Shortening *Silphium integrifolium* Juvenility and Establishing a Sterilization Protocol for Micropropagation

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Abstract

Perennial crops hold great potential to move towards more sustainable agriculture by offering a long-term solution to the ongoing problem of soil erosion and degradation associated with annual cropping systems. A new candidate to perennial agriculture is *Silphium integrifolium* Michx (rosinweed), a native herbaceous perennial plant closely related to sunflower. This project aims at exploring the vernalization process to shorten *S. integrifolium* juvenility, induce precocious flowering, and establish a sterilization protocol for micropropagation to facilitate and accelerate its breeding and domestication process.

Current breeding efforts focus on developing populations with improved agronomic traits such as seed yield, seed size, plant stature, and size and number of flowers. An impediment to breeding efforts of *Silphium* is its delayed flowering, i.e. long term of juvenility. *Silphium* plants when grown from seed usually have to grow and experience vernalization before flowering the next year. Because of the long period of juvenility in *Silphium*, the breeding cycle is lengthy, which involves time needed to break the seed dormancy, rosette forming and developing in the first season, and then bolting and flowering in the following season; thus shortening *Silphium*'s prolonged juvenility period and inducing flowering can help to shorten the breeding cycle and accelerate domestication. This study aims at exploring the vernalization process for *S. integrefolium* and understanding the age response to vernalization in order to achieve dependable early flowering, which will ultimately shorten its juvenility and enable faster breeding cycles and accelerate its domestication process. We discovered that plants required at least three weeks of cold to consistently induce flowering and as the length of cold treatment increased, time to bolting and flowering after cold treatment decreased. We also determined that young plants needed to grow to the stage of 8 to 10 leaves to be successfully vernalized after 4 weeks of cold treatment.

Perennial crops are constantly exposed to fungi and bacteria in their environment, and thus explants from field-grown plants are difficult to disinfect for micropropagation

because of both endophytic and epiphytic microbes. Field grown *S. integrifolium* plants were potted and grown in the greenhouse for five weeks; new stems and anthers along with seeds were tested for *in vitro* sterilization. We report successful sterilization protocols that involved disinfecting seeds with isothiazolone biocides (PPM) and NaClO and sterilizing stems and anthers through vacuum infiltration of PPM. These protocols should be useful for subsequent micropropagation which could be used to facilitate *Silphium* breeding and domestication.

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Introduction

Found in 21 U.S. states, *Silphium integrifolium* Michx (rosinweed) is a native herbaceous perennial plant closely related to sunflower (Van Tassel et al., 2017). Because of its long-lived nature, high quality forage (approaching that of alfalfa) biomass yield comparable to corn, as well as large, edible seeds with high quality oil akin to domesticated oilseed sunflower, this species has piqued interest in the plant science community for its potential to become a domesticated perennial oilseed/forage crop (Vilela et al., 2018). As a perennial crop, *S. integrifolium* has the potential to provide ecosystem benefits: its complex and deep root system allows it to improve soil structure and health as well as increase biodiversity of agricultural landscape. *S. integrifolium* pollen also provides food to a wide range of native pollinating insects (Viela et. al, 2018). Domestication efforts in *S. integrifolium* have been carried out for about 20 years (Vilela et. al., 2018). Experimental trials have been conducted in Minnesota for the past five years on yield and vigor in three different sites in Rosemount, Becker, and St. Paul, involving crop management such as evaluating seeding date, plant density and nitrogen fertilizer's effects on yield and other traits (Schiffner, 2018; Schiffner et al., 2020, 2021). Breeding efforts have focused on developing populations with improved agronomic traits such as yield, seed size, plant stature, size and number of flowers. An impediment to breeding efforts of *S. integrifolium* is its delayed flowering, i.e. long term of juvenility, which is defined as the extended period of post-germination, vegetative development in which flowering is repressed even under otherwise favorable environmental conditions (Nocker & Gardiner, 2014). The achenes (seeds) of *Silphium* germinate after seed dormancy is broken by a cold–moist stratification period. Plants form a rosette with profuse foliage growth in the first year (Vilela et al., 2018). *Silphium* plants grown from seed usually require a period of cold exposure (vernalization) before flowering the next year.

Another challenge to developing *S. integrifolium* as a new crop is generating homogeneous breeding populations for evaluation in replicated genetic studies or field trials. As an obligate outcrosser, propagating by seed cannot achieve true-to-type propagation; propagation through cuttings and divisions is slow, and cannot ensure disease-free production. At present, there does not exist a *S. integrifolium* micrpropagation protocol to achieve rapid true-to-type multiplication. This project aims at exploring the vernalization process to shorten *S. integrifolium* juvenility, induce precocious flowering, and establish a sterilization protocol for micropropagation to facilitate and accelerate its breeding and domestication process.

Problems with Annual Crops and Perennial Agriculture's Benefits

Annual grain crops consist of around 70 percent of all global cropping systems and provide an estimated 80 percent of all the world's food. However, there are many environmental, economic and energy risk factors associated with annual agriculture. These annual crops, such as cereals, oilseeds, and legumes, are often grown in industrialized monocultures which demand many labor, energy, herbicide, and fertilizer inputs. Runoff and pollution from these inputs have negative impacts on biodiversity of species, ecosystem function, water quality, and carbon emissions (Cox et al., 2006). Although there have been attempts to cultivate annual crops with respect to minimizing soil erosion and water runoff, there are still negative impacts on water, soil, air, and wildlife that persist (Foley et al., 2005). In Minnesota, soybeans and corn are the dominant crops. Corn planted in 2021 for all purposes by Minnesota producers is estimated at 8.50 million acres and soybean planted acreage is estimated at 7.70 million acres according to the latest USDA, National Agricultural Statistics Service – Acreage report (Minnesota Ag News, 2021). Both crops utilize annual monoculture systems that tend to lose much more of applied fertilizer in the form of nitrates than perennials; excess runoff of nitrogen and phosphorus from agriculture in Minnesota enters many river basins here and leads to algal blooms and degradation of aquatic systems (MN Pollution Agency, 2013; Westra et al., 2007). Phosphorus stimulates the proliferation of aquatic bacteria and increases hypoxia (lack of oxygen in water) and economic losses due to poor water quality (Pimentel et al., 2012). Nitrogen similarly alters ecosystem productivity,

function, and biodiversity, leading to a loss of fish, invertebrate, and amphibian species (Hernandez et al., 2016). In recent decades, there are increasing concern about N in Minnesota's surface waters because of the toxic effects of nitrate on aquatic life, increasing N concentrations and loads in the Mississippi River that contribute to a large oxygen-depleted zone in the Gulf of Mexico, and the fact that some Minnesota streams exceed the 10 milligrams per liter (mg/l) standard established to protect drinking water sources (MN Pollution Agency, 2013).

