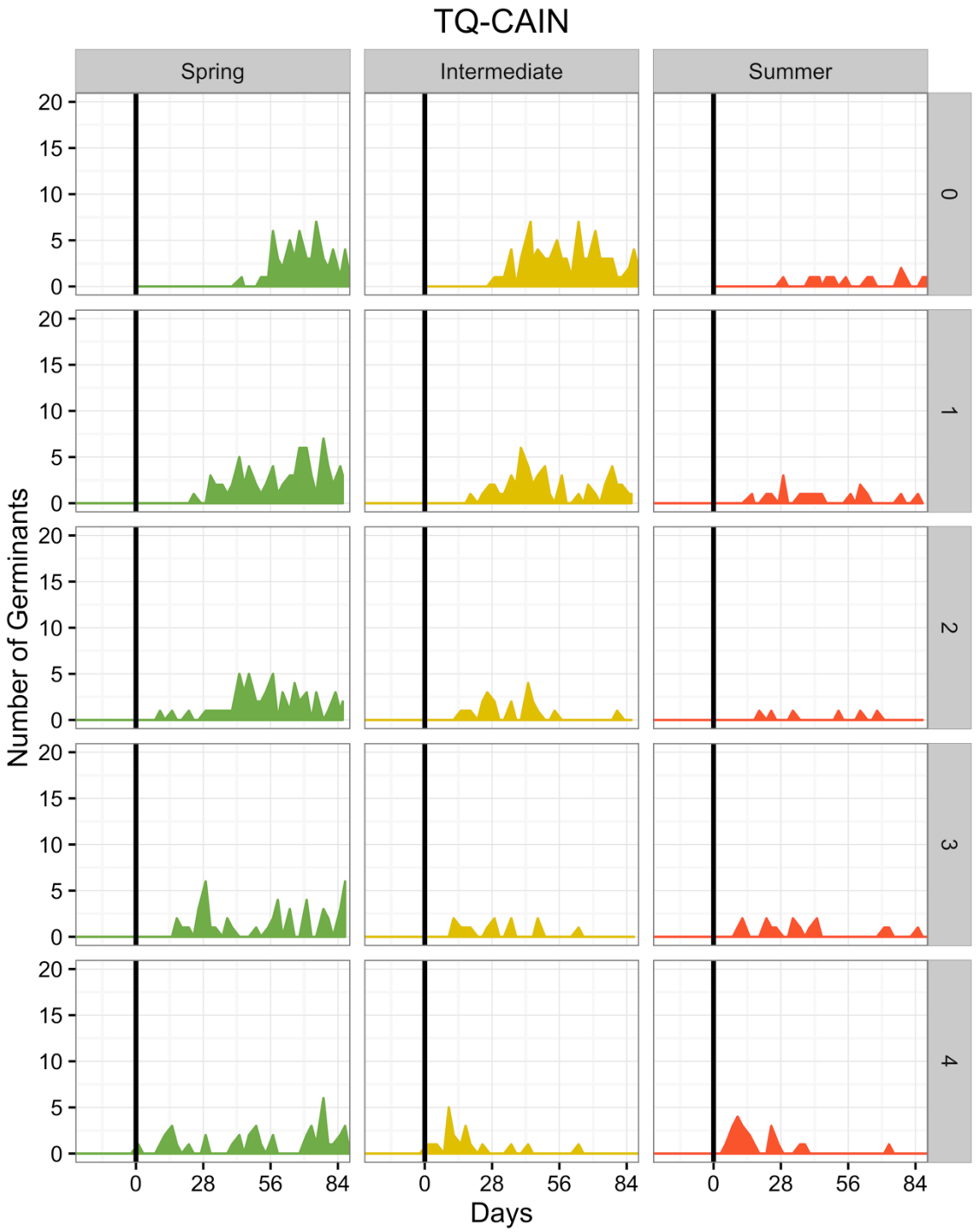


**Figure 7. Distribution of TQ-CAIN germination events over time during the three months spent in the germination incubators. Rows represent the number of months spent in winter stratification. The vertical black line represents the day that each accession was moved to the germination incubator.**

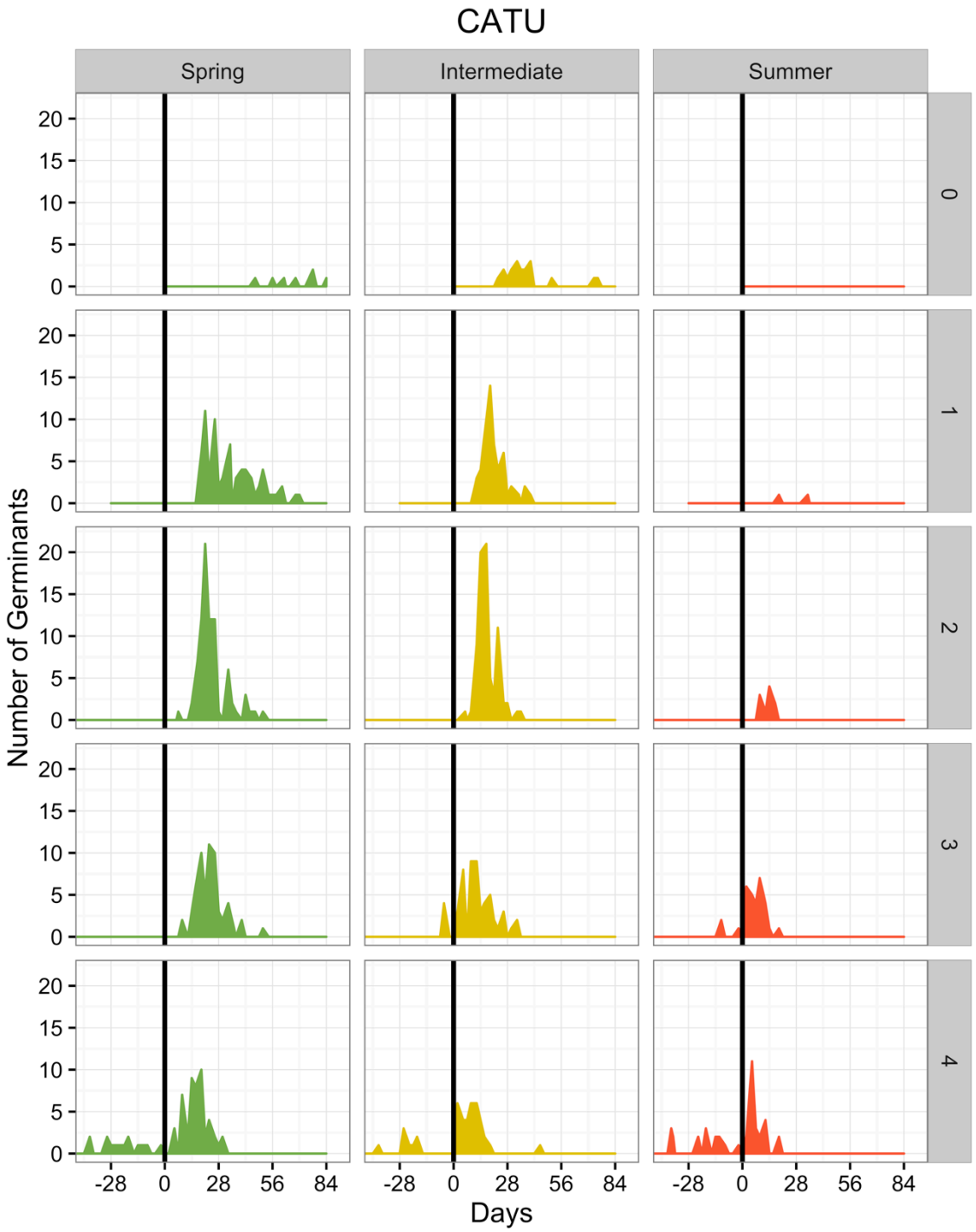


**Figure 8. Distribution of CATU germination events over time during the three months spent in the germination incubators. Rows represent the number of months spent in winter stratification. The vertical black line represents the day that each accession was placed in the germination incubator. Note that the x-axis is shifted in these plots. Some accessions show germination prior to day zero- these are the lots which were affected by the winter incubator malfunction.**

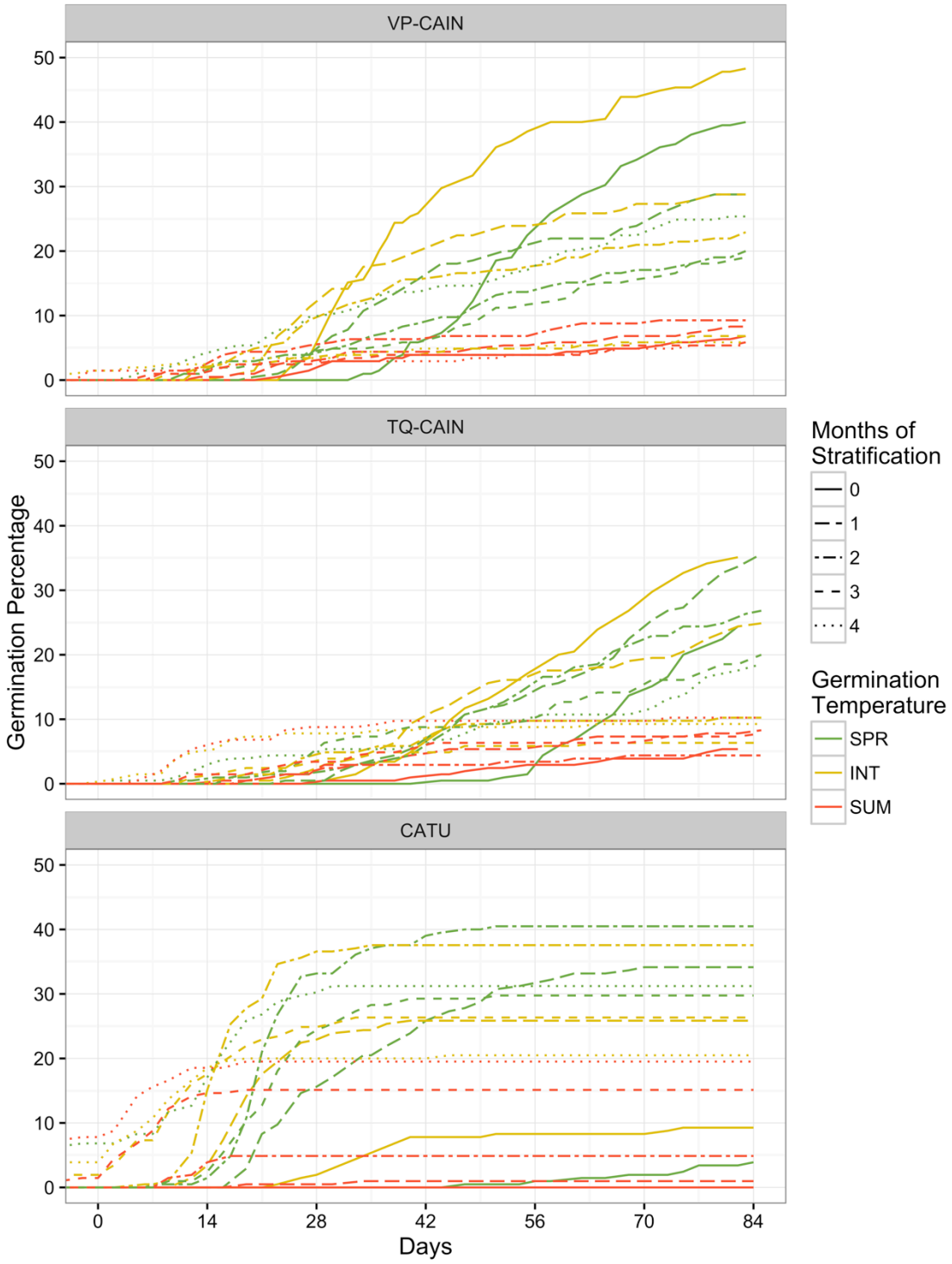


**Figure 9. Cumulative germination percentage for VP-CAIN, TQ-CAIN, and CATU. The number of months in winter stratification is represented by line type. Color represents germination temperature.**

Figures 6 and 7 suggest that germination temperature does not have a strong effect on the timing of VP-CAIN and TQ-CAIN germination. Germination started and finished around the same time for accessions which experienced the same number

of months of winter stratification. The duration of winter stratification, however, did have an effect on CAIN germination timing. As time in winter increased, the number of days to the start of germination appeared to decrease.

Germination was slow for all CAIN accessions (Figure 9). VP-CAIN W0-SPR and W0-INT stand out as two accessions with faster germination rates early on that allowed them to accumulate a higher germination percentage by the end of three months. None of the accessions appear to have finished germinating.

For CATU, germination timing appears to be affected by both germination temperature and stratification. Compared to the W0 accessions, accessions with 1-4 months of stratification had much higher and narrower germination peaks (Figure 8). Germination rate was also affected, as evidenced by the increase in slope from 0-2 months of stratification (Figure 9). The midpoint of these curves (Figure 8) also appeared to shift towards day 0 with increasing stratification. These differences will likely be captured by both uniformity and germination rate.

### *Uniformity*

Uniformity for each accession is displayed in Figure 10. Calculations of uniformity are most meaningful for accessions which achieved high enough germination percentages to be considered for future seed treatments. For VP-CAIN, those were the spring and intermediate accessions without any winter stratification (W0-SPR and W0-INT). Of these two accessions, the intermediate temperature resulted in higher uniformity at 54 days than spring at 93 days. This 39 day reduction without much loss in germination percentage is a meaningful increase in uniformity for nursery professionals.

For TQ-CAIN, uniformity was low in both of the accessions with the highest germination percentages (W0-SPR and W0-INT). Uniformity was higher in many of the other accessions, but these treatments would likely not be attempted because of their lower germination percentages. Generally speaking, uniformity was quite poor for all CAIN accessions.

The uniformity of CATU germination, on the other hand, responded strongly to winter stratification. In spring and intermediate temperatures, uniformity increased with the addition of one, two, or three months of winter stratification. The shortest germination window recorded in an accession with at least 20% germination was 11 days for W2-INT. Other accessions with high uniformity were W3-SPR and W1-INT (16 days) and W2-SPR (18 days). The CATU accessions with the highest uniformity are some of the same accessions that experienced the highest germination percentages, making a strong case for treating CATU seed with 2 months of winter stratification and germination in spring or intermediate temperatures.

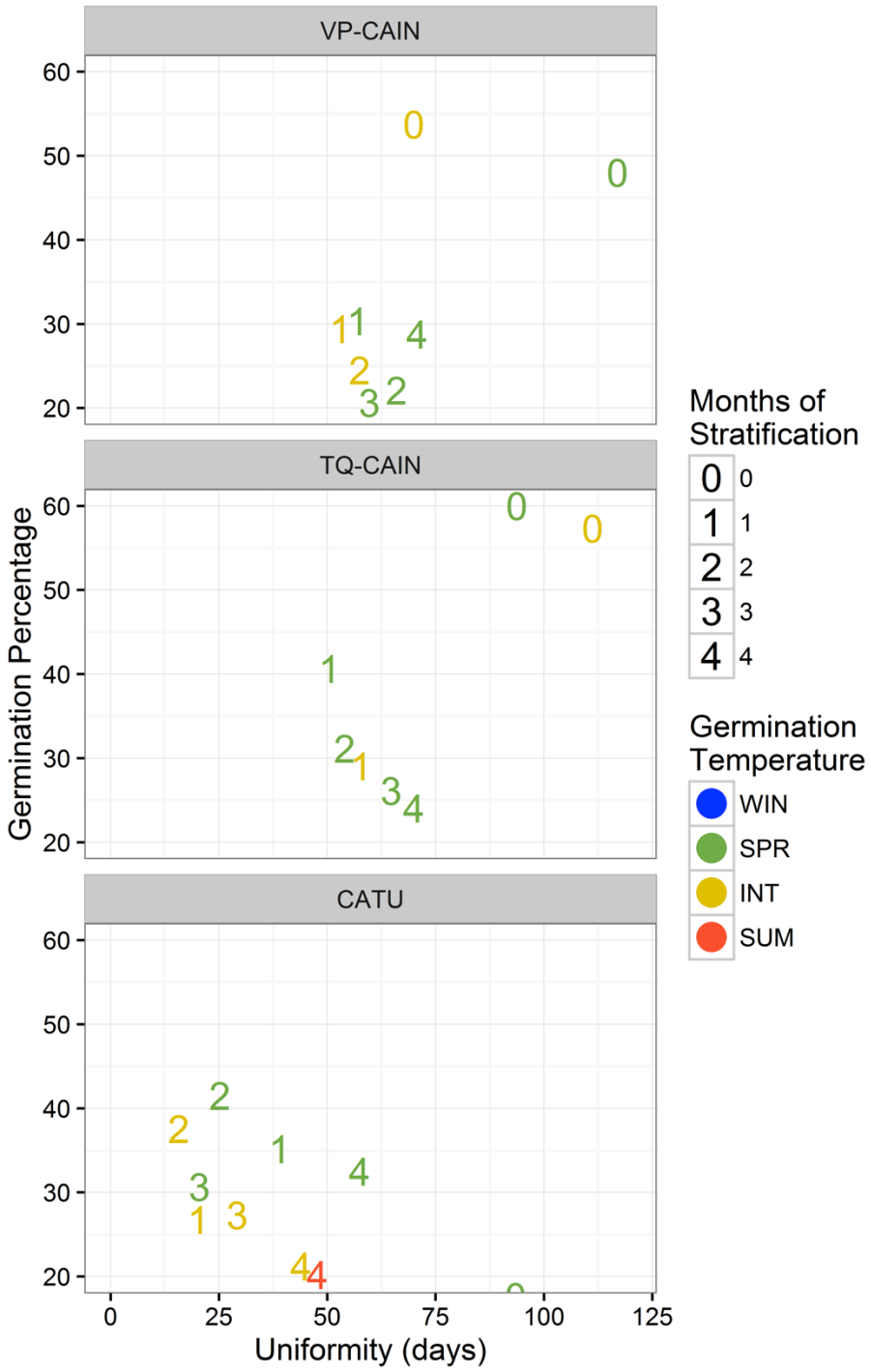


**Figure 10. Uniformity in days for VP-CAIN, TQ-CAIN, and CATU. Each point represents an accession, with months of stratification represented by number and germination temperature represented by color. Uniformity increases as the number of days to 80% germination approaches zero.**

### *Germination Rate*

The germination rate, or time to the germination midpoint (50% relative germination), for each accession that achieved at least 20% (absolute) germination is displayed in Figure 11. As was the case with uniformity, germination rate is best assessed when paired with germination percentage and the most meaningful relationships are drawn from accessions with high germination percentages.

For VP-CAIN and TQ-CAIN, the groups with the highest germination percentages all had slow germination rates. VP-CAIN was faster than TQ-CAIN and the intermediate groups were 20-25% faster than the spring groups, but overall germination was slow. The most ideal combination of germination percentage and germination rate was the W0-INT accession for VP-CAIN, which reached its germination midpoint in 42 days.

For CATU, increased time in winter stratification decreased the time to 50% germination. For the accessions with the highest germination percentages, the germination midpoint was reached in 13-32 days.

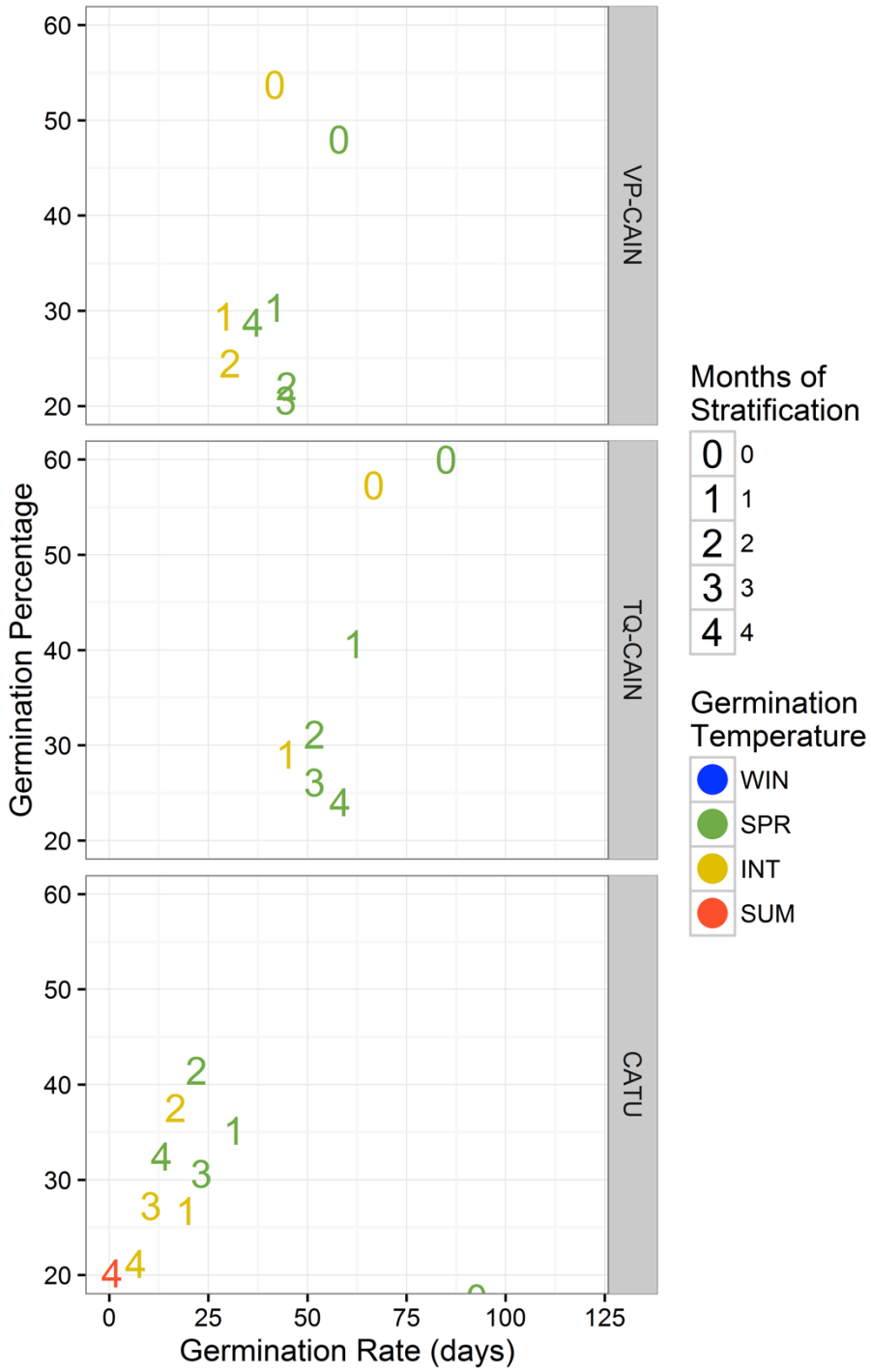


**Figure 11. Germination rate (time to 50% germination) in days for VP-CAIN, TQ-CAIN, and CATU. Each point represents an accession, with months of stratification represented by number and germination temperature represented by color. The winter controls are not displayed.**

## Viability

Figure 12 compares the percentage of viable seeds on the final date of testing to the final germination percentage for each accession. Initial TZ testing results are displayed in

When evaluating viability at the end of the experiment for CAIN accessions, it is difficult to identify any obvious patterns. For VP-CAIN, the lowest viability was observed in the summer control, but other summer lots did not have as much loss in viability. TQ-CAIN showed the biggest reduction in viability in the accessions that experienced 2 months of winter stratification. Viability fluctuated quite a bit within each germination temperature as months of stratification increased.

Table 5. Seed lot viability for VP-CAIN from initial TZ testing (45.1%) is lower than the maximum germination percentage for VP-CAIN (53.7%), suggesting that initial TZ testing was inaccurate. This was the first round of TZ testing in the entire experiment, and was conducted before the protocol was refined for subsequent testing. The maximum seed lot viability recorded was 59.7%, and is a better estimate of initial VP-CAIN viability since viability can only decline over time. Poor sampling (mixing) could be another explanation for this discrepancy. For TQ-CAIN, initial TZ testing results (68.5%) were similar to the highest final seed lot viability recorded (67.1%), suggesting that 68.5% is a reasonable estimate of initial viability for TQ-CAIN.

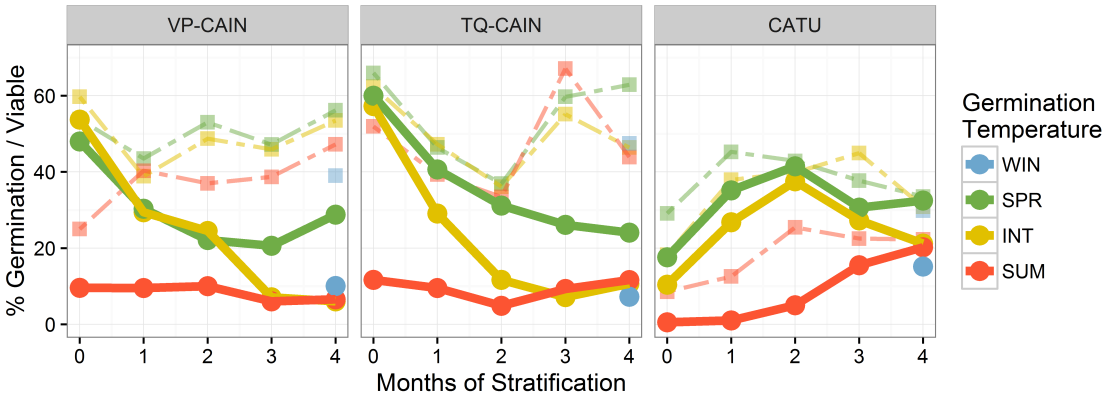


**Figure 12. Final viability compared to germination percentage for each accession. The solid lines represent final germination percentage. Dashed lines represent total viable seed for each accession at the end of the experiment.**

Initial TZ testing was not completed for CATU because poor staining was mistaken for bad seed prep. CATU seeds were dormant prior to a period of winter stratification (see Discussion). Deeply dormant seeds don't stain in the presence of TZ, making it difficult, if not impossible, to distinguish between dormant and intact but non-viable seeds. The highest final seed lot viability recorded for CATU was

45.3%, which is likely representative (or at least a conservative estimate) of the initial viability of this seed lot.

When evaluating viability at the end of the experiment for CAIN accessions, it is difficult to identify any obvious patterns. For VP-CAIN, the lowest viability was observed in the summer control, but other summer lots did not have as much loss in viability. TQ-CAIN showed the biggest reduction in viability in the accessions that experienced 2 months of winter stratification. Viability fluctuated quite a bit within each germination temperature as months of stratification increased.

**Table 5. Estimates of initial seed lot viability from the stratification and germination experiment. Challenges in initial TZ testing resulted in underestimates of viability for VP-CAIN and CATU. The maximum viability recorded during final TZ testing provided a better estimate for initial seed lot viability for these two lots, since viability can only decrease over time.**

| Seed Lot | Initial TZ testing results | Highest recorded final seed lot viability | Best estimate of initial seed lot viability |
|---|---|---|---|
| VP-CAIN | 45.1% | 59.7% | 59.7% |
| TQ-CAIN | 68.5% | 67.1% | 68.5% |
| CATU | NA | 45.3% | 45.3% |

Because seed dormancy complicates TZ testing, it is difficult to evaluate the effects of winter stratification and germination temperature on CATU viability. Instead, these data likely reflect the decreasing state of dormancy in SPR and INT temperatures, as time in cold stratification increases. Seeds in summer are likely in a state of conditional dormancy.

Although dormancy of CAIN and CATU seeds may prevent meaningful comparison of viability after germination testing, one takeaway from this experiment is that both CAIN and CATU have low overall viability. At less than 70% for CAIN and 45% for CATU, neither species should be expected to achieve germination 100% germination in the field or nursery. Propagators should use these measurements of viability to calculate more accurate sowing rates.

## DISCUSSION

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#### *Germination Response*

Both VP-CAIN and TQ-CAIN achieved the highest germination percentages (48-60%) in spring and intermediate accessions with no winter stratification. Germination declined substantially as exposure to winter stratification increased. Germination was equal or higher in spring than intermediate temperatures for all

stratification durations, and low in summer regardless of time in stratification. Germination was also low in winter. Based on germination percentage alone, CAIN should be sown in spring or intermediate temperatures without any prior winter stratification for maximum germination.

CAIN uniformity was not strongly affected by germination temperature or winter stratification. VP-CAIN-W0-INT had the best uniformity (54 days) of the high-germinating CAIN accessions. Although 54 days is poor uniformity by agricultural standards, it is not uncommon for some native species. Slow germination, although frustrating for propagators, may be an important strategy for CAIN. By spreading out germination, the risk (to the population) of germinating too early and losing all seedlings to a late frost (in spring) or a dry spell (in fall) is decreased (Baskin & Baskin, 2014). Despite this advantage in the wild, poor uniformity still poses a risk for loss of diversity in the nursery, as non-uniform crops are difficult to care for.

Germination rate (time to 50% germination) was slow for both CAIN seed lots, although VP-CAIN was faster (42-58 days) than TQ-CAIN (67-85 days) for the highest germinating accessions. The response of germination rate to a particular seed treatment is important for propagators to be aware of, but less critical than germination percentage and uniformity. Nursery personnel can now adjust the sowing date of CAIN seeds with these germination rates in mind.

TQ-CAIN viability (68.5%) was similar to, but slightly higher than VP-CAIN viability (59.7%). Changes in CAIN viability in response to treatment conditions are difficult to interpret. The percentage of viable seeds did not change in response to winter stratification or germination temperature in any clear pattern. TZ testing of CAIN seeds was extremely difficult, as variation in staining intensity was high. Although we did our best to standardize our classifications from one person to another and from the beginning to the end of the experiment, TZ testing is notoriously subjective (Peters, 2000), and results may not be accurate.

TZ testing was developed for agricultural seeds, which are typically bred to have little to no dormancy. The TZ test is most useful if performed to supplement a germination test, identifying seeds that did not germinate but are still viable- by definition, dormant (at least to some degree).

The TZ testing handbook briefly mentions “deep dormancy”, which can be encountered in native species (Peters, 2000). According to the handbook, deeply dormant seeds may not stain because of low respiration rates. Additional pretreatments may be required, such as exposing seeds to drastic temperature alternations (5-35°C), or gibberellic acid. Vivrette and Meyr (2002) suggest extending the prechilling (stratification) time as one method to improve TZ results for dormant native seed. For example, *Iris missouriensis* showed 0% germination/42% dormant seed after 30 days, 47% germination/21% dormant seed (for a total of 68% viable) after 60 days, and 90% germination/5% dormant seed (for a total of 95% viable) after 180 days. The TZ testing handbook warns that

deeply dormant seeds may lead to false negatives, and analysts must learn to interpret a "no stain" or "light stain" in this case. No method is outlined to distinguish between non-viable and deeply dormant seeds.

In these resources, criteria are not given for identifying species with deep dormancy and the exact definition of deep dormancy is not given. Rather, it appears to be a trait assigned to seed lots at the analysts' discretion, which prove difficult to TZ test using standard protocols. Without clear guidelines for identifying deeply dormant seeds and interpreting their staining patterns, the utility of the TZ test for native seeds is limited at best. Results must be interpreted with caution.

### *Seed Dormancy*

Seed dormancy is the next logical topic in a discussion of CAIN propagation. Unfortunately, because this experiment was designed to identify the best propagation protocol for CAIN rather than the germination ecology of CAIN in the wild, there are limitations to what can be said about seed dormancy. For example, I can't comment on the state of dormancy at the time of seed ripening. I can, however, say that seeds were non-dormant when testing began 19-20 weeks later.

After-ripening during warm dry storage may have contributed to the lack of dormancy at the time of testing. McGinnis and Meyer found that 21 weeks of warm dry (22°C, 40-50% RH) storage increased the germination percentage of *Carex pensylvanica*, a close relative of CAIN, compared to seeds stored for only 9 weeks (2011). Other studies have found sedges that have shown both increased and decreased germination with dry after-ripening (Schutz & Milberg, 1997). Future testing could identify the role of after-ripening, if any, on CAIN seed dormancy and germination.

Germination was highest in treatments that did not experience winter stratification, and decreased with increasing time in stratification. This is contrary to the response of *Carex pensylvanica*, which showed an increase in germination after 2 months of cold stratification (McGinnis & Meyer, 2011). Schutz and Rave found that cold stratification significantly increased the germination percentage in 28 of 32 *Carex* species, increasing the odds of germination 61 times on average (1999). The negative response of CAIN to cold stratification appears to be unusual for *Carex*.

For accessions which were exposed to winter, increasing durations of cold stratification increased the level of CAIN seed dormancy. This is illustrated by both the narrowing of the temperature range which stimulated germination and the overall reduction in germination percentage. For VP-CAIN, germination in intermediate temperatures dropped to baseline levels (10%) with 3 or 4 months of stratification. 2-4 months of stratification did the same for TQ-CAIN. Because CAIN never lost the ability to germinate in spring temperatures, this increase in dormancy is best described as conditional dormancy.

Based on the germination response described above, I suspect that CAIN has some level of primary dormancy at the time of ripening, loses that dormancy by the fall, and then regains conditional dormancy during the following winter, cycling through a conditionally dormant and non-dormant state on an annual basis. This pattern could be confirmed with a burial experiment (see Baskin & Baskin, 2014).

### *Germination Temperature*

Although CAIN germination was not tested in the full range of possible temperatures, we can assess the suitability of the four temperature ranges that were tested. If seeds were in fact non-dormant at the beginning of testing (as opposed to conditionally dormant), then summer (24°C/14°C) and winter (5°C) temperatures are unsuitable for CAIN germination. Spring temperatures (15°C/8°C) are the ideal germination temperature range and are suitable even in a state of conditional dormancy. Intermediate temperatures (19°C/11°C) are also suitable when seeds are non-dormant. It is possible that seeds could gain the ability to germinate at higher or lower temperatures with additional dormancy release. A burial experiment would allow us to confirm the significance of these temperature ranges. Testing at additional temperatures (for example, "early spring" (10°C/7°C) or "mid-summer" (28°C/16°C)) would give us higher resolution data about suitable temperature ranges for germination at different points in the annual dormancy cycle.

Temperature is often cited as a primary driver for germination, but the amplitude of daily temperature fluctuations can also influence germination percentage (Baskin & Baskin, 2014). Here, daily temperature fluctuations were not uniform across germination temperature treatments (Table 6). For *Carex*, germination tends to increase with increasing temperature fluctuations (Schutz, 2000). Because germination was lowest in summer (the season with the largest temperature fluctuation), decreased germination was likely due to the high mean temperature rather than the 10 degree fluctuation between the daily high and low.

Low germination in winter could be due in part to the small temperature fluctuation (Table 6). A future study that compared temperature regimes with different amplitudes and the same mean temperature could confirm or deny this pattern. The additional 1°C of fluctuation between SPR and INT could have contributed to the higher germination percentages observed in those accessions, but because it was such a small difference, it was likely not the main driver. Ecologically meaningful differences in temperature fluctuations are often 5-10 (or more) degrees Celsius (Kettenring & Galatowitsch, 2007).

| Temperature Regime | Daily high and low temperature (°C) | Amplitude of daily temperature fluctuation (°C) |
|---|---|---|
| Spring | 15/8 | 7 |
| Intermediate | 19/11 | 8 |
| Summer | 24/14 | 10 |
| Winter | 5/2 | 3 |

**Table 6. Amplitude of temperature fluctuations for each of the temperature regimes used in this experiment.**

Spring (15°C/8°C) is an unusual optimum germination temperature for *Carex*. Many sedges require relatively high temperatures for germination. Of the 32 sedges tested by Schutz and Rave, germination was highest for most at 20 or 25°C (1999), and the fluctuating temperature range 22°C/10°C often yielded higher germination than other temperatures. Only one, *Carex sylvatica*, showed the highest germination at 10°C (when tested at constant temperatures 10, 15, 20, 25, 30°C). Kettenring and Galatowitsch found that 11 of 14 North American *Carex* germinated best at 27°C/15°C, when tested at 27°C/25°C, 22°C/8°C, 14°C/1°C, and 5°C/1°C (2007).

### *Additional Observations*

Although untested, we did observe an interesting pattern for CAIN, which was that dishes which appeared drier often had more germinants than wetter dishes in the same accession. No notes were taken with specific numbers, but germinants were often more numerous and more developed (especially with a longer root and more root hairs) in these dishes. Fluctuating moisture levels could be a gap detection mechanism for seeds, similar to the role of light and fluctuating temperatures. Without precisely controlled watering and careful daily measurements we could not determine whether this pattern was real or perceived, but this observation was made nonetheless.

### *Implications for* Carex inops *ssp.* inops *life history*

Although this study was designed to identify the best protocol for nursery propagation, some inferences can be made about the germination pattern of CAIN in the wild. Knowing that CAIN favors germination temperatures in the "spring" and "intermediate" range and that winter stratification inhibits rather than promotes germination, suggests that CAIN typically germinates in the fall. As discussed above, optimum germination without winter stratification is an atypical pattern for *Carex*. Spring germination, which is ensured by having a stratification requirement, is much more typical.

*Carex* are most commonly associated with wetlands. The Flora of North America contains 480 species of *Carex*, the vast majority of which are typically found in moist to wet habitats (Ball & Reznicek, n.d.). *Carex* of dry upland sites are less common,

and are less well represented in germination literature. Of the four *Carex* that did not positively respond to cold stratification in Schutz and Rave (1999), three are from dry open habitats and one was from a dry forest habitat. Of the 28 that did respond positively to cold stratification, 25 were from wetland habitats (open or forested) and 3 were from dry forested sites.

The unconventional response of CAIN to winter stratification and germination temperature could be related to its preference for dry open habitat, however interpretations of seed dormancy as a result of habitat characteristics must be made with caution. Some have proposed that intra-specific differences in dormancy can be strongly influenced by habitat, while others have found the opposite (Schutz & Milberg, 1997). The environmental conditions experienced by the mother plant can have a large influence on the dormancy of its seeds, so great care must be taken to identify the source of variation in dormancy (Baskin & Baskin, 2014).

Regardless of the reason for fall germination and optimum germination in cool temperatures, this pattern does make intuitive sense given the life history and distribution of CAIN. CAIN seeds ripen in May or June and fall to the ground soon thereafter. A long dry summer usually follows. Some germination could occur immediately if the dormancy state at maturity allows, but late spring would be an unfavorable time to germinate given the hot dry conditions of a typical prairie summer (Table 3). Germination during summer will likely be low. Unless seeds are located in moist microsites, they may have insufficient water available until fall rains return for even the low (10%) level of germination observed in the W0-SUM accession. Survival for any summer germinants is also likely to be low, again, because water is scarce.

The W0-INT and W0-SPR accessions suggest that germination will increase sharply as the temperature begins to drop in September. Although germination rate and uniformity were poor for these accessions, roughly equal germination in both W0-INT and WO-SPR means that slow germination may not be limited by the duration of this germination window, which should last from September through November in a typical year (Table 3).

Fall germination would allow CAIN to take advantage of the low canopy cover at this time of year, when more light would be available to stimulate germination. Controlled burning of the prairies also typically occurs in late summer or early fall, reducing leaf litter and increasing light to stimulate germination. Although CAIN would not be alone in its fall germination strategy, fewer species would likely be germinating at this time than during spring, reducing direct competition from other plants.

The advantages of fall germination come with the risk of subsequent winter exposure to freezing temperatures. Although the average low temperature in winter is above 0°C, freezing temperatures do occur. Microsite conditions could protect some of these young seedlings, but without being well established,

unprotected seedlings would likely not survive a hard freeze. Prolonged soil flooding would also pose a risk, although the well-drained soils which characterize these prairies would prevent this from being the case most of the time.

The differential response of CAIN and *C. pensylvanica* to winter stratification is probably explained at least in part by the different habitats these closely species are found in. Minnesota winters are wholly unsuitable for new seedlings and fall germination would be a poor strategy for any but the fastest growing perennials. Western Washington prairies, on the other hand, are frequented by late summer fires and have temperatures moderated by our coastal climate. With the reduced risk of freezing and increased chances of low-competition canopy gaps, fall germination seems like a reasonable strategy for CAIN.