On the other hand, perennial agriculture holds a promising alternative for diversifying existing agricultural systems while feeding the growing world population. Perennial crops hold great potential to move towards more sustainable agriculture by offering a long-term solution to the ongoing problem of soil erosion and degradation associated with annual cropping systems. Perennial grains only need tilling and sowing approximately every five to six years reduces erosion and saves energy, fertilizer, and herbicide/pesticide inputs which lead to pollution of the local water system (Pimentel et al., 2012). Compared to annual crops, perennials have longer growing seasons, deeper rooting depths, and greater root mass, which offers a more stable surface to reduce soil erosion and makes them more efficient in managing nutrients and water while providing improved wildlife habitat (Glover et al., 2010). Perennial crops generally have advantages over annuals in maintaining important ecosystem functions (Cox et al., 2006; DeHaan et al., 2005).

Perennial-cropping systems require significantly lower amounts of N fertilizer and have greater water and nutrient uptake; thus perennial-crop systems have much low nitrate losses compared with row-crop systems (Brezonik et al., 1999). Based on a four-year study in Minnesota's Cottonwood major watershed, Randall et al. (1997) found that nitrate losses were reduced by as much as 96–98% in perennial cropping systems, compared with the losses from row-crop systems. Nitrate losses in fields of corn and soybean ranged from 202 to 217 kg N/ha, whereas nitrate losses under continuous alfalfa or perennial grass ranged from 4 to 7 kg N/ha. In another study, a cover crop of perennial rye grass in the Mississippi River Basin reduced nitrate losses to surface waters by 29–

94% from November to May (Sainju and Singh, 1997). To reduce excess phosphorus and nitrogen in the waters of Minnesota that contribute to downstream water quality problems, one significant strategy is to promote perennial agriculture such as *S. integrifolium* and establish double cropping of perennials within Minnesota's traditional corn and corn/soybean crops, and such a strategy will ultimately help to reach the final nitrogen reduction goals in Minnesota (MN Pollution Agency, 2014).

S. integrifolium*'s* **Breeding Cycle and Vernalization Requirement**

The *S. integrifolium* group in UMN's Kevin Smith lab has characterized provisional *S. integrifolium* growth stages. The juvenile stage (or stage 1) lasts for the duration of the first growing season and is characterized by a rosette growth form that is low to the ground and lacks any stems. The end of the juvenile stage is marked by bolting in stage 2, in which the first signs of a shoot apex emerging from the rosette is visible, leading to the development of a stalk with several nodes. By inducing early flowering and shortening juvenile period in *S. integrifolium*, the overall time required to create or select a desirable genotype is reduced, thus increasing efficiency by increasing genetic gain per unit of time (see Fig. 1). Creation of breeding populations and generating new hybrids will be faster as more cycles of breeding and selection can be performed. Shortening juvenile period in *S. integrifolium* may be achieved via the implementation of an effective vernalization process.