### *Recommendations for propagation of* Carex inops *ssp.* inops

Restoration professionals should take advantage of this natural germination pattern by sowing CAIN in September. Sowing in sites with low canopy cover or which have been recently burned will maximize light exposure. Seeds should be sown on the surface (not drilled).

Nursery professionals should follow a similar production schedule. Fall sowing should take place in September, allowing for the maximum window of time for germination. Seeds should be surface sown and given ample light exposure. Regular irrigation will ensure that seeds remain imbibed and have water available for early growth. Allowing soil media to dry down somewhat between waterings could replicate the pattern of paper drying which was described above, and potentially increase germination.

Containers should be located where they will experience the full range of daily temperatures, and propagators should be mindful of average daily high and low temperatures to ensure they are not above the "intermediate" range (19°C/11°C). To accomplish this, containers should start outside but could be placed in a greenhouse as temperatures drop below the "spring" range (15°C/8°C), both to protect from occasional freezing temperatures and to potentially extend the germination season. If day length is a significant driver of germination, supplemental light may be required for germination to continue.

## Carex tumulicola

### *Germination Response*

Maximum CATU germination was achieved in the W2-SPR accession (41.5%), although germination was also quite high in W2-INT (37.6%) and W1-SPR (35.2%).

Germination in SPR and INT temperatures increased with one and two months of winter, and then decreased with three and four months. Germination was slightly higher in SPR than INT temps at all cold stratification durations. Summer germination was low with zero or one month of stratification, but increased after two to four months, reaching 20.2% in W4-SUM. Germination reached 15.2% in the winter control.

CATU uniformity showed a strong positive response to cold stratification, increasing with 1-3 months of stratification in both SPR and INT temperatures. The CATU accession with the highest uniformity was W2-INT; 80% of germination was accomplished in 11 days. Other accessions with high uniformity were W1-INT and W3-SPR (both 16 days) and W2-SPR (18 days). When considered in combination with germination percentage, the W2-INT and W2-SPR accessions have both high germination percentage and high uniformity. Propagators will be glad to know that, with proper control of winter stratification and germination temperature, germination will be uniform enough that seedlings can be treated as a group, reducing the time and resources required to produce a genetically diverse crop of CATU.

The CATU germination rate was also positively affected by cold stratification. Time to 50% germination decreased as the duration of stratification increased, regardless of germination temperature. For W2-INT and W2-SPR, which had the highest germination percentage and uniformity, the 50% germination rate was 17-22 days. Based on these data, two months of winter stratification followed by spring or intermediate temperatures will result in quick, uniform, and high yielding CATU germination, with maximum germination around 40%.

The best estimate of initial CATU seed lot viability is 45.3% (accession W1-SPR). Others have documented germination rates around 40% suggesting that this is a reasonable estimate of seed lot viability (Guerrant & Raven, 1998).

Because seed dormancy complicates TZ testing, the effects of winter stratification and germination temperature on seed viability must be interpreted carefully for CATU. Viability appears low for W0-SPR and W0-INT, but likely reflects a conditionally dormant state. One, two, and three months of stratification show decreased dormancy and near maximum viability. According to these data, the decrease in germination percentage in W3-SPR and W3-INT is not due to a decrease in viability or increase in dormancy, supporting the hypothesis that fungi may have played a role in decreasing germination in W3 and W4 accessions. Whether the reduced “viability” in summer and winter is due to dormancy or decreased viability cannot be determined from these data.

### *Seed Dormancy*

CATU seeds were in a conditionally dormant state at the beginning of the experiment. Two months of winter stratification relieved CATU seed dormancy. Additional stratification beyond two months decreased dormancy even more, allowing some level of CATU germination in summer temperatures. As mentioned previously, the loss of dormancy with exposure to cold wet stratification is common in *Carex,* as well as many other species of temperate climates.

### *Germination Temperature*

Of the four temperature ranges that were tested, SPR (15°C/8°C) is the optimum temperature range for CATU germination; germination was always at least slightly higher in SPR than INT temperatures. INT temperatures (19°C/11°C) were suitable for germination in all conditions tested. SUM (24°C/14°C) temperatures stimulated germination after the additional loss of dormancy with 3-4 months of winter stratification. Winter (5°C/2°C), although likely unable to stimulate germination, was able to support germination once dormancy was relieved. The heat shock on the day of the winter incubator malfunction triggered immediate but non-uniform (continuous) germination in the CATU accessions which experienced it. This suggests that CATU could begin to germinate on a warm day in early spring once enough cold days have accumulated to release seeds from dormancy.

### *Implications for* Carex tumulicola *life history*

CATU seeds ripen in September, and are likely in a state of primary conditional dormancy when shed from the mother plant. This dormant state would prevent most seeds from germinating during the first fall. Subsequent winter temperatures release CATU from this dormant state, enabling germination to occur during the following spring. As demonstrated by the winter incubator malfunction, warm winter days may stimulate early germination after dormancy release has occurred. Luckily, germination will likely occur slowly in winter temperatures (as demonstrated by the winter control), allowing CATU to avoid the risk of losing too many germinants to a late frost.

The transition to spring temperatures will happen slowly in most years (compared to the one day transition experienced by seeds in this experiment) so it is difficult to say how uniformly germination will occur in the wild. Regardless of how quickly it occurs, it is likely that germination will occur early in spring and be complete before the canopy fills in with other perennials, an advantage for a species which likely requires ample light to achieve optimum germination. If germination does depend strongly on light availability, CATU will likely take advantage of canopy gaps which can result from fall burning and would still be present in spring.

### *Recommendations for propagation of* Carex tumulicola

Propagators should stratify CATU seeds in a refrigerator at 5°C for the 2 months prior to the target sowing date for maximum control of germination timing. If a lightly protected space is available (such as a hoop house), seeds can be sown in early spring with decreased risk of frost kill. Care should be taken to ensure that daily temperature swings in this growing environment are sufficiently large to stimulate germination (assuming that CATU requires this). If protected space is not available, sowing of stratified seed can be pushed back until around April 1st, the last frost date in Olympia, WA ("Day of the Last Spring Freeze from the 1981-2010 U.S. Climate Normals," n.d.), as near maximum germination will still be achieved in intermediate temperatures. In either case, seeds should be surface sown and exposed to ample light.

Seeds could also be sown directly into containers in fall or mid-winter to receive their stratification requirement outdoors. Although it is uncommon for light to be required during stratification to relieve dormancy (Baskin & Baskin, 2014), this was not tested. Direct sowing outdoors would eliminate the possibility of insufficient dormancy release in a refrigerator.

Restoration professionals should direct sow CATU seed in fall or winter for maximum germination. Seeds should be sown early enough that the full two month stratification requirement is met. Sowing in mid-winter would push spring germination a bit later, reducing the risk of exposure to winter frosts, but also shortening the window of time with ample light (before the canopy fills in) to stimulate germination. The growing season would also be shortened. For more control, seeds could be sown in spring or late spring in areas with low competition after being stratified in a refrigerator.

## CONCLUSION

The response of CAIN and CATU to winter stratification and germination temperature has important implications for nursery personnel and restoration professionals, and provides the baseline for propagation protocols.

Germination percentages were achieved at near maximum levels for all three seed lots. The optimum treatments identified for CATU also increased the uniformity and rate of germination. CAIN germination never achieved uniformity or a fast germination rate. These characteristics of CAIN germination may be improved with additional seed pretreatments. In the next experiment, perigynia removal and the application of smoke are explored as methods to increase the measures of germination described above, as well as seedling vigor.

# SMOKE WATER EXPERIMENT

## BACKGROUND

### Fire and smoke

Native populations traditionally managed Puget Sound prairies through controlled burning (Dunwiddie & Bakker, 2011). Fires were typically set in late summer and early fall, and encouraged the growth of prairie species, many of which were important food crops. Fires also discouraged the encroachment of conifers, which can shade out prairie species and decrease prairie quality. For seeds, fires reduced competition and released nutrients into the soil, providing opportunities for both germination and future growth.

A fire that burns off aboveground biomass changes the environment at the soil surface in a number of ways, including an increase in solar radiation, shifting of the red:far red light ratio, the addition of soil nutrients, increasing fluctuations in water availability, and an increase in the amplitude of daily temperature fluctuations (Nelson, Flematti, Ghisalberti, Dixon, & Smith, 2012). Detection of any of these signals could allow seeds to take advantage of the reduced competition after a fire, but many of these signals would occur with any disturbance that removed the canopy layer. Being able to distinguish a fire from other disturbances might be advantageous, allowing seeds to take advantage of the pulse of nutrients that is unique to this disturbance type.

Indigenous communities in Western Washington, South Africa, and Western Australia have known about the stimulatory effects of fire on plant communities for centuries, but the effects of smoke on germination were not noted in scientific literature until 1990. DeLange & Boucher (1990) found that the application of smoke increased the germination of *Audouinia capitata*, a threatened endemic South African shrub that had proven difficult to propagate from seed. Over the years, the effects of smoke on germination were noted in plants from all over the globe, both those from, and decidedly not from, regions which are frequented by fire (Nelson et al., 2012). It wasn't until 2004 when two groups independently identified the active component in smoke: karrikin (Flematti, Ghisalberti, Dixon, & Trengove, 2004; van Staden, Jager, Light, & Burger, 2004).

Karrikin actually describes a family of compounds. KAR1 was identified first and is the most active stimulator of germination in the karrikin group (Flematti et al., 2004; von Staden et al., 2004). Other germination stimulants have recently been identified in smoke water, but KAR1 remain the most important. Karrikins are universally present in smoke, regardless of the vegetation type burned, and are even present in the smoke of burned filter paper and "Liquid Smoke", a readily available flavoring product (Flematti et al., 2004; Doherty & Cohn, 2000). In addition to

germination stimulants, smoke water also contains compounds which can inhibit germination. Exposing seeds to undiluted smoke water often results in zero percent germination and can even kill seeds (Doherty & Cohn, 2000; van Staden et al., 2004). It is only when smoke water has been sufficiently diluted that the stimulating effects of karrikins can occur.

Smoke water has been shown to enhance germination in the seeds of many species, including Grand Rapids Lettuce (*Lactuca sativa*), rice (*Oryza sativa*), *Arabadopsis thaliana*, and *Brassica tournefortii* (Doherty & Cohn, 2000; Long et al., 2010; Kulkarni et al., 2006; von Staden et al., 2004). The effects of smoke water on germination have been shown to depend on a number of factors. Smoke concentration has already been mentioned, but the state of seed dormancy, imbibition history, duration of exposure to smoke water, smoke water pH, and exposure to light have also been shown to enhance or limit the effects of smoke water on germination.

Imbibition history is relatively straightforward. In *Brassica tournefortii,* seeds which had no history of imbibition showed the greatest sensitivity to smoke water (Long et al., 2010). Germination increased from 0 to 60% with only three minutes of exposure, whereas seeds that had been previously imbibed with water didn't reach 60% germination until 24 hours of smoke water exposure. Imbibition history is not important for all species. In rice, imbibition history did not have an effect on smoke water sensitivity (Doherty & Cohn, 2000).

The duration of exposure to smoke water had an effect on germination percentage in *Brassica tournefortii* and rice (Doherty & Cohn, 2000, Long et al., 2010). These studies both showed that longer exposure times increased germination (some only showed maximum germination after 14 days of exposure). There was no indication that a longer exposure time ever resulted in germination inhibition, however all of these species are fast-germinating, so the longest duration of exposure (which was the full course of the experiment) was only 14 days.

The pH of smoke water solutions used in Long et al. (2010) were found to be highly acidic (pH of 3). Smoke water that was buffered to a more neutral pH had lowered stimulatory effects on germination percentage.

The interacting effects of light and smoke water have been demonstrated in a number of species. In rice and Grand Rapids lettuce, smoke water exposure only has a significant effect on germination when seeds are germinated in darkness (Kulkarni et al., 2006, von Staden et al., 2004).

It is important to note that exposure to smoke water does not relieve dormancy (Baskin & Baskin, 2014). Rather, smoke water is able to enhance germination once dormancy has been relieved. The response of 37 smoke sensitive species of Western Australia has been shown to fluctuate with the seasons (Roche, Dixon, & Pate, 1998).

Producing smoke water requires time and resources that not all propagators will have access too. If Liquid Smoke, which is cheap and available in most grocery stores, can be demonstrated to have the same effect as smoke water on CAIN and CATU germination, smoke treatment may be more accessible for those nurseries.

The concentration of karrikins and germination inhibitors in smoke water and liquid smoke changes from batch to batch (Doherty & Cohn, 2000). Chemical analysis is possible, but smoke is notoriously complex, and it is likely that not all germination stimulants and inhibitors have yet to be identified. Purified or synthesized karrikins are also available, but would add cost to any project, and would omit the potential benefits of additional germination stimulators present in smoke.

The effects of smoke water, if any, might not be detected by an increase in germination percentage. In tomato seeds, exposure to smoke did not enhance germination but did decrease the number of abnormal seedlings and increase the number of lateral roots (Jain and von Staden, 2006). In rice, smoke water enhanced vigor by increasing root and shoot length (Long et al., 2010). Seedling vigor is an important quality that influences the probability that a germinated seed will survive the vulnerable establishment phase.

Based on the extensive literature on the procedural effects of smoke water application and on the prerequisite for dormancy release, a protocol was developed to test the effects of smoke water on CAIN and CATU germination. A pre-trial was also developed to rapidly and cheaply identify appropriate dilutions of smoke water and liquid smoke to test on slow-germinating species. A small proof-of-concept trial was also conducted to assess the effects of smoke on seedling vigor.

### Perigynia removal

All *Carex* seeds are enclosed by a sack of tissue called a perigynia. The size, shape, thickness, and durability of the perigynia varies from species to species. Perigynia removal reduced the time to onset of germination in *Carex stipata* (Hough-Snee & Cooper, 2011). The rate of germination also increased, resulting in a higher final germination percentage. Perigynia removal also increased the germination rate of *Carex nebraskensis* (Hoag, Dumroese, & Sellers, 2001;). Removal of the perigynia for *C. pensylvanica* increased germination from 12% to 32% (McGinnis & Meyer, 2011).

Baskin & Baskin advised that any germination ecology study test seeds in their natural dispersal units (2014). For *Carex*, that includes the perigynia. CATU has a thin papery perigynia that is smaller than the typical mature seed. As the seed grows and ripens, it commonly breaks the perigynia open. Although the perigynia commonly clings to the seed for some time afterwards, it is not uncommon for it to

fall off. It seems unlikely that such a thin and easily shed covering would have significant impacts on the germination of CATU seed.

For CAIN, the perigynia is thick, wrapped tightly around the seed, and has a swollen fleshy portion at the basal end (Figure 11). It does not fall off easily. In fact, manual removal of perigynia from fresh seeds can be quite difficult. Although Baskin & Baskin advise against tampering with seed dispersal units, the goal of this study was to identify ways to increase germination. Because CAIN has such a thick perigynium, it seems likely that its removal could have a significant impact on germination.

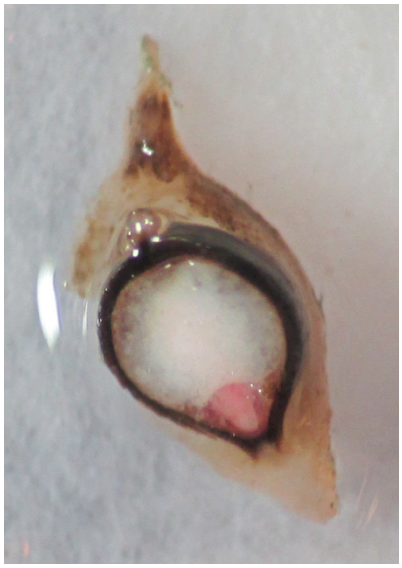

**Figure 13. *Carex inops* ssp. *inops* seed with perigynia. The perigynia (light colored tissue surrounding the dark brown seed coat) was torn slightly when this seed was cut in preparation for TZ testing, but is normally tightly wrapped around the seed. The fleshy base can be seen towards the bottom of the photo.**

The frequency of CAIN perigynia removal in the wild, and the mechanisms by which it would occur, are unknown. The susceptibility of the soft tissue of the perigynia to fungi was observed repeatedly in this experiment; seeds with perigynia intact often had higher rates of fungal infestation that seeds with their perigynia removed. Decomposition of the perigynia by fungus and bacteria could act as a mechanism for perigynia removal.

Herbivory could be another natural mechanism of perigynia removal. During the collection of CAIN at Tenalquot Prairie, ants were occasionally found chewing on the fleshy base of the perigynia when seeds were still attached to the mother plant. If ants regularly seek out and consume CAIN perigynia, this could be another natural method of perigynia removal.

## Summary

This experiment was built upon the first experiment. CAIN and CATU were exposed to the ideal winter stratification and germination temperature regime identified in the first experiment, and given sufficient time to germinate (one month for CATU and three months for CAIN). With dormancy relieved, this experiment focused on the effects of smoke (CAIN and CATU) and perigynia removal (CAIN only) on germination. The experiment described below aims to answer the following questions:

1. Does the application of smoke encourage germination?

2. Is the source of that smoke (whether it be from homemade native smoke water or store-bought Liquid Smoke) important in stimulating germination?
3. Is the concentration of smoke water or liquid smoke important in stimulating germination?
4. Does perigynia removal stimulate germination in CAIN?

The germination response of CAIN and CATU was analyzed by plotting the distribution of germination events over time, and by comparing the percentage, speed, and uniformity of germination between treatments.

## MATERIALS & METHODS

### Materials

Two seed lots, VP-CAIN and CATU, were evaluated in this trial. TQ-CAIN was not included due to the limited quantity of seed; the entire seed lot was used in the previous experiment. This trial began on April 22nd, 2016, 44 weeks after collection for VP-CAIN and 31 weeks after collection for CATU. Seeds were stored at 22°C prior to the start of the experiment.

Smoke treatments were derived from two sources. The first, smoke water (SW), was provided by Carl Elliott, manager of Shotwell's Landing nursery, on April 12th, 2016. This batch of SW was produced on September 25th, 2015 according to the method described in Krock, Smith, Elliott, Kennedy, & Hamman (2016). Native prairie plant chaff was burned over *Quercus garryana* coals and cooled smoke was pulled through DI water by vacuum suction. SW was stored frozen prior to and after pickup.

Liquid smoke (LS), the second smoke source, was obtained on April 17th, 2016 (Wright's Hickory Liquid Smoke, B&G Foods, Parsippany, NJ 07054) and stored frozen after being purchased. The remaining materials in the experiment, including tetrazolium, petri dishes, filter paper, and incubators, were the same as described in the previous experiment.

### Pre-trial to assess smoke water and liquid smoke potency

A pre-trial was designed to assess the potency of SW and LS, with the intention of identifying dilutions which would be appropriate to test on slow-germinating VP-CAIN and CATU. Lettuce (*Lactuca sativa)* was chosen as a test species because seeds are readily available and cheap, and the effects of smoke on lettuce germination have already been documented (van Staden et al., 2004). For lettuce, smoke stimulates germination in the dark, essentially replacing the requirement for light;

these effects have been observed within 24 hours (van Staden et al., 2004). This fast, low cost, and low-tech trial could be repeated by nursery staff in the future to assess changes in SW and LS potency over time, and when new batches are made or purchased.

The pre-trial was conducted one week prior to the full experiment. Lettuce seeds (*Lactuca sativa*, Grand Rapids Lettuce, Non-GMO untreated seed, Lot A, packed for 2016, Lake Valley Seed, Boulder, CO 80303) were obtained from a local farm supply store. SW and LS were thawed to room temperature and mixed thoroughly. A series of dilutions were made from each using DI water (Table 7). 100% SW was the color of weak black tea and 100% LS was the color of strong sun tea. 10% solutions of each still had a tint of color, but the rest were clear. "Smoke" could be smelled down to the 1% solution, and solutions down to at least 0.0001% had a strong to faint "alcohol" smell.