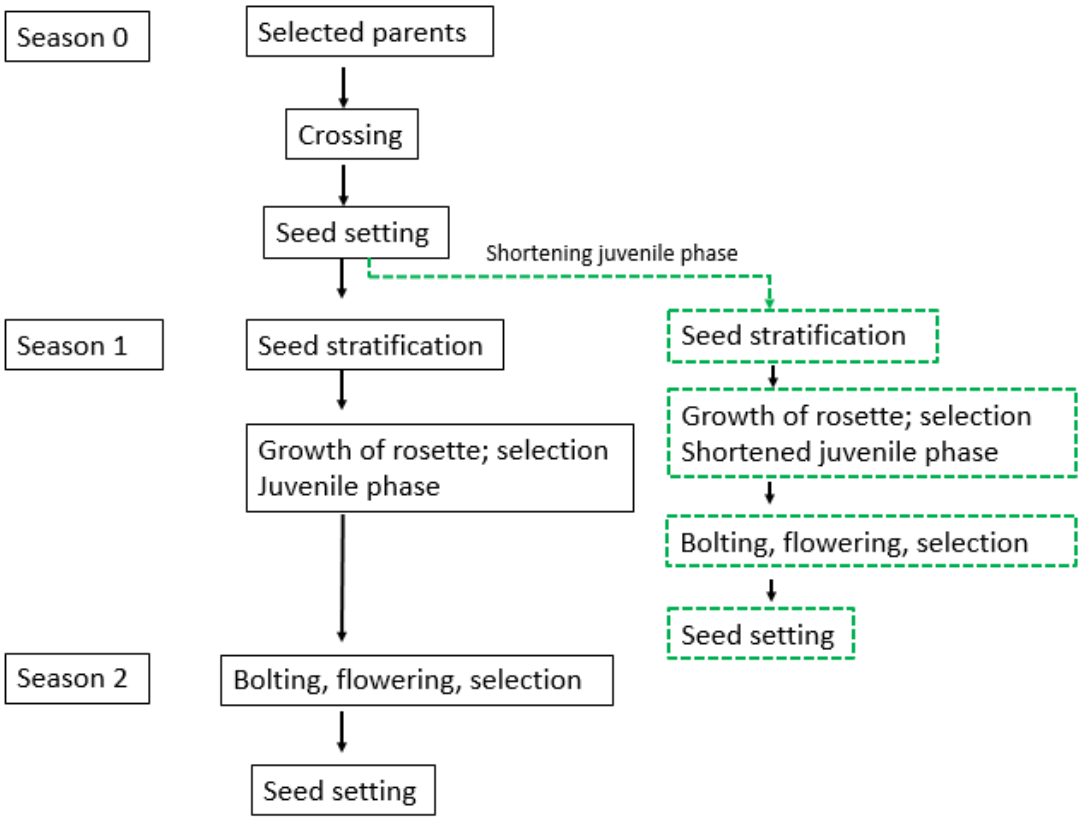

Figure 1.1. Comparison of breeding cycles of *S. integrifolium*. Time scale of a traditional breeding cycle using natural conditions (left) in comparison with a cycle using flower-inducing techniques (dashed box) to shorten the juvenile phase (right).

Vernalization

The reproductive success of a plant such as flowering and seed set is essential for plant species to survive; thus, the timing of flowering needs to be in sync with environmental conditions that facilitate flowering and seed development (Jung & Müller, 2009; Leijten et al., 2018). Plants have the ability to change their development based on environmental stimuli; a major switch is the transition from vegetative growth to flowering (or from juvenility to maturity). In many plant species, this change is determined by environmental cues from seasonal changes which are experienced by the plant. Photoperiod, vernalization, and light quantity are the main environmental cues that plants respond to in order to flower (Cameron et al., 2005; Glover, 2014; Michaels & Amasino, 2000; Runkle, 2006). To optimize the timing of flower production, plants have evolved mechanisms

such as vernalization, to avoid the risk of fall germination leading to flowering in winter (Glover, 2014).

The so-called vernalization is defined as "the acquisition or acceleration of the ability to flower by a chilling treatment" (Chouard, 1960); it is a process by which flowering is promoted when plants sense exposure to a proper length of cold temperatures. In other words, vernalization is the low temperature induction of flowering in responsive plants (Chouard, 1960; Glover, 2014). The vegetative phase of many species is reduced by exposure to low temperatures (Lang, 1965). Vernalization is common in flowering plants for both dicots and monocots. Many crops in temperate regions require vernalization to induce flowering, such as wheat (*Triticum aestivum*), oilseed rape (*Brassica napus*), pea (*Pisum sativum*), and sugar beet (*Beta vulgaris*) (Chouard, 1960).

Genetics of Vernalization

The genetic routes through which plants flower are often referred to as flowering pathways: for example, the photoperiod pathway refers to flowering by responding to inductive photoperiods; other flowering pathways include vernalization pathway and gibberellin-based promotion pathway. The endogenous changes that lead to flowering are sometimes referred to as autonomous pathways, which are independent of environmental cues (Amasino, 2010). In the model plant of *Arabidopsis*, a set of genes, known as flowering-time integrators, is regulated by more than one of the input pathways; in other words, various flowering-time pathways converge on a set of genes (Glover, 2014). Previous studies have shown that the apical meristem is the site of cold perception during vernalization, and that vernalization leads to meristem competency to flower. Meristems successfully vernalized are able to "remember" that they are vernalized; this "memory" is mitotically stable (Michaels & Amasino, 2000). Genes in plants that require vernalziation such as winter annuals account for the presence or absence of a vernalization requirement (Kim & Sung, 2014). The genetic basis of flowering responses to seasonal cues was explored in the model species *Arabidopsis thaliana*. Genetic studies using natural accessions of *Arabidopsis* show that two dominant genes, *FRIGIDA* (*FRI:* At4g00650) and *FLC* contribute to the vernalization requirement (Lee et al., 1993; Clarke and Dean,

1994; Lee et al., 1994; Michaels and Amasino, 1999; Johanson et al., 2000; Strange et al., 2011); the level of *FLC* expression is largely responsible for the vernalization requirement in *Arabidopsis* (Kim & Sung, 2014). In certain plants such as *Arabidopsis*, some accessions of which are winter annuals, the winter-annual trait is conferred by a single dominant gene FRIGIDA (FRI). A dominant allele of another gene, FLOWERING LOCUS C (FLC), is flowering repressing gene necessary for FRI to give a winter annual habit (Sung & Amasino, 2004). Thus, the plant "remembers" vernalization via changes in chromatin structure of FLC. In many accessions of *Arabidopsis*, naturally occurring mutations in *FRI* lead to early flower without vernalization (Johanson et al., 2000; Strange et al., 2011). The level of *FLC* expression is reduced in the absence of active FRI allele; as a result, plants do not require vernalization for accelerated flowering. On the other hand, natural accessions with both active *FRI* and *FLC* alleles require vernalization treatment to flower early, as vernalization triggers repression *of FLC* and thus plants may flower under the inductive photoperiod (Kim & Sung, 2014).

Besides endogenous gene regulators of flowering time as described above, many other factors can also influence the floral transition. For example, in some species, especially long-lived plants and perennials, the floral transition will not occur until the plant is a certain size, irrespective of the environmental cues it has received (Glover, 2014). Such plants have an autonomous pathway which represses flowering until a certain developmental stage has been reached (Bernier & Périlleux, 2005; Glover, 2014). However, even short-lived annuals like Arabidopsis go through certain life stage transitions before flowering. In Arabidopsis, rosette formation is eliminated through the loss of function of genes such as *EMBRYONIC FLOWER 1* (*EMF1*), which transforms the apical meristem into an inflorescence meristem from the point of germination (Aubert *et al*. 2001). *EMF1* is not regarded specifically as a flowering-time gene, and it mainly controls phase transition during development in general; however, *emf1* is a factor that functions as other flowering induction pathways and is necessary for appropriate development and accurate timing of flowering (Glover, 2014).

Vernalization Requirements

Vernalization results in plants' ability to flower by chilling treatment (Chouard, 1960); that is, the cold treatment does not initiate flowering directly; instead, plants acquires the capacity for later flowering (Michaels & Amasino, 2000). In most species, vernalization requires long-term exposure to low temperatures (such as during winter). However, within a given species, there can be variations in terms of the extent to which vernalization is required for flowering, such as winter wheat and spring wheat (Amasino, 2004). In the natural conditions of temperate areas, highly predictable factors such as the annual change in day length and the period of winter cold are called primary flowering time controlling factors. Less predictable climatic factors, such as ambient temperature, light and water availability, are usually viewed as secondary factors that can only modulate the effects of primary factors. Two primary factors (day length and vernalization) can substitute for each other, and can also be replaced by secondary or tertiary factors (Bernier & Périlleux, 2005).

Vernalization is an adaption for plants in the Northern hemisphere in temperate regions that have a winter-annual or biennial habit; plants begin growing from seed in spring in Northern hemisphere temperate regions and then flower next spring. It is crucial that plants are not "tricked" into flowering by cold temperatures during fall so that their flower buds are not killed by freezing temperatures. For example, although there can be a brief period of cold exposure in fall followed by a short return of warm temperatures, plants cannot flower under such conditions that do not promote fertilization and seed development. As a result, plants must not only sense cold exposure but also have a mechanism to measure or sense the prolonged periods of cold during winter, so that plants only permit flowering after a sufficient cold exposure to ensure that winter has passed (Amasino, 2004; Jung & Müller, 2009).

Vernalization requires plants to be able to withstand freezing temperatures during winter; however, vernalization must occur in cold, but non-freezing temperatures as metabolic activity is still required (Sung et al., 2004). The optimal vernalization temperature for most plants range from 6-10 °C (Chouard, 1960), but for plants that flower with cold

promotion, the range of effective temperature is generally 1-7 °C with 4 °C as typical; some cereals can be vernalized at temperature as low as -6°C, while plants native to warm regions, such as olive, can flower after cold treatment at temperature as high as 13 °C (Michaels & Amasino, 2000; Leijten et al., 2018). In addition, the consistency of cold treatment is another factor that affects the effectiveness of vernalization, which is also species dependent (Michaels & Amasino, 2000). Plants also need to be cold treated in light. Experiment shows that when some varieties of Chrysanthemum were vernalized in darkness, the effect of vernalization can be eliminated with several days of 30-40°C heat treatment, which indicates a requirement for a threshold level of metabolic activity before the state of vernalizaton can be "fixed" (Michaels & Amasino, 2000).

On the other hand, time required for the vernalization process to complete varies by species, with 1-3 months as typical (Michaels & Amasino, 2000). The length of vernalization needed by a plant depends on whether the species has an obligate or facultative vernalization requirement. Plants with short vernalization period (20 to 30 days) are facultative. In facultative vernalization species, usually photoperiod is the primary stimulus for floral induction and vernalization is either a substitute for or speeds up the flowering process. Plants that require longer vernalization periods for complete induction for flowering are obligate; in this case, vernalization is needed for flowering (Engelen-Eigles & Erwin, 1997). For example, winter wheat has an obligate vernalization requirement, and it must be planted in fall so that the crop can be exposed to cold in order to flower. On the opposite, spring wheat has a facultative vernalization requirement, and there is no need for cold exposure in order to flower (Michaels & Amasino, 2000). According to studies at Michigan State University that examined over 400 herbaceous perennials and their flowering requirements, most perennials have obligate vernalization requirement for flowering (Padhye et al., 2006).

Another vernalization requirement is a mechanism to sense the increasing daylengths to ensure spring does come (Amasino, 2004). Cold exposure is usually not sufficient to induce flowering. Knott (1934) found that day length is perceived by leaves. Short-day plants will only flower when the uninterrupted darkness or night length exceeds a critical length, while long-day plants will flower as day length increases (Amasino, 2010).

Vernalization must also be paired with an additional stimulus such as increased day length for most species (Woods et al., 2017). Plants with the obligate vernalization requirement can be divided into two groups based on their photoperiod response after vernalization; these include plants that are day neutral and will flower in any photoperiod after vernalization and plants that require long days after vernalization (Padhye et al., 2006). The process of vernalization may be compared to forcing, in which plants are induced to flower in a different time period (usually earlier) rather than their usual time. Some aster plants such as Coreopsis will flower if exposed to 10 weeks of cold (5 °C) and then 14 hours or more of day length (Nordwig, 1999). Although many perennials or biennials that grow naturally in cold climates have a vernalization requirement, others do not. For example, *Campanula carpatica* is hardy to at least USDA hardiness zone 3 (-40F), but does not have a vernalization requirement and will flower based solely on exposure to long days (Padhye et al., 2006). For other perennials, the amount of light a plant receives during the day can interact with the chilling requirement or even override the need for chilling (Cameron et al., 2005). This dual control of flowering via vernalization and daylength ensures that plants will not flower during a sudden warm period in winter and that it will only flower in summer when daylengths are correct.

Finally, plant age also determines whether plants may flower after the vernalization treatment, and young seedlings must reach a critical developmental age before vernalization can occur. Besides environmental cues such as changes in temperature and day lengths that plants use to provide input on when to initiate flowering, endogenous cues are also significant for plants to initiate flowering. For example, the juvenile to adult transition is a system that involves endogenous cues, which affects whether plants are mature enough to flower; at the juvenile phase, plants are not competent to flower even if inductive environment cues exist (Amasino, 2010). The juvenile phase for annuals can be short—some plants can flower after forming a few leaves, but a perennial's juvenile phase can be as long as several decades (Amasino, 2010). Before being vernalized, juveniles of some facultative biennial species must grow to a minimum pre-vernalization size before the cold season, which can vary even within populations of a species (Reekie et al. 1997; Wesselingh et al. 1997). One example is *biennial Hyocyamus niger*, which

doesn't respond to cold treatment before growing for 10 days in normal growing conditions (Lang, 1986). The other example is Aquilegia, which usually responds to a cold treatment for flowering, but most seedlings with four to six leaves do not flower after a cold treatment; however, seedlings with a total of eight to ten leaves before cooling flower uniformly after the cold treatment (Runkle, 2016).