25 lettuce seeds were placed on top of two pieces of dry filter paper per petri dish, and 2.5 ml of solution were applied to each. Dishes were quickly covered, wrapped in aluminum foil to exclude light, and incubated in the dark at 24-29°C for roughly 24 hours. The germinants in each dish were then counted and qualitatively compared for differences in vigor. Three dilutions were identified, based on their stimulation of lettuce germination in this pre-trial, for their potential to stimulate germination in *Carex* (Table 7, in bold). For full results of the pre-trial, see Appendix 10.

**Table 7. Dilutions of smoke water and liquid smoke assessed in the lettuce seed pretrial. Dilutions in bold were chosen for testing on CAIN and CATU, based on their stimulation of lettuce seeds in the pre-trial.**

| Dilutions Tested | Percentage SW or LS (%) |
|---|---|
| Full strength | 100 |
| 1:10 | 10 |
| 1:100 | 1 |
| 1:1000 | 0.1 |
| **1:10000** | **0.01** |
| 1:100000 | 0.001 |
| **1:1000000** | **0.0001** |
| 1:10000000 | 0.00001 |
| **1:100000000** | **0.000001** |
| 1:1000000000 | 0.0000001 |
| Control | 0 |

### Experimental Design

Seven treatments were chosen for testing on both VP-CAIN and CATU (Table 8). This included a DI-water only control and three dilutions of SW and LS (all prepared

with DI water). One additional accession, in which the perigynia was removed (called NP, for "No Perigynia"), was also tested on VP-CAIN. The NP accession was not repeated on CATU because its perigynia are so thin and fragile that they commonly fall off of mature seeds.

**Table 8. Descriptions of the accessions tested in the smoke experiment. Dilutions of SW and LS were made with DI water. *The "no perigynia" treatment was only tested on VP-CAIN.**

| Accession Code | Smoke Source | Concentration (%) | Concentration Code | Perigynia |
|---|---|---|---|---|
| Control | - | 0 | - | intact |
| NP* | - | 0 | - | removed |
| SW-A | SW | 0.01 | A | intact |
| SW-B | SW | 0.0001 | B | intact |
| SW-C | SW | 0.000001 | C | intact |
| LS-A | LS | 0.01 | A | intact |
| LS-B | LS | 0.0001 | B | intact |
| LS-C | LS | 0.000001 | C | intact |

Dry VP-CAIN and CATU seeds were mixed thoroughly and then separated into 14 groups of 200 (7 groups per species). Perigynia were removed from one additional group of VP-CAIN by rubbing dry seeds with 80 grit sandpaper in a 35mm soil sieve. Crushed seeds (there were a few) were removed. Each group was then placed in a 10" x 10" piece of mesh screen (no-see-um netting) and tied shut with a rubber band.

200ml of each SW and LS dilution and the DI water only control (Table 8) were prepared and placed in open-topped glass jars. Mesh bags were clipped to the inside of the jars in order to fully submerge seeds. Each jar contained two bags (one from each species) except the DI water only control, which contained three bags (VP-CAIN-Control, VP-CAIN-NP, and CATU-Control). Two 4-valve aquarium bubblers were used to aerate these solutions while the seeds imbibed at room temperature. See Appendix 9 for photos of this set-up.

Mesh bags were removed 56 hours later, quickly rinsed with tap water, swirled in a 10% concentrated bleach solution for 60 seconds, and then rinsed again in tap water for 10 seconds. Seeds were then divided into groups of 50, placed on two fresh pieces of filter paper (with 50 seeds per dish), watered with DI water, and placed in either the intermediate (VP-CAIN) or winter incubator (CATU). CATU was moved to the intermediate incubator 8 weeks later. Dishes were checked three times a week; germinants were tallied and removed, and DI water was applied when necessary.

### Vigor Experimental Set-up

Assessment of vigor took place during the smoke experiment, starting on week 6. A sub-sample of germinants was collected each week and seedling growth was monitored closely. To obtain this sub-sample, seeds were checked as usual on Mondays. One additional day of evaluation was added on Tuesdays, which allowed us to identify seeds that had germinated within the last 24 hours. Germinants found on Tuesdays (following the same definition mentioned previously, with a cotyledon and a radicle, both being large enough to be identifiable), were removed and placed on a slant board in the intermediate growth chamber (details of the slant board set-up can be found in Appendix 13). Fully germinated seeds which had partially germinated prior to this date were included in the sub-sample, but their status as a partial germinant was noted.

Cotyledon and radicle length were measured with an electronic caliper (Mitutoyo Absolute Digimatic, Model #CD-6"CSX) as they were removed from petri dishes (Day 0, Tuesdays), roughly 24 hours later (Day 1, Wednesdays), two days after that (Day 3, Fridays), and three days after that (the following Day 6, Mondays), for a total of seven days. Slant boards are designed to encourage straight radicle and cotyledon growth, however our *Carex* did not always grow straight. We made our best effort to measure the total root and shoot length, despite these curves. Length was measured to the closest 0.5mm. Seeds were discarded after their final meaurements to make room for the next week's vigor sample.

### TZ Testing

After 12 weeks of incubation, ungerminated seeds were TZ tested according to the protocol described in the previous experiment. A batch of 200 seeds from each seed lot was also TZ tested at the beginning of the smoke experiment to establish a baseline rate of seed lot viability.

### Analyses

Germination counts, vigor measurements (root and shoot growth), and TZ data were compiled in a relational database (Microsoft Access, 2013). Data were then processed further in R (version 3.3.1). The distribution of germination events over time was plotted in the same way as the first experiment, both as the number of germinants per day and cumulative germination percentage over time. Three metrics were calculated to describe the response of VP-CAIN and CATU: germination percentage, uniformity, and germination rate. Definitions for these metrics are the same as those explained in the stratification experiment. The percentage of viable seeds was also calculated for each seed lot and accession.

## RESULTS

### Percent Germination

VP-CAIN germination ranged from 26.5% to 39.7% ( Figure 14). The VP-CAIN-Control germinated at 28.8% and the highest concentrations of SW and LS (0.01%) germinated at similar levels. As the concentration of smoke decreased, germination increased. SW-B and SW-C both achieved roughly 35% germination, and the highest germinating accession was LS-C, at 39.7%. The VP-CAIN-NP accession also experienced increased germination compared to the control. At 38.7%, it did nearly as well as the best germinating smoke-treated accession (LS-C). None of the treatment conditions decreased germination substantially from that achieved by the control. Rather, the treatment conditions either increased or had neutral effects on germination percentage.

Germination percentage appeared to be more strongly affected by smoke concentration than smoke source, although a slightly higher germination percentage was achieved by LS than SW. Also, perigynia removal and the lowest concentrations of SW and LS had similarly large effects on germination.

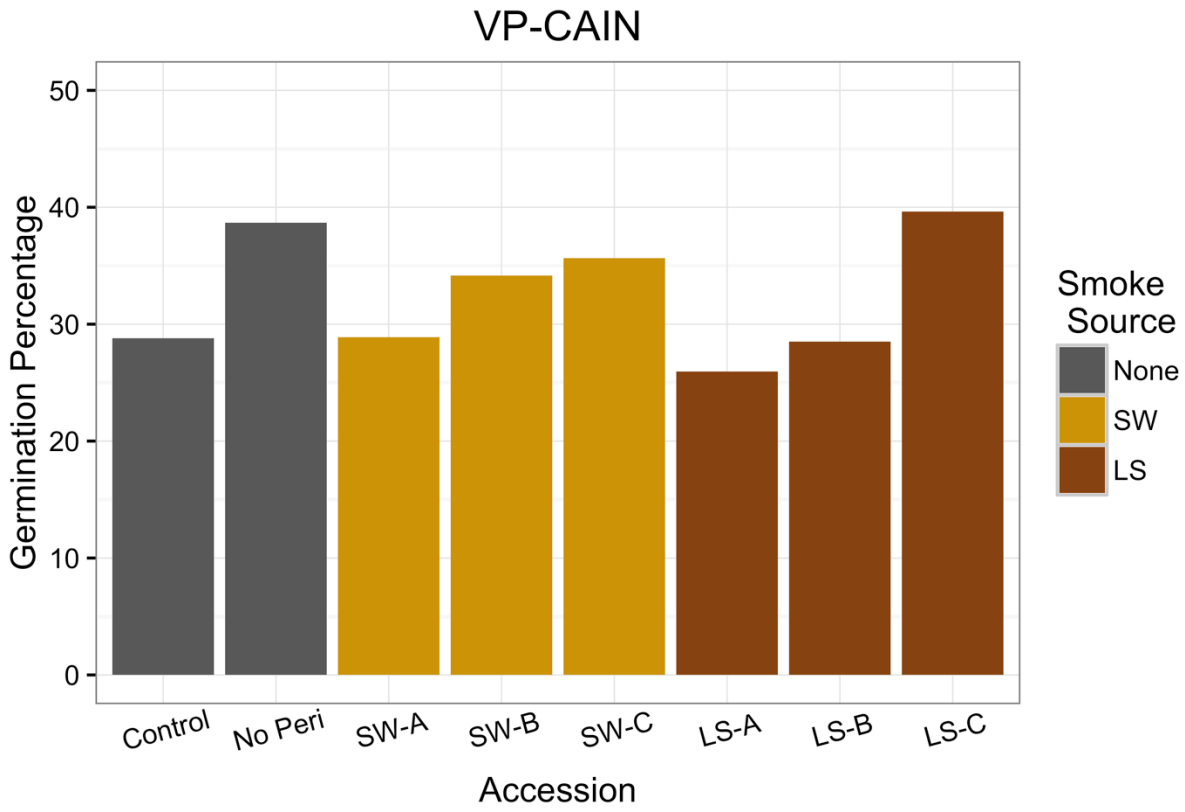


**Figure 14. Final germination percentages of VP-CAIN with smoke treatment and perigynia removal. Bars represent the mean final germination percentage for the four dishes in each accession.**

CATU showed a similar response to smoke application (Figure 15). None of the treatments decreased germination percentage; increases were observed with both SW and LS. Germination ranged from 26.6% to 38.2%. The DI-water only control was the lowest germinating accession. A slight but likely insignificant increase was observed in the high concentration SW and LS accessions (SW-A and LS-A). The biggest increase in germination was in SW-B and SW-C (both germinating at roughly 37%) and LS-B (38.2%). As was the case with VP-CAIN, smoke concentration appeared to have a bigger effect on germination percentage than smoke source, as both smoke sources achieved germination percentages within the same range (30-38%).

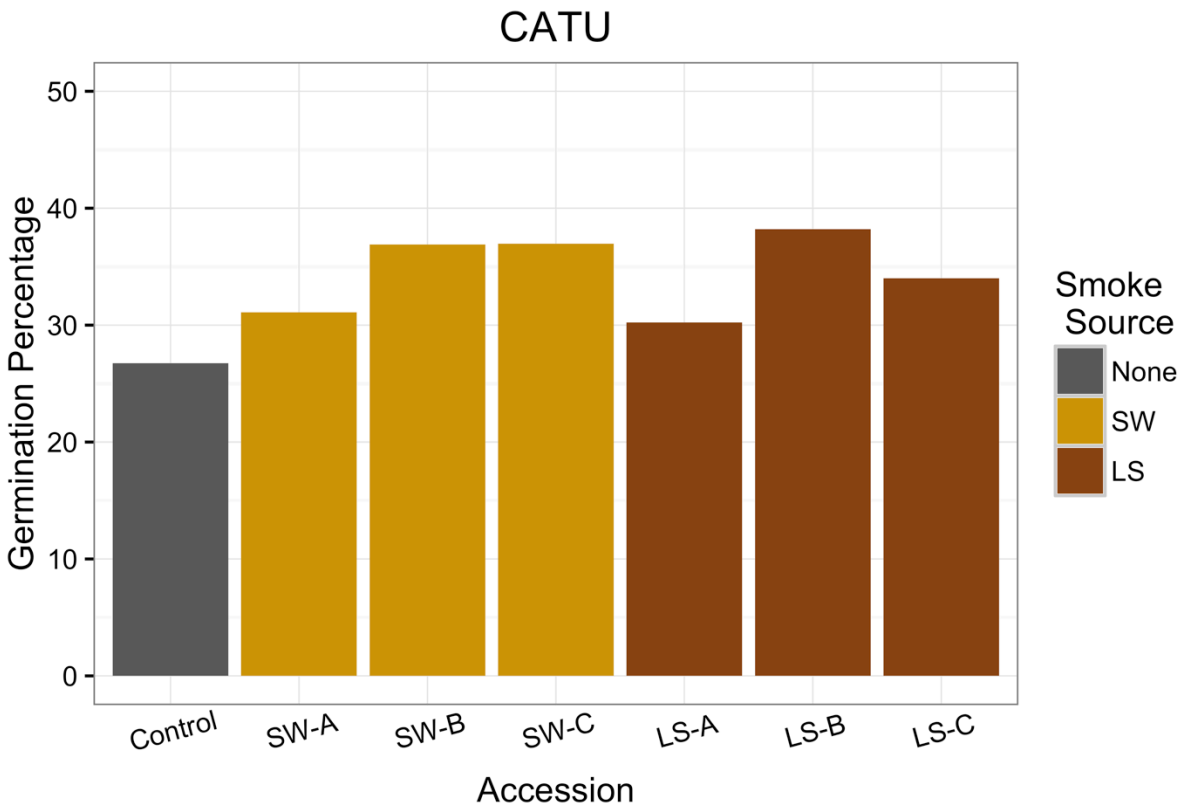


**Figure 15. Final germination percentages of CATU after smoke treatment. Bars represent the mean final germination percentage for the four dishes in each accession. Bar color indicates smoke source.**

### Germination Timing

For VP-CAIN, all accessions showed a similar distribution of germination events over time (Figure 16). Germination began after roughly 28 days, coming on slowly at first, and then increasing during the third month (after 56 days). For a few accessions, germination appeared to be slightly bimodal, with a higher density of germination events around the midpoint of month two and towards the end of

month three, although no clear germination peak existed for any of these accessions. No accessions appear to have completed germinating by the end of the experiment.

For CATU, all accessions had a relatively normal, if not slightly right skewed, distribution of germination events over time (Figure 16). The onset and peak of germination appear to have occurred at roughly the same time for all accessions. Germination may not be complete, but the majority of the initial germination peak appears to have reached its end for all CATU accessions.

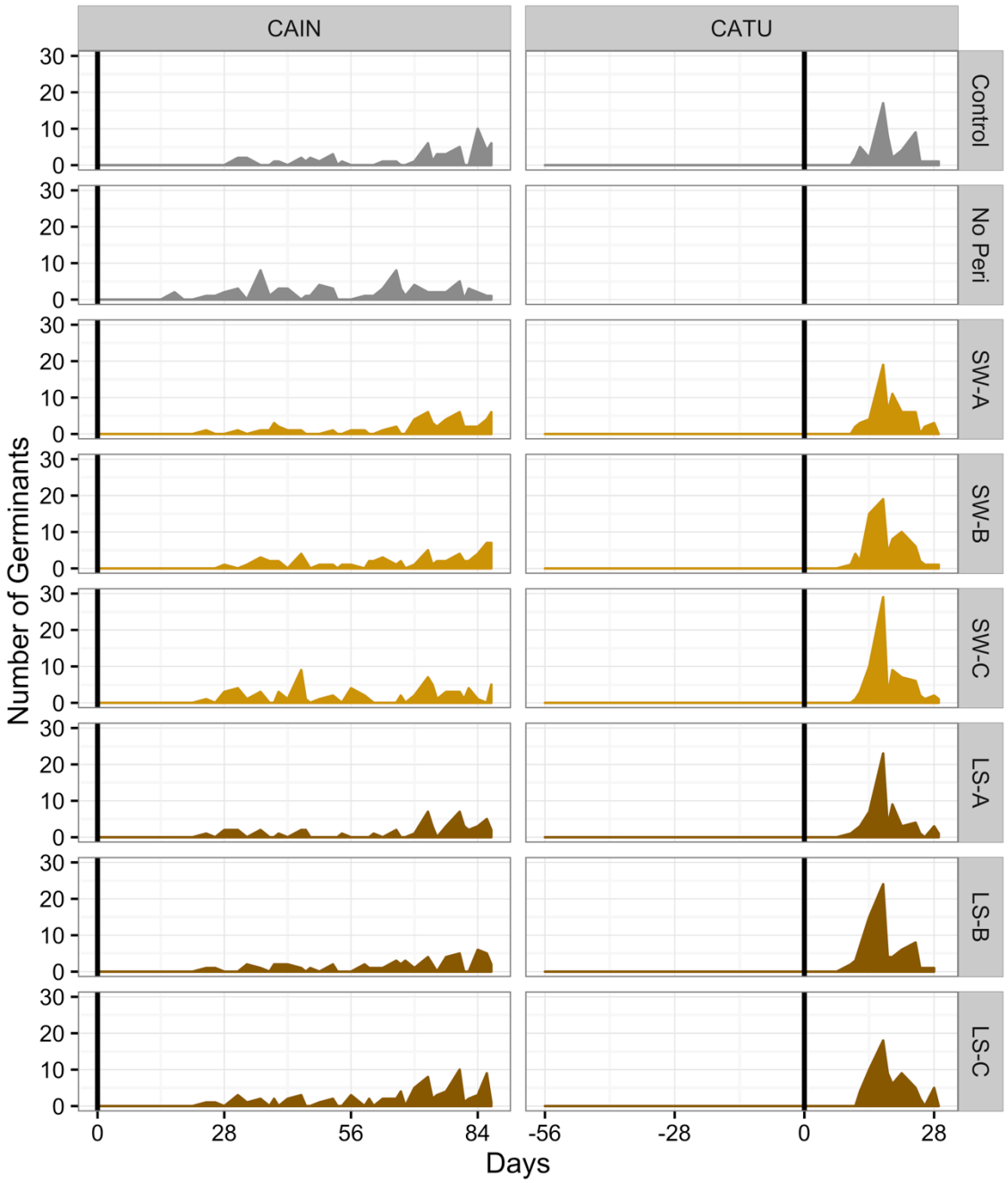


**Figure 16. Distribution of germination events over time for VP-CAIN and CATU in the smoke experiment. The bar at day zero represents the day that the accession was placed in the intermediate incubator. The number of germinants is the sum of the germinants from all four dishes on each day.**

Figures 17 and 18 give us a different view of the timing of germination. For VP-CAIN, the NP accession started germinating first (Figure 17). SW-C started at roughly the same time as the rest of the accessions, but at a faster rate, quickly catching up with NP. These two accessions continued to germinate at a steady rate until the end of the experiment. LS-C started slowly but picked up speed around day 70, catching up to the germination percentage of NP and SW-C by the end. The other accessions fell into two groups. SW-A, LS-A, and the Control slowed down quite a bit

around day 50, and then increased their germination rate substantially around day 70, mirroring the germination rate of NP, SW-C, and LS-C. SW-B and LS-B, on the other hand, maintained a steady rate of germination from onset until the end. Despite these subtle differences in germination rate, all of these slower accessions reached similar final germination percentages.

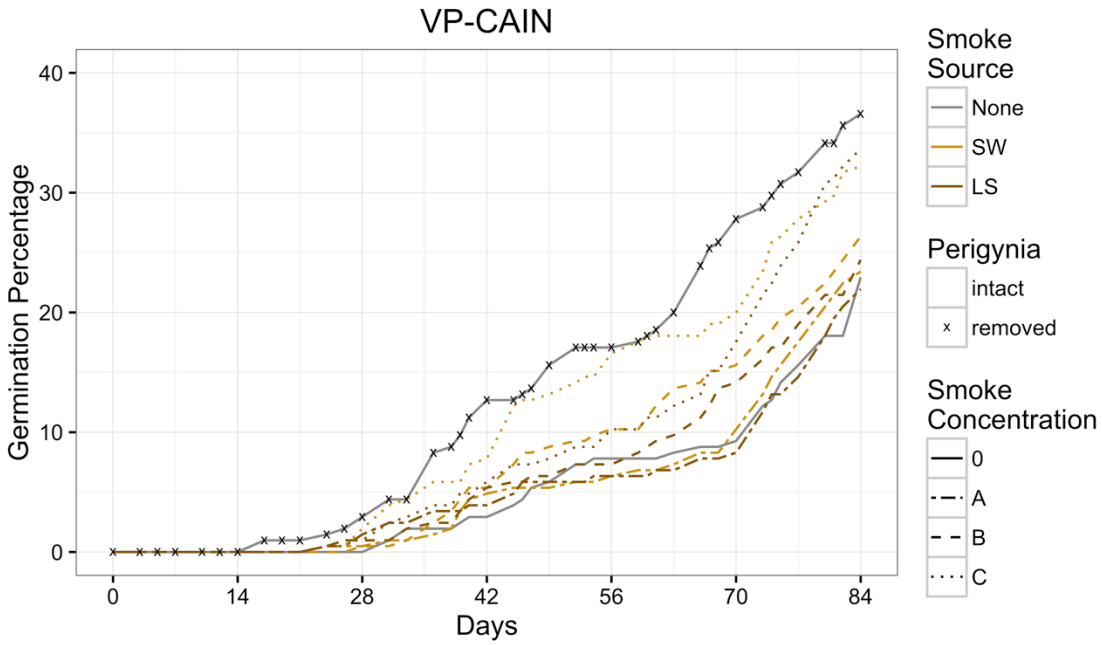


**Figure 17. Cumulative germination percentage for VP-CAIN accessions after smoke treatment and perigynia removal. Lines represent sum of all seeds germinated per accession prior to and including that day, divided by the total number of seeds per accession.**

Differences in absolute germination percentage differed substantially during the course of the experiment. At 56 days, accessions occupied a 10% range in absolute germination percentage (with SW-A and LS-A at roughly 7% and NP and SW-C at roughly 17%). At 70 days, that grew to a 20% range in absolute germination percentage (from 8% to 28%).