Plants Vernalization in the Asteraceae Family

Genetics of flowering time is extensively studied in the plant model species *Arabidopsis thaliana*; however, this knowledge is still limited in most crops (Leijten et al., 2018). Although many members of Asteraceae family are important crops and many of those require vernalization to induce flowering, the molecular basis for vernalization in this family is not yet well-understood (Cullerne et al., 2021). Although *S. integrifolium* has not been studied for vernalization requirements, there are vernalization studies on other species of the Asteraceae family. For example, even very small plants (such as plants with only 1 pair of true leaves) of *Polymnia canadensis* can respond to vernalization. Such vernalized plants will then flower under both long and short days. However, they can only flower once they have reached a minimum size after vernalization has ended. In addition, plants of this species can actually be de-vernalized if they are exposed to high enough temperatures post-vernalization (Bender et al., 1999). Another member of Asteraceae, winter-hardy Safflower (*Carthamus tinctorius*) can be induced to flower faster if their seeds are exposed to prolonged cold temperatures during their germination. Vernalization's impact was quantitative for this species; increasing the duration of cold increased flowering to a greater extent until the response to vernalization became saturated after 2 weeks of cold exposure (Cullerne et al., 2021). This may explain why young plants of *S. integrifolium* may bolt when planted in the fall (and exposed to cold spells before the killing frost). In *Rhodanthe floribunda*, an Australian native flower species in Asteraceae family, seedlings of four age groups (1, 7, 14, 28 days old) were exposed to different cold periods (0, 3, 7, 14 and 21 days) at 20/10 °C under short day (11h). It was found that *R. floribunda* has a facultative requirement for flowering in response to vernalization, and can respond to vernalization at a very early stage, indicating a short juvenile phase of this species. Additionally, the longest cold exposure

and the oldest seedlings prior to chilling had faster development rate and were more floriferous (Ha and Johnston, 2013).

In *Chrysanthemum superbum*, which is reported to be a long-day plant (requiring long days to flower), flowering was insignificant in plants that were not exposed to cold temperatures but were exposed to long days. In plants that were exposed to 4.5 °C temperatures then 18 °C temperatures, flowering increased to 100% after 16 weeks of chilling. However, other cultivars of *C. superbum* are capable of flowering in long days without experiencing any chilling temperatures (Damann et al., 1996). In *C. grundifolia* 'Sunray', no plants flowered without vernalization in any short-day or long-day conditions, and flowering was not influenced by daylight integrals but generally increased with vernalization duration (Niu et al., 2002). In *Coreposis grandiflora*, another long day species, plants of the cultivar 'Single Mayfield Giant' treated with 7 weeks of 3 °C temperatures in continuous light flowered 30% more than plants grown in normal temperatures in short days. Seedlings were able to flower after being chilled in 3 °C in long days for 42 days with 100% floral induction. However, other cultivars of this species can still flower (ranging from 70 to 90% flowering) when grown under continuous long day conditions without chilling temperatures (Damann et al., 1996).

It is currently unknown if *S. integrifolium* has a requirement for longer day lengths to flower, though it is likely as *S. integrifolium* in its natural habitat begins flowering as day lengths increase during late spring and summer. One challenge for *S. integrifolium* breeding is the long juvenile periods; thus it is necessary to accurately predict *S. integrifolium*'s developmental response to low temperatures, as the vegetative phase of many species is reduced by exposure to low temperatures (Lang, 1965). There have been rare observations of *S. integrifolium* flowering without vernalization in the first season, whether in the greenhouse or in the field. It is hypothesized that some individuals may have a genetic basis for a low threshold for vernalization; it is also possible that these individuals encountered some kind of environmental condition that facilitates flowering such as a brief period of cold temperatures or longer light exposure from non-natural sources.

Micropropagation, Its Benefits, and Plant Tissues Sterilization

Tissue culture is commonly used as a collective term to describe all kinds of *in vitro* plant cultures (George, 2008). As a tissue culture technique, micropropagation is defined as *in vitro* regeneration of plants from organs, tissues, cells or protoplasts (Beversdorf, 1990); it's a method of *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions in order to produce true-to-type clones of pathogen-free plants (George, 2008; Loyola-Vargas & Ochoa-Alejo, 2018). Used in 1960 by George Morel for the production of orchid plants at a commercial level, micropropagation is seen as a basic step in non-conventional improvement of crop plants. This section focuses on establishing a sterilization protocol for micropropagation in order to facilitate and accelerate its breeding and domestication process.

Micropropagation using axillary bud meristems allows for the recovery of genetically stable, true-to-type elite progeny (George & Sherrington, 1984; Rangappa & Aind, 2013; Sairkar et al., 2009); in many crops, regeneration from multicellular callus have been successful (Greco et al., 1984). Theoretically, micropropagation produces genetically identical plantlets; however, present tissue culture techniques do not permit this in every case, and irregularities (somaclonal variants) do occur sometimes, although a large percentage of success can be achieved (Larkin and Scowcroft, 1981; George, 2008). With large amount of plants being produced and maintained at any time of the year in small spaces, micropropagation facilitates plant breeding by speeding up multiplication and improving uniformity. Moreover, *in vitro* culture methods contribute to improvement of the plant breeding process through conserving germplasm, *in vitro* pollinating, flowering, and fertilizing, embryo rescuing, and somatic embryogenesis—the development of an embryo derived from somatic cells under an appropriate artificial environment (*in vitro*) (Gunn & Day, 1986; Zulkarnain et al., 2015). Because of *In vitro* techniques' convenience and the potential of decreasing the breeding cycle, they are now applied extensively in breeding programs; however, the lack of well-established *in vitro* techniques often limits its practical use (Zulkarnain et al., 2015).