The number of days it took to reach absolute germination thresholds like 10% and 20% also differed substantially between accessions. NP reached 10% germination first, at around 39 days, followed by SW-C at 44 days. The rest of the accessions didn't reach 10% germination until 54-72 days, a full 2-5 weeks after NP. NP also reached 20% germination first (at 63 days) followed by SW-C (70 days) and LS-C (72 days). The rest of the accessions reached 20% germination at 77-82 days.

For CATU, germination appeared to have three phases, indicated by the curves in Figure 18. Between days 7-14, differences in early germination rate established an 8-9% range in absolute germination percentage. From 14-17 days, all accessions proceeded at roughly the same germination rate (indicated by equal slopes) and germination for all accessions was rapid. After 17 days, germination started to slow, but at different times and rates for each accession. LS-B, SW-B, and SW-C

maintained a faster rate of germination through the end, resulting in a higher final germination percentage.

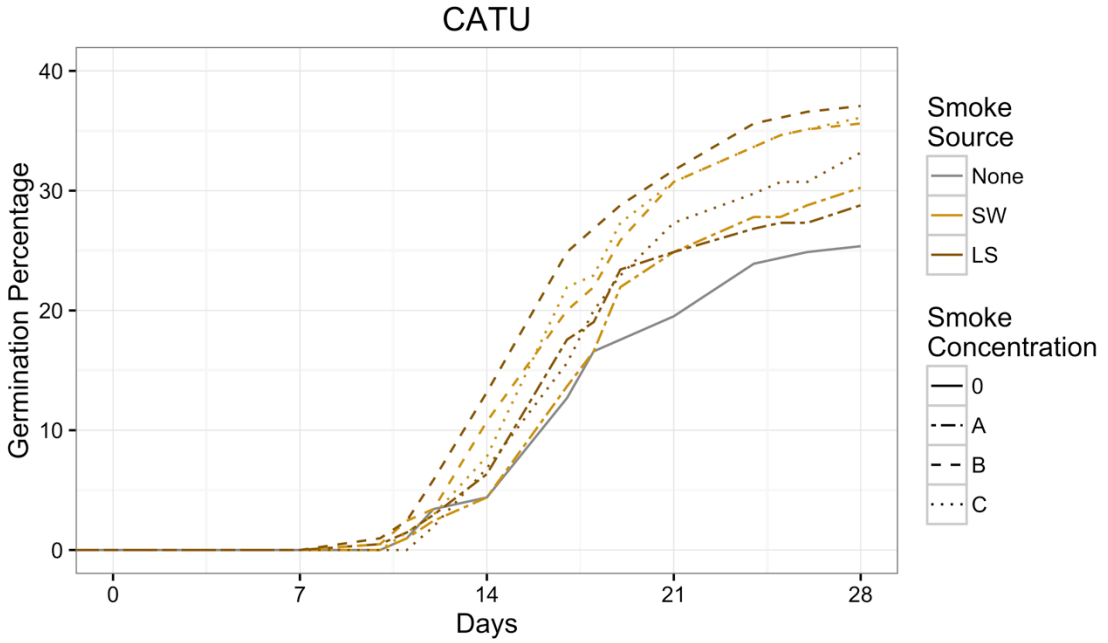


**Figure 18. Cumulative germination percentage for CATU accessions after smoke treatment. Lines represent sum of all seeds germinated per accession prior to and including that day, divided by the total number of seeds per accession.**

The range in absolute germination percentage differed at times for CATU accessions, but never substantially more than the 12% range in germination percentage at the end of the experiment. Differences in the number of days to germination thresholds like 20% and 25% were smaller than that observed for VP-CAIN. All accessions reached 20% absolute germination within a 6 day range (16-22 days), and 25% absolute germination within an 11 day range (17-28 days).

### Days to 50% Germination

Time to 50% (relative) germination ranged from 56-76 days (8-11 weeks) for VP-CAIN (Figure 19). The fastest times to 50% germination were in the NP and SW-C accessions (at 8 and 8.5 weeks), confirming the pattern observed in Figure 17. The rest of the accessions were all in the 10-11 week range, and were likely not significantly different from each other. These times are all slower than that observed for the VP-CAIN-W0-INT accession in the first experiment, which reached 50% germination in 42 days (6 weeks).

Smoke did not have an effect on days to 50% germination for CATU (Figure 19). Results were extremely uniform, as all accessions reached 50% germination in 17-18 days. CATU-W2-INT (from the first experiment) also reached 50% germination in 17 days.

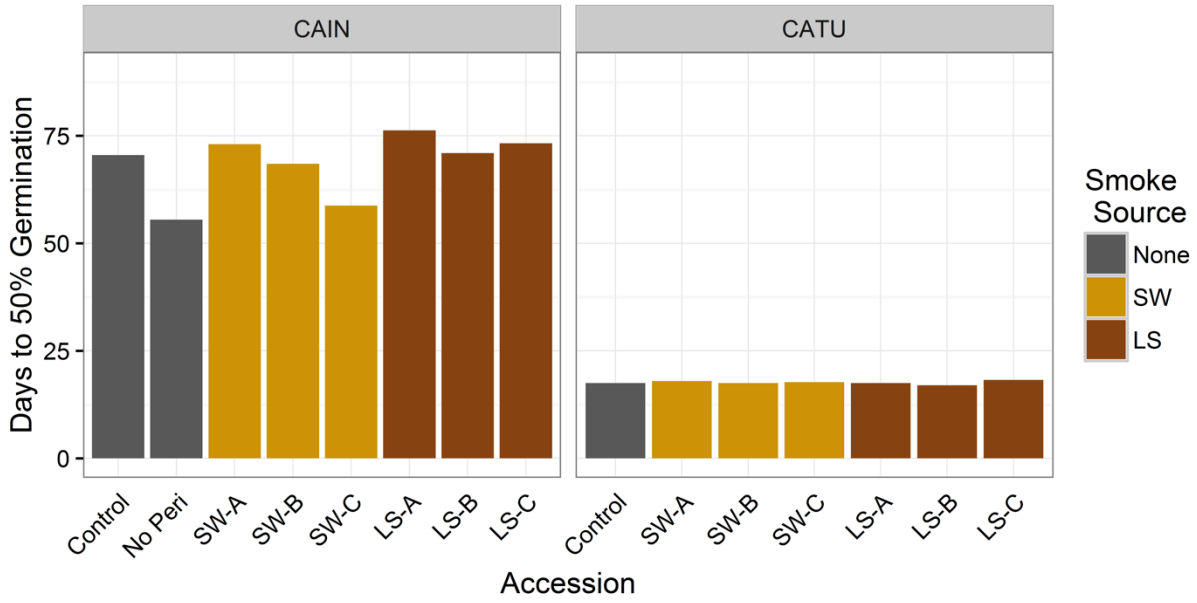


**Figure 19. Germination rate (days to 50% germination) for VP-CAIN and CATU with smoke treatment and perigynia removal. Bars represent the mean number of days to 50% of final germination percentage (the germination midpoint) for the four dishes in each accession.**

## Uniformity

VP-CAIN Uniformity ranged from 39-48 days (5.5-7 weeks) (Figure 20). The highest uniformity was observed in the Control and SW-A accessions, but all accessions were within this 1.5 week range, which is likely not large enough to be meaningful to nursery professionals. The increased uniformity in the Control and SW-A accessions is likely because these accessions had a delayed start to germination, so the time from start (10% germination) to finish (90% germination) was truncated. All accessions were slightly more uniform (1-2.5 weeks) than the VP-CAIN-W0-INT accession from the first experiment at 54 days (8 weeks).

CATU accessions all showed high uniformity, ranging from 10-12 days to complete 80% of observed germination (Figure 20). Uniformity was not affected by smoke application, source, or concentration. CATU-W2-INT from the first experiment had the same value for uniformity- 11 days.

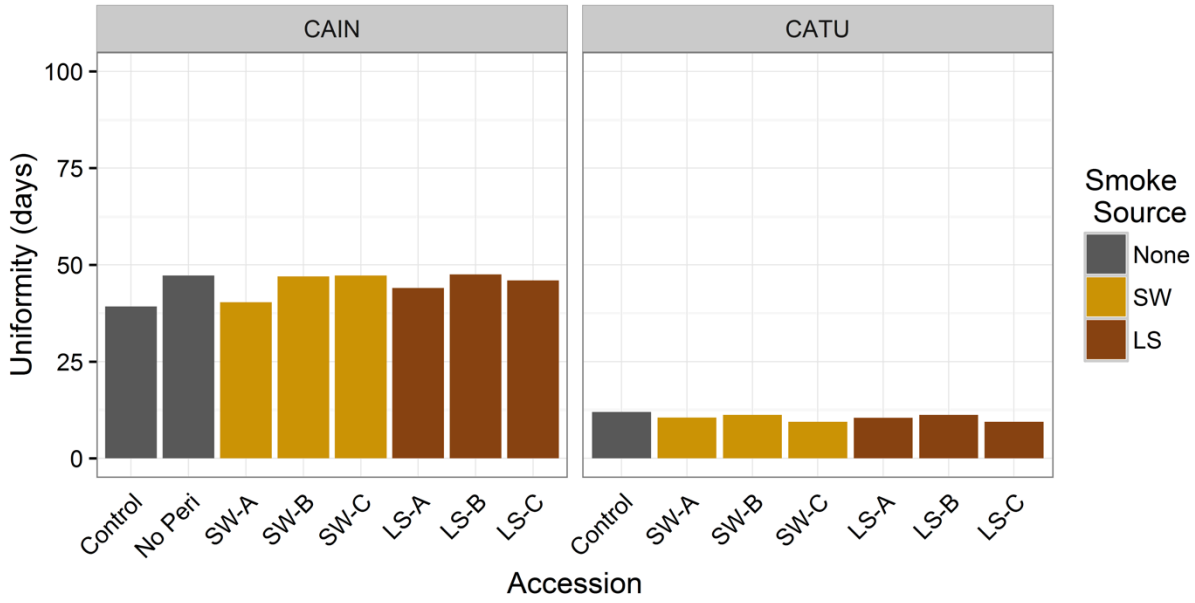


**Figure 20. Uniformity in days for VP-CAIN and CATU after smoke treatment and perigynia removal. Bars represent the mean number of days from 10% to 90% of final germination percentage for the four dishes in each accession.**

### Viability

Viability of VP-CAIN accessions at the end of the experiment varied quite a bit, ranging from 41.0%-56.3% (Figure 21). The Control, NP, and SW-B accessions all had high final viability, between 53.5-56.3%. For SW-A and LS-A viability was much lower, at 41.0 and 41.4%.

Almost all smoke-treated accessions had reduced viability compared to the Control and NP accessions. Smoke source did not affect viability, as both SW and LS resulted in a similar range in final viability (41.0-53.5% for SW and 41.4-49.7% for LS). Smoke concentration did appear to affect viability; the highest concentrations (A) for both SW and LS had a roughly 15% reduction in viability compared to the Control.

For each accession, a significant proportion of seeds remained ungerminated at the end of the experiment. The control had the highest proportion, with nearly half of viable seeds remaining. SW-A had the lowest proportion, but still had nearly one third of viable seeds remaining ungerminated at the end of the experiment.

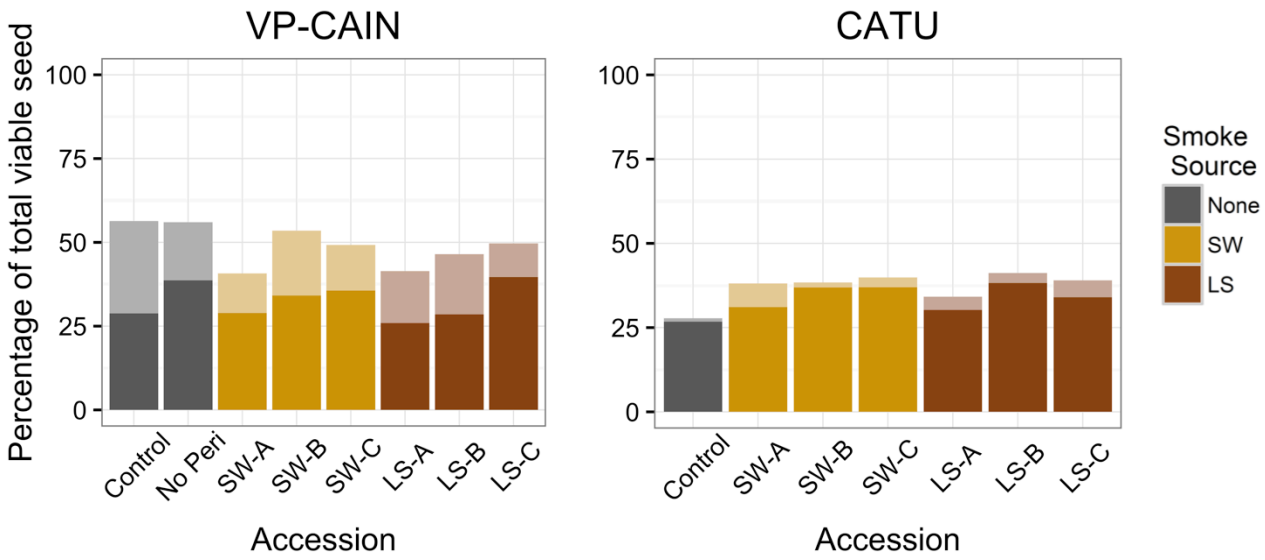


**Figure 21. Comparison of germination percentage to final viability of VP-CAIN and CATU accessions at the end of the smoke experiment. The lower (full saturation) portion of each bar represents the mean final germination percentage for the four dishes in each accession. The upper (half tone) bar represents the mean of the additional viable seeds identified in TZ testing. Each full bar represents the total viable seed at the end of testing for each accession.**

Final CATU viability was relatively uniform (Figure 21). The Control had the lowest viability at 27.7%, followed by LS-A, which was 34.2% viable. All other accessions were between 38-41% viable. Most viable seeds germinated. The accessions with the largest proportion of ungerminated viable seeds were SW-A and LS-C. For these two accessions, the lower germination percentage (compared to SW-B, SW-C, and LS-B) cannot be attributed to reduced viability.

Results of initial TZ testing of VP-CAIN and CATU are presented in Table 9. As was the case in the previous experiment, results from initial TZ testing did not predict the total percentage of viable seeds at the end of this experiment.

**Table 9. Seed lot viability for VP-CAIN and CATU at the beginning of the smoke experiment.**

| Seed lot | Initial TZ testing results | Highest recorded final seed lot viability | Best estimate of initial seed lot viability |
|---|---|---|---|
| VP-CAIN | 38.3% | 56.3% | 56.3% |
| CATU | 8.0% | 41.2% | 41.2% |

In this experiment, initial TZ testing determined that VP-CAIN viability was 38.3%, whereas the final TZ test (when considered with germination data) identified two accessions (NP and Control) with total viability around 56% (Table 9). For CATU, accessions had between 27.7%-41.2% total viable seed at the end of this experiment, whereas initial TZ testing determined CATU was only 8.0% viable. The accessions with the highest final viability were used here as the best estimate of initial seed lot viability.

These final viability estimates are similar to those from the first experiment, where VP-CAIN was estimated to be 59.7% viable, and CATU 45.3% viable. Interestingly, the initial TZ test results for VP-CAIN were also similar, with 45.1% from the first experiment and 38.3% here. Initial TZ testing was not completed for CATU in the first experiment, but would likely have been less than 10%.

### Vigor

Seeds were subsampled and measured for the vigor experiment from week 6-12. Data are available from this trial, but have been omitted here. One observation that was made during this trial is that the average CAIN seedling is much longer than the average CATU seedling (after 6 days in the slant-board setup). CATU seeds were usually around 1 cm long after 6 days, and rarely longer than an inch. CAIN seedlings often approached 2-3 inches in length by day 6. Nursery staff may be happy to hear that, if given adequate moisture and ideal temperatures, CAIN is likely to thrive once it germinates. Alternatively, the lower vigor observed in CATU may help explain the poor germination percentages to date. Perhaps seeds are germinating, but failing to become established due to their low seed vigor (at least relative to CAIN).

## DISCUSSION

### Carex inops ssp. inops

#### *Germination Response*

For VP-CAIN, the highest germination percentages were recorded for NP, SW-C and LS-C. Differences in absolute germination percentage between the different accessions were substantial after 42 days, and especially after 56 days. NP and SW-C had higher absolute germination than all other accessions from day 35-70, the bulk of the experiment. LS-C caught up in the end with a late increase in germination rate.

NP and SW-C had the fastest time to 50% germination, due to both the earlier onset of germination and the faster rate of germination maintained from start to finish, illustrated in Figure 17. NP and SW-C also had the poorest uniformity of all accessions. Control and SW-A had the highest uniformity.

All VP-CAIN accessions would likely have continued to germinate if the experiment had not been terminated at 12 weeks. For accessions which are still actively germinating, the date range used to calculate uniformity is necessarily affected by

the termination of the experiment. This metric may not be the best way to quantify differences between accessions for VP-CAIN. For example, accessions with the highest uniformity (Control and SW-A) also had the lowest final germination percentage, due to the delayed onset of germination. Time to 50% germination captures both the onset of germination and the distribution of germination events over time in a way that, when paired with final germination percentage, allows accessions to be compared in a meaningful way. Absolute germination and the number of days to germination thresholds, determined from germination curves like those in Figure 17 and Figure 18 also give valuable insight.

For VP-CAIN and other species with indeterminate germination (where germination occurs slowly over an extended period of time), earlier onset of germination and a faster rate of germination (early on) are desirable traits. The importance of these metrics is amplified when you consider that not all growing seasons will have 84 days of ideal temperature and moisture. For VP-CAIN, speeding up the timing of germination may be the best way to increase the final germination percentage. If seasonal conditions were fully under the control of nursery personnel, this might not be the case, but the reality of nursery propagation and field planting is that seasons change, and temperature, light, and moisture availability change with them. The duration of "ideal" conditions will always be limited. In this experiment NP, SW-C, and LS-C all had high final germination percentages, but NP and SW-C are the more desirable treatments because of high germination percentages and rates throughout.

Viability of CAIN accessions ranged from 41.0%-56.3%. The Control, NP, and SW-B accessions had the highest viability; SW-A and LS-A had the lowest viability. Viability appeared to be negatively affected by the application of smoke; almost all smoke-treated accessions had reduced viability compared to the Control and NP accessions. Smoke source does not appear to matter, as both SW and LS resulted in a similar range in final viability. Smoke concentration, on the other hand, does appear to matter, as the high concentration accessions (SW-A and LS-A) had the lowest viability for both SW and LS. These data suggest that the application of smoke could both stimulate germination and reduce viability, although the effects may differ depending on concentration.