Stages of Micropropagation

As a tissue culture technique, micropropagation consists of stages such as selection of explants, aseptic culture establishment, multiplication, rooting and acclimatization of plants. Micropropagation starts with preparing plant tissues or explants—the small organs or pieces of plant tissue. There are many different kinds of explants, and the correct choice of explant material can significantly affect the success of micropropagation (George, 2008). Important micropropagation cultures include the following: meristem cultures with the extreme tip of the shoot or lateral buds to free plants from virus infections, shoot tip or shoot cultures that start from excised shoot tips or buds (larger stem apices or lateral buds), node cultures of separate lateral buds with each carrying on a small piece of stem tissues, isolated root cultures from which a branched root system may be obtained, and embryo cultures with embryos being dissected and cultured to grow into seedlings. Explants need to be treated with disinfecting chemicals in order to be free from microbial contaminants when they are cultured on a nutrient medium. Plant materials need specialized media in order to grow *in vitro*, which usually consist of a solution of salts along with various vitamins, amino acids, and an energy source such as sucrose. The media can be solidified or liquid, although solidified media is widely used for explant establishment.

Micropropagation for S. integrifolium and Its Relatives

Because *S. integrifolium* is an outcrossing species, producing homogenous genotypes for replicated testing is difficult. The ability to clonally propagate *S. integrifolium* would allow such replication and improve the ability to accurately measure the genetic merit of breeding material and select superior individuals or populations. Tissue culture techniques such as micropropagation may complement and facilitate the breeding of *S. integrifolium*. Micropropagation may be used to propagate particular genotypes and obtain disease-free homozygous plants (Cavallini & Lupi, 1992). Currently, *S. integrifolium* is propagated via seed, cuttings, and divisions. As an obligate outcrosser, propagating by seed cannot achieve true-to-type propagation; propagation through cuttings and divisions is slow, and cannot ensure disease-free production. At present, there does not exist a *S. integrifolium* micrpropagation protocol to achieve rapid true-to-

type multiplication. This project aims at exploring the optimal approach (es) to shorten *S. integrifolium* juvenility, induce precocious flowering, and establish a sterilization protocol for micropropagation in order to facilitate and accelerate its breeding and domestication process.

Although *S. integrifolium* has never before been micropropagated, several of its close relatives including those in the sunflower genus as well as other perennial *Asteraceae* have been successfully tissue cultured. Another species in the *Silphium* genus, *S. perfoliatum* L. (cup plant), has been studied on the processes of seed sterilization and micropropagation from apical explants of seedlings in *in vitro* cultures (Tomaszewska-Sowa & Figas, 2011). In sunflower, the cytokinin 6-benzylaminopurine (6-BAP, or simply BAP) was able to induce callus tissue from leaf, cotyledon, shoot apices, and hypocotyl tissues while the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) produced poorly developed, nodular calli. BAP is capable of generating green, compact callus tissue, which can then generate many shoots and can even flower *in vitro* (Greco et al., 1984). Another efficient method of obtaining multiple plants from tissue culture is the usage of cotyledons, yielding up to 10 shoots per cotyledon. Many factors influence both quantitative (frequency of response) and qualitative (response type) aspects of tissue culture, including hormone balance, nitrogen supply in the medium, and explant type, age, and genotype (Knittel et al., 1991). At least in sunflower, physical culture conditions play only a minor role in the successful regeneration of plants. The time required from isolation of explant material to harvest of seeds was 4 to 6 months (Knittel et al., 1991). Successful regeneration was obtained from cultures of tuber tissue of *a Helianthus tuberosus* x *H. annuus* cross (Witrzens et al., 1988). Shoot multiplication from cultured shoot apices are possible in sunflower, and usage of benzylaminopurine (BAP) alone induced callus on hypocotyls, leaves, cotyledons, and shoot apices, and plantlet regeneration was achieved. Sunflower (*Helianthus annuus*) protoplasts have also been cultured to form colonies of cells and eventual plants. However, these plants/plantlets exhibited premature flower initiation and high losses when transplanted. These problems were reduced or eliminated by using phloridzin, naringin, and esculin *in vitro* prior to transplanting *ex-vitro* (Witrzens et al., 1988).

According to Dr. Van Tassel from TLI, cuttings require greenhouse conditions and have a slow growth rate. Division of crowns demands intensive labor, requires a large (and thus older) plant, and may only produce a limited number of plants from each mother plant (Personal Communication, April 2020). *S. integrifolium* can also be propagated via seedlings, but *S. integrifolium* is an obligate outcrosser, which means that it cannot achieve true-to-type propagation from seed. As a tissue culture technique, micropropagation is the true-to-type propagation of a selected genotype using *in vitro* culture techniques, which allows inexpensive, mass production possible (Debergh & Read, 1993). The creation of true breeding lines is an important step for cultivar development; micropropagation makes it possible for the production of completely homozygous breeding lines from gametic cells in a shorter time frame in comparison with conventional propagation methods. In most self-compatible crops, breeders employ back-crossing and selfing to produce homozygous lines. The genetic phenomenon necessary to creating homozygous lines requires the formation of haploid gametes via meiosis. This process can be manipulated in a tissue culture environment to produce a double-haploid plant from a gametic cell, which is genetically equivalent to a stable line produced via inbreeding (Morrison & Evans, 1988).

In addition to mass producing true-to-type genotypes, the technique of micropropagation also provides other benefits for crop improvement. Micropropgation technique serves as a pre-requisite for many crop improvement programs, and it is necessary for a breeding program to develop a feasible micriopropagation protocol for regeneration *in vitro* of species (Sathyanarayana & Varghese, 2007). Micropropagation allows disease free plants in large numbers to be produced in a short period at any time of the year, and plants can be maintained in small spaces (Bhatia & Sharma, 2015). Micropropagation particularly provides a good alternative for those plant species that show resistance to practices of conventional bulk propagation via cuttings or division (Bhatia & Sharma, 2015). As a supplementary tool in plant breeding, tissue culture techniques such as micropropagation not only allows for faster clonal propagation through regeneration of plant tissues from plant organs, but also achieves early screening for resistances to diseases (Wenzel et al., 1985). Micropropagation technique combined with new technologies, such as the genome

editing, are currently promising alternatives for the highly specific genetic manipulation of interesting agronomical or industrial traits in crop plants (Loyola-Vargas & Ochoa-Alejo, 2018). Micropropagation can open up avenues to techniques that are beneficial to breeding, such as embryo rescue and anther culture. As a tissue culture method, micropropagation will allow for *in vitro* mutagenesis (Nocker & Gardiner, 2014), as well as serve as a basis for embryo rescue to bypass seed's dormancy stage in order to shorten breeding cycles (Bhatia et al., 2015). With sterilizing and micropropagation protocols established, they will also allow other breeding efforts such as generating a wide range of genetic variation in plant species, and conducting *in vitro* selections for desirable traits with minimal environmental interaction in a considerably shortened time frame (Jain, 2001).

Sterilizing Plant Tissues for Micropropagation

Biotic contamination in micropropagation is caused by pathogens and other microbial contaminants on or in explants, along with environmental and human-associated microorganisms introduced during the *in vitro* culture stages (Cassells & Doyle, 2006). Plants growing in the external environment are contaminated with micro-organisms and pests, which are mainly confined to the surfaces of the plant, although some microbes and viruses may be systemic within the tissues (Cassells, 1997; Cassells & Doyle, 2006). Explants from field-grown plants or from plant parts which are located close to or below the soil may be difficult or impossible to disinfect due to both endophytic and epiphytic microbes (Leifert el al.. 1994). Epiphytic (external) and endophytic organisms can cause severe losses to micropropagation plants (Reed & Tanprasert, 1995). The most important step for aseptic culture establishment is sterilization of explants, and an effective way of preventing bacterial contamination *in vitro* is the elimination of bacteria from the initial plant explants that are introduced into the culture (Mihaljević et al., 2013). The methods for reducing contaminations include the use of explant of donor plants under a strict sanitary regime and reduction of the size of the initial explants just to apical meristem (Mihaljević et al., 2013). Explants must be free from microbial contaminants when they are cultured on a nutrient medium, which involves treating plant material with disinfecting chemicals and sterilizing the tools used in tissue culture (George, 2008). It is

of utmost importance for tissues used in plant micropropagation to be sterile and aseptic; surface sterilization of tissues is the most important step for micropropagation as controlling fungal and bacterial contamination from field sources are difficult (Mihaljević et al., 2013). A plant tissue removed from its mother plant is lacking in many major phytochemical and physical defenses, such as cuticular waxes which may have been partially removed during the sterilization process of the tissue, and the tissue requires time to adjust to its new environment before growing; thus any pathogen such as a single bacteria cell or fungal spore will be able to grow and replicate exponentially when exposed to aseptic tissue culture conditions, usually always faster than the plant bud's growth (Mihaljević et al., 2013).

Plant tissue sterilization can be done in many ways and is usually a multi-step process, involving a preliminary wash with soap and water to remove debris and organic matter as well as dead material from the plant tissue and then washing with more powerful sterilizing compounds such as bleach, hydrogen peroxide, alcohol, and broad-spectrum biocides/fungicides such as PPM (plant preservative media) in various concentrations. Other compounds include calcium hypochlorite, sodium dichloroisocyanurate (DICA), mercuric (II) chloride, and silver nitrate. It was found that silver nitrate was the best surface sterilizer for 'Oblačinska,' a cultivar of sour cherry (Mihaljevic et al., 2013). Introduction of microorganisms due to poor aseptic technique or improperly sterilized equipment can be corrected with improvements in equipment handling, but eliminating internal contaminants is more difficult (Reed & Tanprasert, 1995). Bacteria contaminants are often difficult to detect because they are mostly within plant tissue; if contamination is detected, antibiotics may be added to the culture medium as an attempt to eliminate the contaminant (Cassells, 2012).

Plant Preservative Mixture (PPM), a proprietary mixture of two broad-spectrum isothiazolone biocides, is a prophylactic antibacterial agent widely used in plant tissue culture; a single use of PPM can substitute for many combinations of antibiotics, and PPM is generally less expensive than antibiotics. However, it is difficult to eradicate endophytic bacteria with PPM because PPM cannot directly target microbial cells, but

greater absorption of PPM can be achieved through vacuum infiltration. In the context of plant tissue culture and *in vitro* work, vacuum infiltration is often used in Agrobacterium-mediated transformation in which the application of a vacuum on plant tissues allows for Agrobacterium to enter the air spaces within plant tissues, allowing deeper penetration of Agrobacterium into the plant than a simple soaking of tissues in an Agrobacterium solution (Tague et al., 2006). This is usually done by placing plant tissues into a dish of solution containing Agrobacterium then placing both into a vacuum chamber where a vacuum is applied for several minutes. Air bubbles will be released from plant tissues as the solution enters the tissues; the vacuum is then slowly released and the materials removed. Similarly, these procedures can be duplicated for applications in plant sterilization to allow a sterilizing compound to penetrate deeper into tissues for more thorough cleaning than compared to soaking tissues into the sterilizing compound (Tague et al., 2006). PPM was also found to infiltrate younger axillary buds more effectively because younger plant tissues are softer and thinner (Miyazaki et al., 2010). In *Petunia hybrid*, axillary buds, including attached nodal tissues, were immersed in micropropagation medium containing 5 ml l^{-1} PPM and treated with vacuum infiltration for 2–3 minutes before incubation in PPM solution for a further 15 minutes; after this treatment, the axillary buds were transferred onto medium without PPM. This protocol was found to successfully eradicate *S. paucimobilis* in contaminated *Petunia* cultured young axillary buds—the uppermost node (Miyazaki et al., 2010).