Regardless of the effects of SW and LS on viability, all accessions had ungerminated seeds remaining at the end of the experiment. In the first experiment, VP-CAIN-W0-INT had a final viability of 59.7% and a final germination percentage of 53.7%. The Control accession in this experiment had a final viability of 56.3% and a final germination percentage of 28.8%. The application of smoke appeared to close the gap in seed germination and viability, but at least part of that was due to a reduction in viability.

### *Smoke source and concentration*

Smoke source (SW vs. LS) did not affect final germination percentage for VP-CAIN. Both sources had a neutral to positive effect that resulted in an additional 0%-7% (SW) or 0%-11% (LS) increase in final germination percentage. Smoke source did have an affect on the timing of germination. SW stimulated earlier germination than LS, especially for the most dilute (C ) concentrations.

The concentration of SW and LS cannot be directly compared because the concentrations of the active components (karrikins, other germination stimulants, and germination inhibitors) are unknown. However, within each smoke source, concentration is important. For LS, the lowest concentration resulted in the highest germination percentage. For SW, the lowest concentration improved both germination percentage and timing. The positive response of VP-CAIN to smoke suggests that CAIN may utilize this signal in the wild to identify the occurrence of a fire. The importance of smoke concentration suggests that fire intensity or distance from fire may affect the response of CAIN germination.

Krock et al. (2016) tested the effects of smoke water on *Carex inops* ssp. *inops* seeds that were collected from the same prairies as VP-CAIN. Smoke water, produced in exactly the same manner as the smoke water used in this experiment (but from a previous batch), was diluted to 1:100 with DI water. Seeds were imbibed for 24 hours on filter paper and then incubated at 15°C/6°C with 12 hours of daylight (on that same filter paper) for six weeks. Less than 2% of seeds germinated. Besides the longer exposure to smoke water (on the smoke water soaked filter paper), the biggest difference between these two experiments was smoke concentration.

In the lettuce pre-trial conducted at the beginning of this experiment, a 1:100 dilution of SW was strong enough to inhibit lettuce germination relative to the control (Appendix 10). In fact, three out of four samples treated with 1:100 SW showed no germination at all, and 1:1000 SW treated lettuce seeds had reduced germination in two out of four samples. No two batches of homemade smoke water will ever be the same, but if the composition and concentration of these two batches was similar, a 1:100 dilution may have been strong enough to inhibit germination. CAIN in Krock et al. (2016) was not subjected to a TZ test after germination testing, so dormancy or reduced seed viability could also explain these differences (the seed was older and stored under variable conditions).

Replicating the positive effects of smoke water on germination may be difficult. Concentration is important, and no two batches of SW will be the same. Repeating and refining the lettuce pretrial may help professionals identify appropriate dilutions of future batches of smoke water. The smoke water used in this experiment could also be tested directly for the concentration of karrikins and known germination inhibitors, to provide a reference for future batches.

### *Perigynia Removal*

Perigynia removal resulted in earlier and faster germination compared to the Control accession. Final germination percentage increased by 10%, and at earlier points in time the NP accession more than doubled the Control in terms of germination percentage. Perigynia removal shows promise as a seed pre-treatment, especially in the context of propagating VP-CAIN outdoors where the growing season may be shorter than the 84 days tested here.

The mechanism for increasing germination with perigynia removal is unknown. The perigynia could contain germination inhibitors, which no longer have an effect on the seed once removed. Perigynia removal could also increase exposure to light, as has been suggested by others (Hoag et al., 2001; McGinnis & Meyer, 2011). Perigyia removal could also reduce exposure to fungi. The NP accession appeared to host far less fungi than the Control and other accessions with the perigynia intact.

It should be noted that the perigynia could have a positive effect on seed viability or germination in a more natural setting. Seeds in this experiment always had access to water, but in a less controlled environment, moisture availability would likely fluctuate quite a bit with changes in temperature, humidity, and precipitation. The perigynia swells visibly when imbibed with water, especially in the large fleshy portion at the basal end. This tissue could act as a water storage device, moderating the effects of fluctuating environmental conditions. In a nursery, where water can be controlled, this may not matter. In the wild (and when seeds are direct sown on the soil surface), this may be an important function of the perigynia. Alternatively, the increased exposure to fluctuations in moisture availability could be the mechanism for stimulating germination, rather than increased light exposure.

### *After-ripening and warm dry storage*

The effects of after-ripening on seed viability and dormancy were not directly tested in this experiment, but due to the time that elapsed between experiments, some informal observations can be made.

VP-CAIN seed was harvested in May and stored warm (22°C) and dry before and between experiments. Testing for the first experiment began in September and VP-CAIN responded well to SPR and INT temperatures with zero months of prior stratification. The timing of this experiment corresponded well with the conditions VP-CAIN would have experienced in the wild. Seeds would ripen and fall to the ground sometime in May or June and then have an extended period of warm and dry conditions during the summer months, allowing for a natural period of after-ripening. Fall rains would start in September, exposing seeds to the conditions represented by the INT incubator. Germination would likely begin and continue

until temperatures got cool enough or the days got short enough to inhibit germination.

Seeds were not tested for dormancy at the time of collection, so the role of the first 19 weeks of after-ripening cannot be determined from this experiment. It appears likely, however, that after-ripening is important for reducing CAIN dormancy. Seeds that ripen in spring and germinate in fall often require exposure to high summer temperatures, and this can often be accomplished by storing seeds dry at room temperature (Baskin & Baskin, 2014).

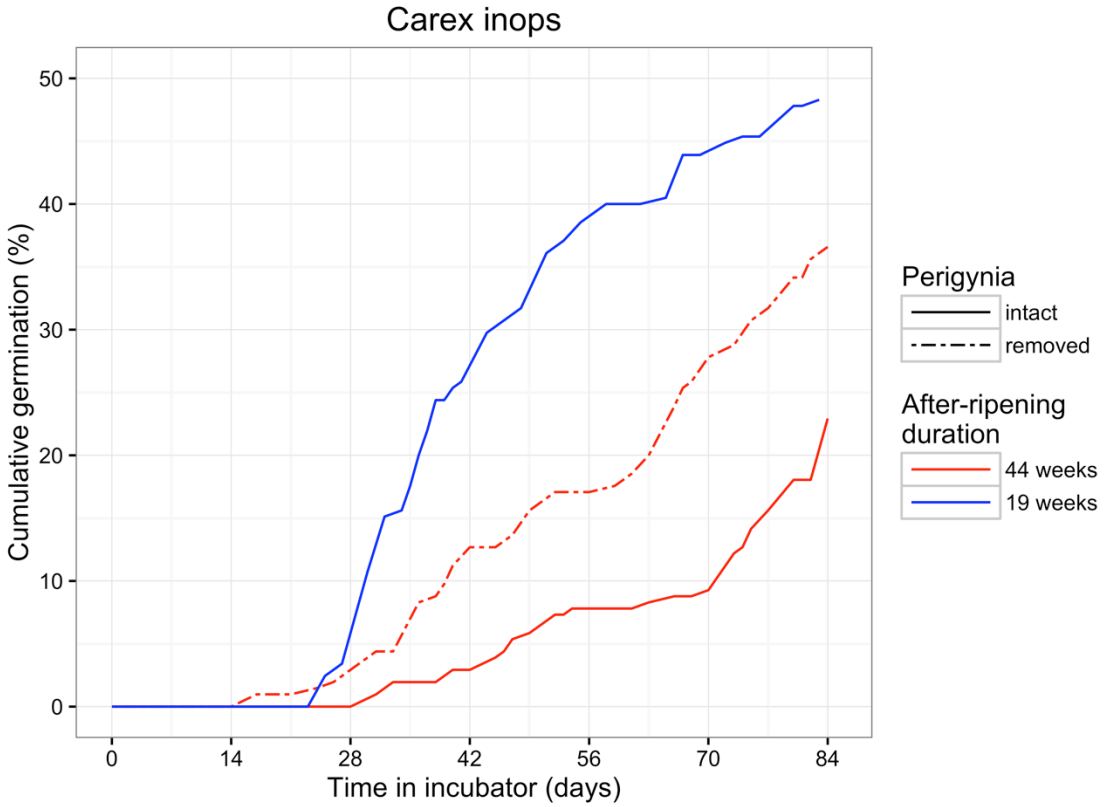


**Figure 22. Effects of after-ripening and warm dry storage on CAIN germination. The W0-INT control from the first experiment is shown in blue. This accession received 19 weeks of warm dry storage prior to being placed in the intermediate incubator. The red lines represent the Control (solid) and NP (dashed) accessions from the second experiment, which both received 44 weeks of warm dry storage prior to being placed in the intermediate incubator.**

In the second experiment, all accessions were given the same conditions identified as most favorable in the first experiment (no winter stratification and germination in the INT incubator for three months). The only difference was the additional 25 weeks of warm dry storage. Germination of the Control accession in the second experiment was significantly lower than VP-CAIN-W0-INT (Figure 22). For example, at 56 days, the Control accession still had not reached 10% germination, while VP-CAIN-W0-INT was at nearly 40%. Perigynia removal seemed to close this gap to some degree, but the NP accession still had substantially lower germination

than VP-CAIN-W0-INT throughout the 84-day germination period. Seed lot viability was similar at the start and end of both experiments, so the difference in germination percentage cannot be explained by a reduction in viability. Rather, these data suggest that the extended period of after-ripening contributed to an increase in dormancy.

These data demonstrate that the duration of warm dry storage is important for CAIN. Insufficient warm dry storage could be one reason why propagators struggled with low CAIN germination in the past, but longer than ideal warm dry storage may be able to induce secondary dormancy. Future work should focus on dry storage time and temperature as an additional mechanism for controlling dormancy in CAIN.

### *Implications for the natural history of Carex inops ssp. inops*

The positive response of VP-CAIN to smoke water indicates that CAIN may show an increase in germination following a fire. Faster rates of germination could help CAIN take advantage of opportunities for reduced competition. For a fall-germinator, speeding up germination is especially important, as plants would have time to become more established before winter.

The intensity and proximity of the burn may affect the germination response, due to the importance of smoke concentration. The sensitivity of CAIN to smoke concentration may be another way that seeds are retained in the soil seed bank. Smoke deposition in the soil would likely not be uniform across the landscape following a fire.

The ecological role of perigynia removal is less clear. Without knowing how perigynia are naturally removed from seeds, it is hard to say when this signal might be important. The possibility that a perigynium could inhibit germination until it decomposes, delaying germination for a year or more, supports the hypothesis that CAIN is well adapted to bank seeds in the soil.

### *Recommendations for propagation of* Carex inops *ssp.* inops

Although perigynia removal and smoke water application were not tested in combination, using both pretreatments could result in even faster germination rates and higher germination percentages. The signals could also be redundant, but it is unlikely that they would result in poorer germination if used together. In the interest of maximizing germination, these pretreatments should be used together.

If perigynia removal is to be attempted on a large scale, a more efficient removal process will have to be developed. During exploratory seed cleaning trials,

perigynia were easier to loosen from seeds after seeds had been pre-moistened in water for roughly one hour. Rubbing moistened seeds with a rubber kitchen gripper on a #35 soil sieve loosened perigynia from seeds more easily than if seeds were dry. Future imbibation times will need to be considerably longer than one hour to achieve the desired effects of smoke water on germination, and this extended imbibition will likely soften up the seed coat as well as the perigynia. Care should be taken not to crush seeds during the perigynia removal process.

Each year, freshly harvested seeds should be allowed to after-ripen for roughly 19 weeks (until mid September) at 22°C. Dry seeds should then be imbibed in smoke water that has been diluted to approximately 1:100,000,000 with DI water. If a different (or old) batch of smoke water is being used, a lettuce pretrial should be conducted first to identify an appropriate dilution to use on CAIN- one of the weakest dilutions that shows increased germination compared to the control should be used. Seeds should imbibe in this solution for up to 56 hours with proper aeration. After imbibing, perigynia should be removed and rinsed from seeds. Seeds should then be sown on the soil surface as described previously. A more detailed protocol can be found in the next section.

### *Future directions*

Future work on the germination ecology of CAIN should look into the role of after-ripening on seed dormancy and germination. Identifying the ideal length of time in warm dry storage prior to imbibing would be a useful addition to propagation protocols.

The effects of combining perigynia removal and smoke water as seed pretreatments would also be useful to assess in a controlled environment. If these pre-treatments are redundant, perigynia removal may be the more reliable way to stimulate germination, if an easy method can be developed for removing perigynia. If the effects are additive, it will likely be worth the effort to do both.

The effects of perigynia removal on germination in a less controlled environment would also be worth exploring, especially if seed production efforts reach the point where direct sowing at restoration sites becomes common practice. The role of this structure could be to protect the seed or to delay germination. Identifying which is the case in a field setting would clarify seed pre-treatments in direct sowing applications.

## Carex tumulicola

### *Germination Response*

For CATU, the highest germination percentages were recorded for SW-B, SW-C, and LS-B. Germination was not reduced by any smoke treatment. Differences in absolute germination percentage before the end of the experiment were never substantially larger than the 12% range in final germination percentage.

There was very little variation between accessions in terms of germination timing. All accessions reached their germination midpoint (days to 50% germination) in 17-18 days and the bulk of germination (measured by uniformity) occurred within a 10-12 day range. Because germination occurred rapidly for all accessions, germination in the nursery or restoration site will likely not be limited by the duration of the spring season. Unlike VP-CAIN, germination rate during active germination is not important for CATU.

Most smoke-treated accessions had a final viability around 38-41% except for LS-A at 34.2%. The Control accession had a final viability of 27.7%. This was unexpected. All seeds received two months of winter stratification, which should have relieved dormancy equally for all accessions. Because all accessions were in the same non-dormant state, dormancy shouldn't have affected TZ results (or at least all accessions should have been affected equally). Any remaining viable but lightly dormant seeds should have been detected in TZ testing. These data suggest that this treatment reduced viability in the Control accession, but it is difficult to envision how the application of DI water in conditions ideal for germination (the INT incubator) could result in reduced viability.

Vivrette & Meyr (2002) suggest that poor staining in deeply dormant seeds is due to slow metabolism. Perhaps the mechanism of smoke stimulation is to increase seed metabolism, both encouraging germination and increasing the ability of TZ to detect ungerminated viable seeds. This could explain why LS-A also had lower final viability- germination inhibitors were too concentrated to stimulate seed metabolism. The other possible explanation is that the Control and LS-A accessions having fewer viable seeds to begin with due to random chance. Regardless of this complication, it appears that the application of SW and LS at the tested concentrations does not reduce CATU viability. Most of the viable seeds germinated during the 28 day incubation.

### *Smoke source and concentration*

CATU responded positively to the application of smoke, demonstrated by an increase in final germination percentage. The concentration of smoke was

important for CATU. As with VP-CAIN, the highest concentration of SW and LS (A), had a neutral effect on germination. The more diluted concentrations (B and C) had a positive effect. For SW, equally high germination was achieved by concentrations B and C, suggesting that SW can achieve these higher gains in germination percentage as long as SW is diluted to at least 1:1,000,000.

Smoke source (SW vs. LS) did not affect final germination percentage for CATU. Both sources increased germination to roughly the same degree, resulting in an additional 3-11% compared to the control. The application of smoke did not have an effect on germination timing, regardless of source or concentration. These data suggest that the benefits of smoke to CATU germination may be achieved through the application of either SW or LS, as long as they are sufficiently diluted.

### *Warm dry storage*

Again, warm dry storage was not directly tested in this experiment, but informal observations can be made due to the timing of the first and second experiment. The Control accession germinated to a lower percentage here than the CATU-W2-INT accession in the first experiment (27.7% vs 41.5%), despite receiving identical pre-treatments (Figure 23). Only the highest germinating accessions in the second experiment approached this level of germination. Seed lot viability was similar at the end of the each experiment, so reduced viability is not responsible for the reduction in germination percentage.

Extended warm dry storage did not appear to have an effect on the timing of CATU germination. The onset, midpoint, and uniformity of germination appear to be unchanged between CATU-W2-INT and the accessions in this experiment.

Reduced germination is likely due to the additional 27 weeks spent in warm dry storage. Evidence from the first experiment suggests that CATU germinates in the spring. If CATU does not germinate in its first spring and is to avoid germination in subsequent fall seasons, when temperature and moisture are similar to spring conditions, secondary dormancy would be required to prevent germination. It would not be uncommon for this state of dormancy to build during summer months. Burial experiments have shown that dormancy increases in *Carex elongata, Carex remota*, and *Carex canescens* during summer, not being relieved until sufficient winter stratification has occurred (Schutz, 1997). The same simulated summer (long period of warm dry storage) that likely reduced dormancy in CAIN prior to the first experiment may have increased dormancy for CATU. Because CATU germination suffered from extended warm dry storage, seeds should be stored cold and dry. Cold dry storage maximizes long-term viability for most orthodox seeds (Baskin & Baskin, 2014).

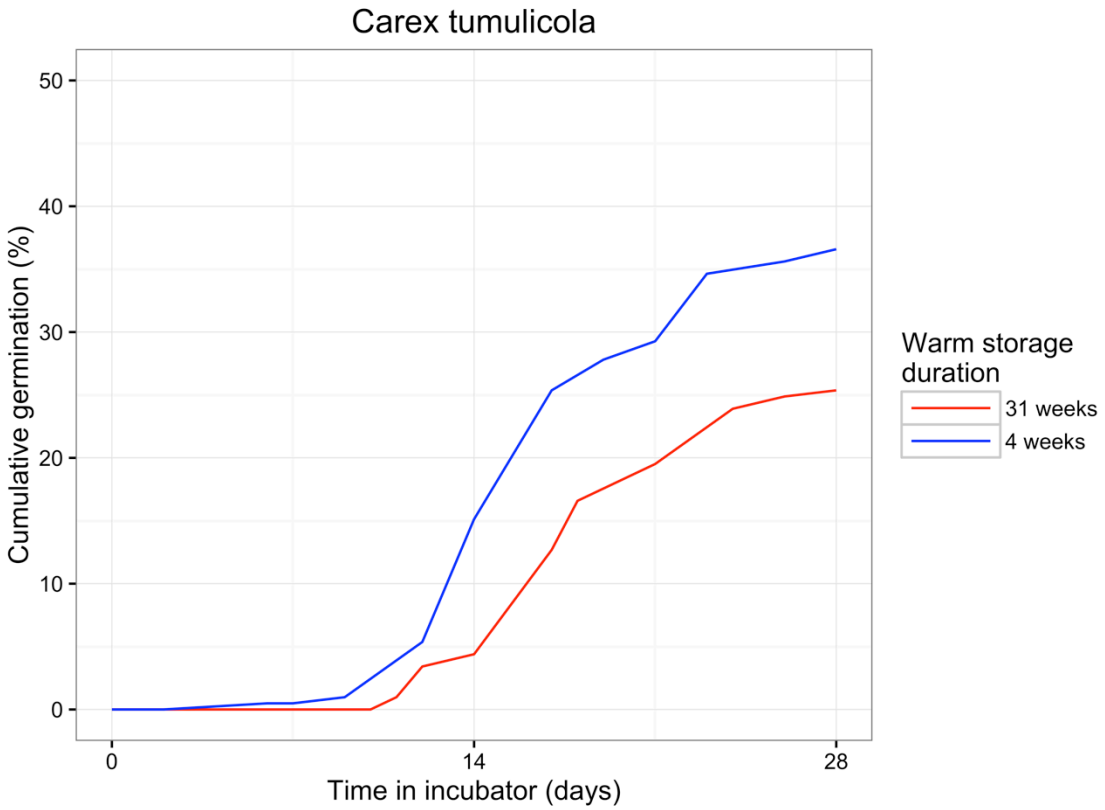


**Figure 23. Effects of time in warm dry storage on CATU germination. The W2-INT accession from the first experiment is shown in blue. The Control accession from the second experiment is shown in red.**

The role of the 4 weeks of warm dry storage prior to the beginning of the first experiment is unknown. Published and unpublished protocols for CATU do not list after-ripening as an important seed pre-treatment (Bartow, 2004; Guerrant & Raven, 1998). It is unlikely that this period of storage had a large effect on the dormancy or viability of CATU, but future work could clarify this.

### *Implications for* Carex tumulicola *life history*

For CATU, germination isn't limited by time, and germination timing doesn't change with treatment as long as dormancy has been relieved. Seeds germinate rapidly and uniformly after sufficient winter stratification with or without smoke application.

Smoke does have the potential to improve germination success by increasing germination percentage. In this experiment, smoke improved germination percentage relative to the Control. However, the Control reached a much lower germination percentage than CATU-W2-INT from the first experiment, and no accession, regardless of smoke treatment, exceeded CATU-W2-INT. The implications of this are unclear. When CATU seeds are sown at the appropriate time of year (in spring after two months of winter stratification), smoke water imbibition

may not have a detectable effect on germination percentage, especially in the nursery where light, temperature, and water can be maintained within ideal ranges.

The positive effects of appropriately diluted smoke water may not come into play until conditions are less than ideal for stimulating germination (i.e. when dormancy has been relieved but other germination cues are absent). Smoke water stimulates *Lactuca sativa* germination in the dark (van Staden et al., 2004). If CATU has a light requirement, a recent fire could stimulate germination in buried seeds. Smoke could also replace the need for other disturbance signals like high-amplitude daily temperature fluctuations or an altered red:far red light ratio. The existence of these relationships are only hypothesized, and will need to be confirmed with future testing.

### *Recommendations for* Carex tumulicola *propagation*

Seeds should be allowed to after-ripen for no more than 4 weeks. If seeds are not sown right away, they should be put into cold dry storage. Before cold moist stratification (whether seeds are stratified outdoors or in a refrigerator), seeds should be imbibed with smoke. The concentration of SW or LS should be confirmed with a lettuce pretrial prior to imbibing. If time and cost are limiting, LS can be used as the smoke source, and should be diluted to 1:1,000,000 (or the equivalent dilution of future batches of LS). If SW is readily available, it should be used as the smoke source, and diluted to between 1:1,000,000 and 1:100,000,000 (again, after confirming the concentration with a lettuce pre-trial). Although both smoke sources can stimulate germination, CATU may be less sensitive to differences in concentration for SW. This flexibility may be advantageous when new batches of SW are produced. After imbibing, seeds should be stratified as described previously. A complete protocol follows in the next section.

### *Future directions*

This experiment demonstrated that smoke can have a positive effect on CATU germination, but that those effects may be mediated by other factors. Future work should explore the relationship between smoke and other signals of disturbance. It would be helpful to clarify the role of after-ripening in CATU germination and dormancy. Finally, a burial experiment would identify the annual dormancy cycle of CATU in the wild, and confirm or deny the pattern of spring germination and summer/fall dormancy proposed here.

## CONCLUSION

Seeds in this experiment demonstrated a much narrower range of responses to treatment conditions than in the first experiment, because all accessions (within each species) were in an equal state of reduced dormancy. Differences in germination response, where present, were due to the ability of the treatment condition to inhibit or stimulate germination. Smoke is a promising pre-treatment for increasing the timing and rate of CAIN germination. Perigynia removal is also a potentially useful treatment for CAIN. For CATU, smoke may be able to stimulate germination when conditions are less than ideal. After-ripening or warm dry storage may have important effects on reducing or inducing seed dormancy for both species. Future work will be required to clarify these relationships, and the context in which they are important.

## PROPAGATION PROTOCOLS

Propagation protocols for *Carex inops* ssp. *inops* and *Carex tumulicola* are described below. These protocols are based on the literature reviewed for this paper, data collected and analyzed in these experiments, and informal observations made along the way. The conditions in a nursery or at a restoration site are very different than those in a germination chamber, and these protocols will surely need to be adapted and refined. Hopefully they at least provide a useful starting point for future propagation efforts.

### *Carex inops* ssp. *inops*

1. Allow seeds to after-ripen for roughly 19 weeks after harvest at room temperature (22°C). Do not place them in cold storage. Plan for a sowing date in mid September.
2. Prepare a 1:100,000,000 dilution of thawed and well-mixed smoke water prepared from native vegetation per the protocol described by Krock et al. (2016) and deionized water.
3. If a new or aging batch of smoke water is to be used, conduct a lettuce pre-trial first to determine the correct dilution of smoke water. Follow this protocol:

    a. Thaw and thoroughly mix smoke water.

    b. Remove 10 ml of 100% smoke water and place in a clean container. Label as 100% smoke water.

    c. Remove 1 ml of 100% smoke water and place in a new container. Add 9ml of water (DI water if available). Mix well. Label as 10% smoke water.

    d. Repeat step c for each dilution and label appropriately. Make at least 8-10 dilutions, each 10-fold weaker than the one before it.

    e. Also prepare and label a water only control.

    f. Prepare 3 petri dishes for each dilution, each with 25 Grand Rapids lettuce seeds on top of two pieces of dry filter paper. Label each dish.

    g. Aliquot 2.5ml of the appropriate solution onto the filter paper for each dish. Quickly cover the dish with aluminum foil to exclude light. The effects of smoke on lettuce germination can only be observed if seeds

are germinated in the dark. Exposing imbibed seeds to light will compromise this trial.

h. Once all dishes have been prepared, incubate them at 25°C for 24 hours.

i. Cover and refrigerate the leftover dilutions.

j. 24 hours later, remove all dishes from the incubator and count germinants. Compare the number of germinants for each dilution to the water only control. For CAIN, choose one of the more diluted solutions that still shows increased germination compared to the control.

k. Use the appropriate leftover solution to make your chosen dilution for imbibing CAIN. For example, if you want to make 1000ml of a 1:100,000,000 dilution, take 1ml of the 1:100,000 solution and add 999 ml of (DI) water. Mix well.

4. Imbibe seeds in this solution for 56 hours with proper aeration (using an aquarium bubbler is a cheap and easy way to accomplish this).

5. Remove seeds from the solution and gently rub with sandpaper or some other abrasive surface to remove perigynia.

6. Sow seeds on the soil surface.

7. Place containers outside of protective structures in early fall, where they will be exposed to maximum sunlight and daily temperature fluctuations. Move containers into a hoop house later in the season, as temperatures start to fall below the 15°C/8°C ("spring") range. Protect seeds from predators.

8. Keep media moist, but not overly wet.

9. If multiple germinants occur in a cone-tainer, don't hesitate to transplant seedlings into empty cells. They transplant with a high rate of survival.

## *Carex tumulicola*

1. Allow seeds to after-ripen for up to 4 weeks, then place in cold dry storage.

2. In early February, prepare a 1:1,000,000 dilution of well-mixed smoke water prepared from native vegetation per the protocol described by Krock et al. (2016) and deionized water.

3. If a new or aging batch of smoke water is to be used, conduct a lettuce pre-trial first to determine the correct dilution of smoke water. Follow this protocol:

    a. Thaw and thoroughly mix smoke water.

    b. Remove 10 ml of 100% smoke water and place in a clean container. Label as 100% smoke water.

    c. Remove 1 ml of 100% smoke water and place in a new container. Add 9ml of water (DI water if available). Mix well. Label as 10% smoke water.

    d. Repeat step c for each dilution and label appropriately. Make at least 8-10 dilutions, each 10-fold weaker than the one before it.

    e. Also prepare and label a water only control.

    f. Prepare 3 petri dishes for each dilution, each with 25 Grand Rapids lettuce seeds on top of two pieces of dry filter paper. Label each dish.

    g. Aliquot 2.5ml of the appropriate solution onto the filter paper for each dish. Quickly cover the dish with aluminum foil to exclude light. The effects of smoke on lettuce germination can only be observed if seeds are germinated in the dark. Exposing imbibed seeds to light will compromise this trial.

    h. Once all dishes have been prepared, incubate them at 25°C for 24 hours.

    i. Cover and refrigerate the leftover dilutions.

    j. 24 hours later, remove all dishes from the incubator and count germinants. Compare the number of germinants for each dilution to the water only control. For CATU, choose a dilution is in the middle of the range of dilutions that show increased germination compared to the control.

    k. Use the appropriate leftover solution to make your chosen dilution for imbibing CATU. For example, if you want to make 100ml of a

1:1,000,000 dilution, take 1ml of the 1:10,000 solution and add 99 ml of (DI) water. Mix well.

4. Imbibe seeds in this solution for 56 hours with proper aeration (using an aquarium bubbler is a cheap and easy way to accomplish this).
5. Place seeds in cold moist stratification for 8 weeks. Monitor for fungi and early germination.
6. In early April, sow seeds on the soil surface.
7. Place containers outdoors where seeds will be exposed to maximum sunlight and daily temperature fluctuations. Protect seeds from predators.
8. Keep media moist, but not overly wet.
9. If multiple germinants occur in a cone-tainer, don't hesitate to transplant seedlings into empty cells. They transplant with a high rate of survival.

## CONCLUSION

This project has identified basic information about the dormancy and germination requirements of *Carex inops* ssp. *inops* and *Carex tumulicola*. Two potentially beneficial seed pre-treatments, smoke water and perigynia removal, were also tested. This work brought up many new questions about the dormancy and germination of these species that have yet to be answered. My hope is that this project will provide a useful starting point for future research, and enough information in the meantime that nurseries can begin to produce these species in desired quantities.

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# APPENDIX

## Appendix 1. Germination data sheet

| *accession code* | Day 1:<br>Days in Winter:<br>Days in _____: | Total % Germination:<br>% Live Seed: | 1 |
|---|---|---|---|

| Day # | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Date<br># seeds | | | | | | | | | | | | | | | | | | | | | | | | |
| **1** | | | | | | | | | | | | | | | | | | | | | | | | |
| **2** | | | | | | | | | | | | | | | | | | | | | | | | |
| **3** | | | | | | | | | | | | | | | | | | | | | | | | |
| **4** | | | | | | | | | | | | | | | | | | | | | | | | |
| Total Germ | | | | | | | | | | | | | | | | | | | | | | | | |

| Day # | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Date<br># seeds | | | | | | | | | | | | | | | | | | | | | | | | |
| **1** | | | | | | | | | | | | | | | | | | | | | | | | |
| **2** | | | | | | | | | | | | | | | | | | | | | | | | |
| **3** | | | | | | | | | | | | | | | | | | | | | | | | |
| **4** | | | | | | | | | | | | | | | | | | | | | | | | |
| Total Germ | | | | | | | | | | | | | | | | | | | | | | | | |

| *accession code* | Day 1:<br>Days in Winter:<br>Days in _____: | Total % Germination:<br>% Live Seed: | 2 |
|---|---|---|---|

| Day # | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Date<br># seeds | | | | | | | | | | | | | | | | | | | | | | | | |
| **1** | | | | | | | | | | | | | | | | | | | | | | | | |
| **2** | | | | | | | | | | | | | | | | | | | | | | | | |
| **3** | | | | | | | | | | | | | | | | | | | | | | | | |
| **4** | | | | | | | | | | | | | | | | | | | | | | | | |
| Total Germ | | | | | | | | | | | | | | | | | | | | | | | | |

| Day # | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Date<br># seeds | | | | | | | | | | | | | | Total Germ |
| **1** | | | | | | | | | | | | | | |
| **2** | | | | | | | | | | | | | | |
| **3** | | | | | | | | | | | | | | |
| **4** | | | | | | | | | | | | | | |
| Total Germ | | | | | | | | | | | | | | |

TZ *Start* Date and Time: *Stop* Date and Time:
TZ Evaluation Date:

| Lot # | #Seeds Tested | Viable | 50:50 | Non-viable | Oth |
|---|---|---|---|---|---|
| 1 | | | | | |
| 2 | | | | | |
| 3 | | | | | |
| 4 | | | | | |
| Total | | | | | |

## Appendix 2. Results from winter stratification and germination temperature experiment: Germination percentage

Final germination percentage for each accession. Percentages are the mean of percent germination for the four dishes in each accession (number of seeds germinated/total number of seeds). Columns represent the number of months initially spent in winter stratification. Rows represent the germination temperature which followed stratification. Accessions shaded in grey were affected by the winter incubator malfunction, which may have increased the final percent germination. Combinations of winter stratification and germination temperature in dark grey were not tested. Note that for the control accessions (W0-SPR, W0-INT, W0-SUM, and WIN) these data represent germination percentages for the full seven months of incubation.

| **VP-CAIN** | 0 | 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|---|
| spring | 48.0% | 30.3% | 22.1% | 20.6% | 28.8% | |
| intermediate | 53.7% | 29.4% | 24.5% | 7.1% | 6.0% | |
| summer | 9.5% | 9.5% | 10.0% | 6.0% | 6.6% | |
| winter | | | | | | 10.0% |

| **TQ-CAIN** | 0 | 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|---|
| spring | 60.0% | 40.6% | 31.1% | 26.1% | 24.0% | |
| intermediate | 57.3% | 29.1% | 11.7% | 7.1% | 10.6% | |
| summer | 11.7% | 9.5% | 4.9% | 9.3% | 11.6% | |
| winter | | | | | | 7.2% |

| **CATU** | 0 | 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|---|
| spring | 17.6% | 35.2% | 41.5% | 30.7% | 32.5% | |
| intermediate | 10.4% | 26.8% | 37.6% | 27.3% | 21.2% | |
| summer | 0.5% | 1.0% | 5.0% | 15.5% | 20.2% | |
| winter | | | | | | 15.2% |

## Appendix 3. Results from winter stratification and germination temperature experiment: Uniformity

Uniformity of germination for each accession. Values represent the mean number of days elapsed from 10% to 90% of final germination percentage. Uniformity was not calculated for accessions with less than 20% final germination. Columns represent the number of months initially spent in winter stratification. Rows represent the germination temperature which followed stratification. Accessions shaded in grey were affected by the winter incubator malfunction, which may have decreased uniformity. Combinations of winter stratification and germination temperature in dark grey were not tested. Note that for the control accessions (W0-SPR, W0-INT, W0-SUM, and WIN) these data represent uniformity for the full seven months of incubation.

| **VP-CAIN** | 0 | 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|---|
| spring | 93 | 48 | 58 | 54 | 65 | |
| intermediate | 54 | 46 | 48 | 42 | - | |
| summer | - | - | - | - | - | |
| winter | | | | | | - |

| **TQ-CAIN** | 0 | 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|---|
| spring | 80 | 44 | 47 | 63 | 62 | |
| intermediate | 99 | 47 | - | - | - | |
| summer | - | - | - | - | - | |
| winter | | | | | | - |

| **CATU** | 0 | 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|---|
| spring | - | 35 | 18 | 16 | 50 | |
| intermediate | - | 16 | 11 | 22 | 33 | |
| summer | - | - | - | - | 39 | |
| winter | | | | | | - |

## Appendix 4. Results from winter stratification and germination temperature experiment: Germination rate

Germination rate for each accession. Values represent the mean number of days until 50% of final germination percentage was achieved, with day zero being the day the accession was moved out of stratification and into the germination incubator. Germination rate was not calculated for accessions with less than 20% final germination. For all of the controls, which never moved incubators, day zero is the day the experiment began. Columns represent the number of months initially spent in winter stratification. Rows represent the germination temperature which followed stratification. Accessions shaded in grey were affected by the winter incubator malfunction, which may have decreased uniformity. Combinations of winter stratification and germination temperature in dark grey were not tested. Note that for the control accessions (W0-SPR, W0-INT, W0-SUM, and WIN) these data represent germination rate for the full seven months of incubation.

| **VP-CAIN** | 0 | 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|---|
| spring | 58 | 42 | 45 | 45 | 36 | |
| intermediate | 42 | 29 | 31 | 23 | - | |
| summer | - | - | - | - | - | |
| winter | | | | | | - |

| **TQ-CAIN** | 0 | 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|---|
| spring | 85 | 62 | 52 | 52 | 58 | |
| intermediate | 67 | 45 | - | - | - | |
| summer | - | - | - | - | - | |
| winter | | | | | | - |

| **CATU** | 0 | 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|---|
| spring | - | 32 | 22 | 23 | 13 | |
| intermediate | - | 20 | 17 | 11 | 7 | |
| summer | - | - | - | - | 1 | |
| winter | | | | | | - |

## Appendix 5. Results from winter stratification and germination temperature experiment: Viable seeds

Final seed lot viability for each accession. Values represent the mean percentage of viable seed ((# germinants + # viable seeds from final TZ testing)/total # seeds). Columns represent the number of months initially spent in winter stratification. Rows represent the germination temperature which followed stratification. Combinations of winter stratification and germination temperature in dark grey were not tested.

| **VP-CAIN** | 0 | 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|---|
| spring | 53.9% | 43.5% | 53.0% | 47.2% | 56.2% | |
| intermediate | 59.7% | 38.9% | 48.8% | 45.9% | 53.5% | |
| summer | 25.0% | 40.3% | 37.0% | 38.7% | 47.2% | |
| winter | | | | | | 39.0% |

| **TQ-CAIN** | 0 | 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|---|
| spring | 66.0% | 46.4% | 36.9% | 59.7% | 62.9% | |
| intermediate | 62.4% | 47.3% | 36.1% | 55.2% | 46.4% | |
| summer | 51.9% | 39.3% | 33.4% | 67.1% | 43.9% | |
| winter | | | | | | 47.5% |

| **CATU** | 0 | 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|---|
| spring | 29.1% | 45.3% | 42.9% | 37.7% | 33.5% | |
| intermediate | 18.3% | 37.9% | 39.8% | 44.9% | 30.8% | |
| summer | 8.5% | 12.6% | 25.4% | 22.5% | 22.3% | |
| winter | | | | | | 29.8% |

## Appendix 6. Tetrazolium (TZ) testing protocol

TZ Prep Steps

1. Take a final germination count. Remove germinants and record on data sheet as usual.
2. Any partially germinated seeds that remain should also be removed from the petri dish and added to the final germination count on today's date. Note whether each of these seeds has a leaf only (mark with a "C" for cotyledon) or root only (mark with an "R" for root).
3. Take photos of each dish. These can be referred to later to confirm a count if a seed is lost, or to evaluate the degree of fungal infestation at the end of the experiment.
4. Count all remaining seeds and record this final count under "# seeds tested" on TZ portion of germination datasheet.
5. Moisten one 9 cm piece of filter paper with DI water and place paper on a clean TZ plate. TZ plates are regular petri dished with a labeled 25 cell grid drawn on the bottom plate, which makes it easier to keep track of seeds during analysis. It is important that filter paper is at least as big around as the diameter of the petri dish used. Otherwise TZ solution will evaporate during incubation and may not rewet the paper.
6. Peel one label off of the old petri dish and stick to the bottom of the new TZ plate. If there are more than 25 seeds to analyze, add a letter "A" to this label, and a letter "B" to the label for the second TZ plate.
7. Using a fresh single edge razor blade cut a seed in half longitudinally (through the center of the embryo). Tweezers are helpful for holding seeds in place while cutting, especially CAIN seeds.
8. Identify the half of the cut seed which contains more of the embryo (this should be the larger half if the seed was cut longitudinally through the embryo, but could be the smaller half if the cut was off). If both halves contain equal amounts of the embryo, choose the half with a cleaner cut edge. Place the chosen half on the TZ plate in a grid cell with the cut side facing down. Place the other half of the seed in a discard pile.
9. Repeat steps 7 and 8 for each seed until you fill plate A (25 seeds). Place the remaining prepared seeds on plate B.
10. Keep the filter paper in the TZ grids moist as you work by adding deionized (DI) water when necessary. Prepare all plates that will be incubated on this date before proceeding to the next step.
11. Prepare a 1% TZ solution with 1 g TZ and 100 ml DI water. Mix until all TZ has dissolved. Store any excess solution in the refrigerator.
12. Use a paper towel to blot off excess DI water on each TZ plate, being careful not to pick up any seeds on the paper towel. Then add about 1.5 droppers full of 1% TZ solution to each dish (just enough to create a visible film of TZ solution across the paper, but not enough that seeds float on the solution).
13. Wrap the edge of each petri dish with parafilm. This prevents the

evaporation and spilling of TZ solution.

14. Set up the TZ incubator. Incubation at 30-35°C is ideal. Keep the incubator below 40°C.
15. Place TZ plates in the incubator, making sure that all dishes are as flat as possible, and let incubate for 48 hours. Note the date and time of the start of TZ incubation on the germination datasheet.

TZ Analysis Steps

1. Open incubator and record temperature.
2. Remove TZ plates, peel off parafilm, and then remove excess TZ solution with a dropper or paper towel.
3. Rehydrate filter paper with DI water. Keep filter paper and seeds sufficiently moist at all times.
4. Record the time and date that TZ incubation was stopped.
5. If you cannot evaluate seeds right away, place them in the winter incubator (or a refrigerator) for up to a few days.
6. When you are ready to evaluate, flip all of the seeds in one TZ plate over to expose the cut surface.
7. Place the TZ plate under a dissecting microscope and evaluate each seed individually. For each seed, note which of three (or four) categories best describes seed viability: Viable, 50:50, Not Viable (or Empty). See the TZ Testing section under the Materials and Methods section of the winter stratification and germination experiment for descriptions of these categories. Make sure the cut surface is moist when making this evaluation. Dry seeds will crack and dry tissue can obscure staining. Prick embryos out of seeds to observe more clearly if necessary.
8. Use a TZ evaluation data sheet (with grids that match the TZ plates) to record the viability for each seed (see Appendix 7).
9. Add up totals for TZ plates A & B, and record on germination data sheet.

## Appendix 7. Data sheets for TZ evaluation

| Species/Treatment: | Dish #: | Evaluator: | Date: | Time: |
|---|---|---|---|---|
| | | | | |

| | A | | | | | | B | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | 1 | 2 | 3 | 4 | 5 | |
| A | | | | | | A | | | | | | A |
| B | | | | | | B | | | | | | B |
| C | | | | | | C | | | | | | C |
| D | | | | | | D | | | | | | D |
| E | | | | | | E | | | | | | E |

| Totals | Yes | 50:50 | No |
|---|---|---|---|
| A | | | |
| B | | | |

| Species/Treatment: | Dish #: | Evaluator: | Date: | Time: |
|---|---|---|---|---|
| | | | | |

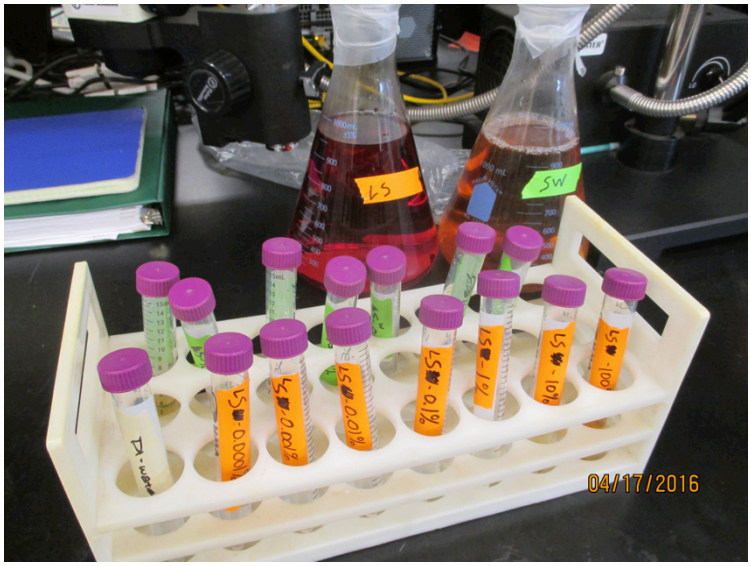
| | A | | | | | | B | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | 1 | 2 | 3 | 4 | 5 | |
| A | | | | | | A | | | | | | A |
| B | | | | | | B | | | | | | B |
| C | | | | | | C | | | | | | C |
| D | | | | | | D | | | | | | D |
| E | | | | | | E | | | | | | E |

| Totals | Yes | 50:50 | No |
|---|---|---|---|
| A | | | |
| B | | | |

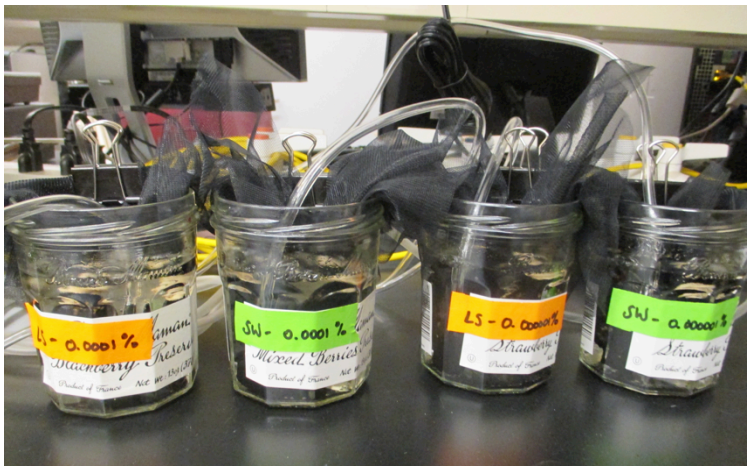
## Appendix 8. Smoke Water production process, from (Krock et al., 2016)

Smoke-water was made based on the protocol of de Lange and Boucher (1990), which consisted of a 2-part process. 1) A combustion chamber (20 l [5.3 gal]) burned dry plant chaff from *Festuca roemeri* (Pavlick) Alexeev (Roemer's fescue; Poaceae), *Collinsia parviflora* Lindl. (maiden blue eyed mary; Scrophulariaceae), and *Eriophyllum lanatum* (Pursh) Forbes (common woolly sunflower; Asteraceae)(3 kg [6.6 lb]) over coals of native *Quercus garryana* Douglas ex Hook.(Oregonwhite oak; Fagaceae)(~4 kg [8.8 lb]), and air-flow regulation resulted in a slow, cool burn (130–170 °C [266–338 °F] internal chamber temperature). 2) The smoke flowed by convection into a cooling chamber(60 l[15.9 gal]) and was then drawn through 5 l (1.3 gal) of de-ionized water in an aspirator bottle for 1 h by vacuum suction. The dark-brown solution (termed plant-derived smoke-water) was stored in a laboratory freezer (–12 °C [53.6 °F]) until use.

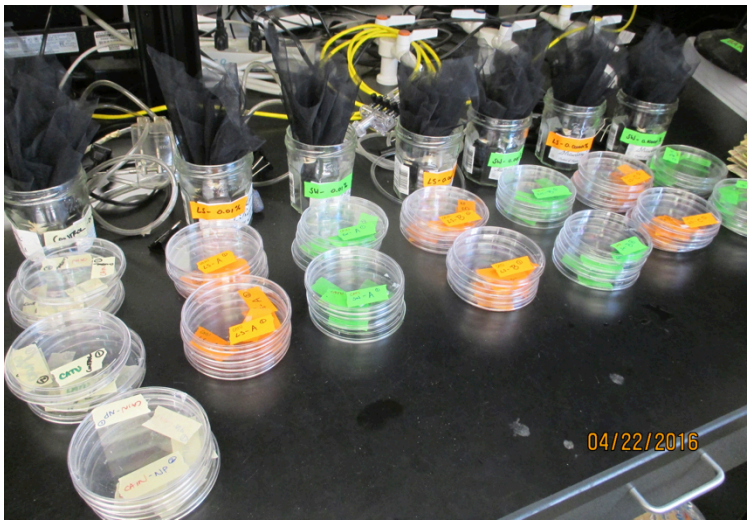
## Appendix 9. Photos of smoke water treatment process




The series of dilutions of smoke water and liquid smoke that were prepared for the lettuce pre-trial. A similar series of dilutions was prepared and then scaled up to make the 200 ml solutions that seeds were imbibed in for the smoke water experiment.



Seeds were wrapped in mesh (secured shut with rubber bands) and submerged in diluted smoke water and liquid smoke. An aquarium bubbler kept the seeds aerated during imbibition. Binder clips kept the mesh bags submerged. CAIN and CATU were imbibed together.

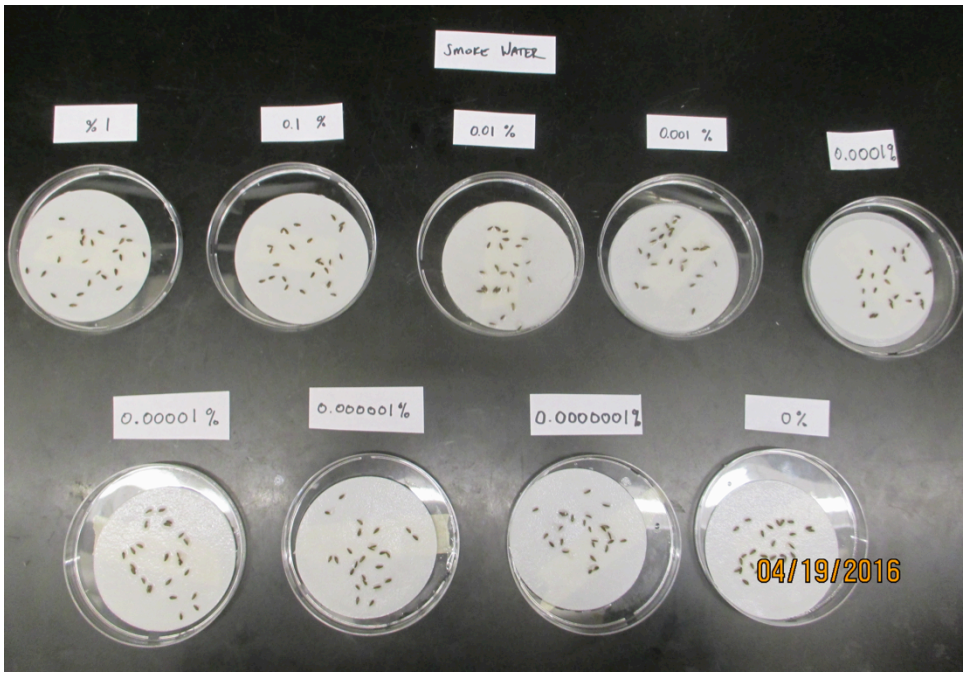



Imbibed seeds were rinsed in tap water and washed in diluted bleach for 60 seconds. Here, they are about to be plated and moved into the germination incubators to begin the smoke and perigynia removal experiment.

## Appendix 10. Lettuce pre-trial results

The effects of a range of smoke water (SW) and liquid smoke (LS) concentrations on lettuce seed germination were tested on two different days. The percentage of seeds that germinated in the dark after 24 hours at 24-29°C are recorded below. Each dish contained roughly 25 seeds and two replicates were prepared for each concentration on each day. The percentages below represent the average of these two percentages. Concentrations which resulted in a germination percentage greater than the control are highlighted in yellow.

| | Liquid smoke | | Smoke Water | |
|---|---|---|---|---|
| Concentration (percent) | Day 1 | Day 2 | Day 1 | Day 2 |
| 100 | 0.0% | - | 0.0% | - |
| 10 | 0.0% | - | 0.0% | - |
| 1 | 0.0% | 0.0% | 10.0% | 0.0% |
| 0.1 | 10.0% | 4.0% | 26.0% | 4.0% |
| 0.01 | 25.5% | 4.0% | 40.0% | 28.0% |
| 0.001 | 24.0% | 8.0% | 34.0% | 16.0% |
| 0.0001 | 31.1% | 12.0% | 32.0% | 10.0% |
| 0.00001 | - | 20.0% | - | 12.0% |
| 0.000001 | - | 22.0% | - | 26.0% |
| 0.0000001 | - | 22.0% | - | 34.0% |
| 0 | 20.6% | 8.0% | 20.6% | 8.0% |




Photograph of smoke water treated lettuce seeds on day 2 of the lettuce pretrial.

## Appendix 11. Smoke experiment results: *Carex inops* ssp. *inops*

VP-CAIN germination percentage, uniformity, and germination rate after the application of SW and LS, and perigynia removal.

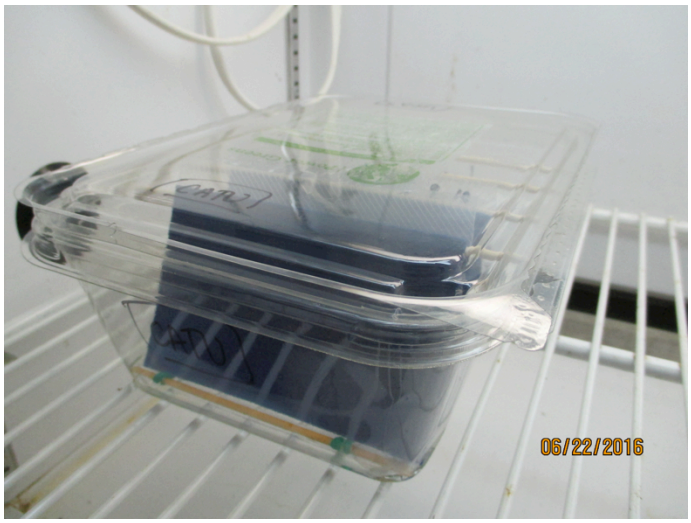
| Accession Code | Smoke Source | Smoke Concentration | Perigynium | Percentage germinated (total seeds) | Percentage viable | Days to 50% Germination | Uniformity (days) |
|---|---|---|---|---|---|---|---|
| Control | NA | 0 | Intact | 28.8% | 56.3% | 71 | 39 |
| NP | NA | 0 | Removed | 38.7% | 56.0% | 56 | 47 |
| SW-A | SW | High | Intact | 29.0% | 41.0% | 73 | 41 |
| SW-B | SW | Med | Intact | 34.3% | 53.5% | 69 | 47 |
| SW-C | SW | Low | Intact | 35.7% | 49.2% | 59 | 47 |
| LS-A | LS | High | Intact | 26.5% | 41.4% | 76 | 44 |
| LS-B | LS | Med | Intact | 28.5% | 46.5% | 71 | 48 |
| LS-C | LS | Low | Intact | 39.7% | 49.7% | 73 | 46 |

## Appendix 12. Smoke experiment results: *Carex tumulicola*

CATU germination percentage, uniformity, and germination rate, after the application of SW and LS.

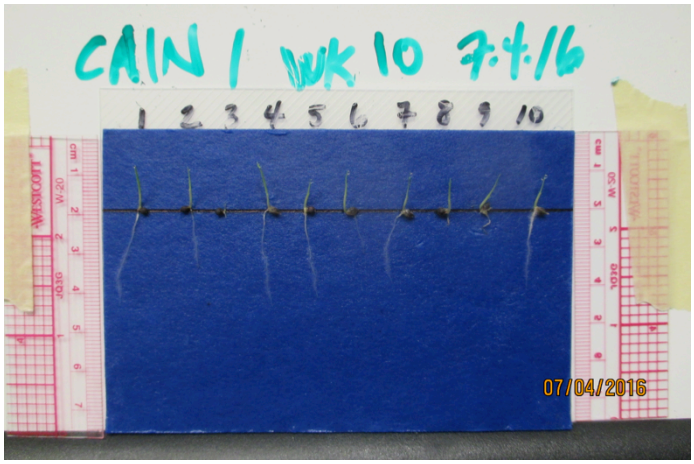
| Accession Code | Smoke Source | Concentration | Perigynium | Percentage germinated (total seeds) | Percentage Viable | Days to 50% Germination | Uniformity (days) |
|---|---|---|---|---|---|---|---|
| Control | NA | 0 | Intact | 26.6% | 27.7% | 18 | 12 |
| SW-A | SW | A | Intact | 31.0% | 38.1% | 18 | 11 |
| SW-B | SW | B | Intact | 37.0% | 38.5% | 18 | 11 |
| SW-C | SW | C | Intact | 36.9% | 39.9% | 18 | 10 |
| LS-A | LS | A | Intact | 30.2% | 34.2% | 18 | 11 |
| LS-B | LS | B | Intact | 38.2% | 41.2% | 17 | 11 |
| LS-C | LS | C | Intact | 34.0% | 39.0% | 18 | 10 |

## Appendix 13. Slant board set-up for vigor trial




The slant board was composed of three main parts. The box itself was an empty, clean lettuce box from the grocery store. The frame within the box, which held the panels in a slanted position (roughly 15 degrees from vertical), was made from BBQ skewers. Skewers were pushed through the top edge of the box to form the upper half of the frame, which the top edge of each panel rested on. The lower portion of the frame was free-floating, and sat in the bottom of the box. It was built from BBQ skewers and twist ties, and provided an edge for the bottom of each panel to rest on (the skewers were not poked through the walls because the box needed to hold water). The panels consisted of rectangular pieces of plastic, made from old plastic folders, and pieces of blue germination paper (Seedburo Seed Germination Blotter .025" thick, www.seedburo.com) cut to fit each plastic back. A line was drawn on the paper with a ball point pen to guide seed placement. Numbers drawn on the plastic panel identified each seed below, and corresponded with rows on the vigor datasheet (Appendix 14). Enough water was added to the bottom of the box so that the paper was able to reach the water and wick moisture up to the seeds.

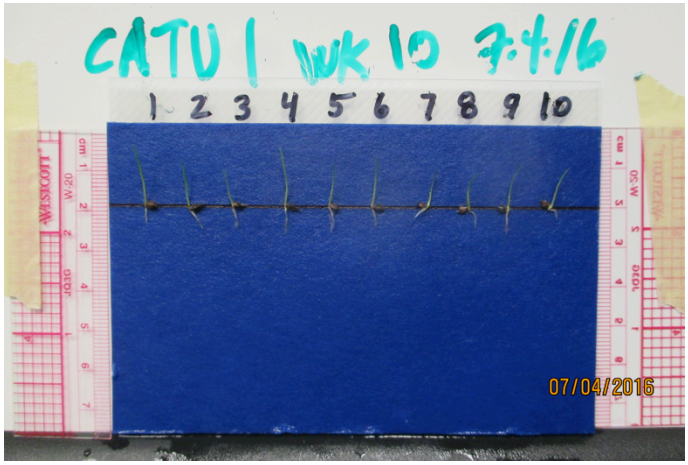
The slant board setup was cheap and easy to build, but did have its problems. The BBQ skewers had to be replaced each week because they were often covered in mold by the end of the week. The box and panels were cleaned with soap, water, and bleach each week to prevent contamination of the new batch of seeds. We had two boxes that we would rotate through, so that cleaning the box didn't slow down the turnaround time. Using plastic, metal, or some other non-porous material for the frame would be an improvement to make on future versions, but mold will probably always be an issue, since mold spores will probably always come in with each batch of seeds.




Panels were removed from the slant box on each day that they

were measured so that a photograph could be taken of the seeds. Rulers were placed next to the panel for scale. Photos were originally intended to be used with analytical software which can measure root and leaf length through image analysis. Due to the small scale of this trial, that software was never used and measurements were taken by hand.

Seeds were positioned on the slant board with leaf up and root down, and not moved for the next 6 days (seeds were measured on the paper). These photos are representative of how straight roots usually grew on the slant board. Both of these photos were taken 6 days after germination (on the final day of measurement). By day 6, CATU roots were usually much shorter than CAIN roots, as can be seen in these photos.



## Appendix 14. Vigor Data Sheet

**Vigor Datasheet** Sheet __ of __

Species: _____

Week #: _____ Date of Day 0: _____

| Treatment | Dish # | Seed # | Was it a partial germinant ? | Number of roots | Day 0 (Tuesday) Date: Time: _____-_____ | | Day 1 (Wed.) Date: Time: _____-_____ | | Day 3 (Friday) Date: Time: _____-_____ | | Day 6 (Monday) Date: Time: _____-_____ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Leaf Length (mm) | Root Length (mm) | Leaf Length (mm) | Root Length (mm) | Leaf Length (mm) | Root Length (mm) | Leaf Length (mm) | Root Length (mm) |
| | | 1 | | | | | | | | | | |
| | | 2 | | | | | | | | | | |
| | | 3 | | | | | | | | | | |
| | | 4 | | | | | | | | | | |
| | | 5 | | | | | | | | | | |
| | | 6 | | | | | | | | | | |
| | | 7 | | | | | | | | | | |
| | | 8 | | | | | | | | | | |
| | | 9 | | | | | | | | | | |
| | | 10 | | | | | | | | | | |
| | | 11 | | | | | | | | | | |
| | | 12 | | | | | | | | | | |
| | | 13 | | | | | | | | | | |
| | | 14 | | | | | | | | | | |
| | | 15 | | | | | | | | | | |
| | | 16 | | | | | | | | | | |
| | | 17 | | | | | | | | | | |
| | | 18 | | | | | | | | | | |
| | | 19 | | | | | | | | | | |
| | | 20 | | | | | | | | | | |

Photo taken on Day 0:_____ Day 1:_____ Day 3:_____ Day 6:_____