Perennial crops are considered to be locally, if not systemically, endophytically contaminated, and the endophytes are mainly bacteria entering through natural openings on the plant surface, or through wounds (Cassells, 2012). In another experiment, perennial *Centella asiatica* explants were prepared from field-grown well-established plants that were highly exposed to fungal and bacterial contamination. After removing leaves and roots, nodal segments (part of the stolon) were washed with detergent using an anti-bacterial sponge. After rinsing with tap water, nodal pieces (about 2.5 cm in length) were excised from the stolons and treated with cetrimide, bavistin, trimethoprim, and mercuric chloride. Plant materials were then rinsed with autoclaved sterilized distilled water, which was followed by 60 min soaking of the explants in PPM 2% and finally the

addition of 2 mL/L of PPM into the medium. The nodal explants were later trimmed from both ends to about 1.5 cm prior to inoculation on the culture medium. Results from this sterilization protocol produced very successful results (clean culture 90 ± 1.33%). Furthermore, shoots cultured on full-strength MS medium containing 0.5 mg/L indole-3-butyric acid (IBA) generated optimum rooting frequency (95.2 ± 0.81%), the number of roots/shoot (7.5 ± 0.107), and the mean root length (4.5 ± 0.133 cm). Later the acclimatized plantlets were successfully established with almost 85% survival (Moghaddam et al., 2011).

Different plants have different tolerances to cleaning chemicals and some plants are able to withstand higher concentrations and longer durations of cleaning chemical treatment compared to other plants. In addition, the origin, type, and storage time of the plant tissue used for micropropagation has impacts on how easily they can be thoroughly sterilized (George, 2008). Usually, plant tissues collected from the field (which is subject to all kinds of various fungal spores, bacteria, and contaminants from the open air) tend to be more difficult to thoroughly clean; however, plant tissues obtained from plants grown in more controlled environments (such as plants grown in sterile commercial potting mixes in a closed greenhouse) tend to be easier to clean (George, 2008). The type of plant tissue used is also important; a bud tissue obtained from a subterranean crown, rhizome or bulb is usually harder to clean than a bud or meristem obtained from an aboveground stem or leaf. In addition, the longer a plant tissue is stored (such as in cold storage) before being sterilized, the more difficult it is to sterilize as many bacteria and fungi will still be able to grow while the plant cannot (George, 2008).

It is difficult to determine standard sterilization procedures that apply to all plants. As *S. integrifolium* is so new to micropropagation, many factors such as which type of bud tissue should be used, its tolerance to different cleaning agents, and which cleaning agents would be best for its sterilization are unknown. In *S. perfoliatum*, success with sterilizing seeds for tissue culture was achieved by using a combination of 70% ethanol, then 5% calcium hypochlorite with 2-3 drops of the surfactant Tween, 3% hydrogen peroxide/or 30% sodium hypochlorite (bleach). In addition, high temperature sterilization

(placing seeds in 96% alcohol then burning them for a few seconds) is also effective in surface-sterilization of seeds (Tomaszewska-Sowa & Figas, 2011). The embryos excised from seeds were placed onto Murashige and Skoog medium (MS) diluted to half-strength and sprouting occurred within the next few weeks. Germination process occurred under 16-hour photoperiod at around 25 degrees C and with humidity of 70%. Apical meristems of seedlings were isolated and were placed onto new media containing growth regulators NAA (an auxin), BAP (a cytokinin), and ABA (abscisic acid) (Tomaszewska-Sowa & Figas, 2011).

Chapter 2 Determining *S. integrifolium*'s Flowering Response to Cold Treatment

Introduction

S. integrifolium Michx. (silflower, siphium, rosinweed) is an herbaceous, cold-tolerant perennial indigenous to 21 states in central and eastern United States. The species has naturally large seeds and has been considered a potential new oilseed crop because its fatty acid profile is similar to sunflower (Kowalski & Wiercinski, 2004; Van Tassel et al., 2014; Tassel et al., 2017). *S. integrifolium* grows from a fibrous root system containing small rhizomes, and it begins growth as a basal rosette in the first year (Vilela et al., 2018). A first-year plant can reach 20-69 cm with an average of 32.7 leaves (Kowalski, 2004). As a perennial, *S. integrifolium* has coarse taproots that can use water stored deep in the soil so that it can withstand short-term droughts and could provide greater returns by reducing cost of irrigation. In addition to becoming a potential dual-purpose perennial crop both as oilseed and biomass crop, *S. integrifolium* can also be grown in unfavorable environments with unreliable soil moisture, nitrogen leaching, and irrigation problems (Vilela et al., 2018). As a native herbaceous perennial plant in the family of Asteraceae, *S. integrifolium* also provides ecosystem benefits as a source of nectar and pollen for a wide range of pollinating insect (Van Tassel et al., 2017; Vilela et al., 2018). Agronomic and breeding research has commenced to help realize the potential for *Silphium* to become a new perennial oilseed crop.

Selection and field trials have been carried out at The Land Institute in Salina, Kansas and University of Minnesota for longer than 15 years (Vilela et al., 2018). Breeding efforts have focused on improving agronomic traits such as yield, number of flowers, and seed size. However, *S. integrifolium* has a long juvenile phase, which is defined as the vegetative period following germination when flowering does not occur and the bud meristem is not "competent" to respond to seasonal environmental inductive cues (Samach, 2011). For perennial plants, the duration of the juvenile phase is the main factor driving the length of the breeding cycle (Nocker & Gardiner, 2014). *S. integrifolium*

remains vegetative during the 12 months following transplantation (Vilela et al., 2018), and this long juvenile phase slows the rate of breeding progress.

Shortening the lengthy juvenility period of *S. integrifolium* can be achieved through an effective vernalization process. Vernalization is "the acquisition or acceleration of the ability to flower by a chilling treatment" (Chouard, 1960). A plant's ability to flower by chilling treatment is an adaptation for plants in the Northern hemisphere in temperate regions to withstand freezing temperatures during winter (Chouard, 1960; Amasino, 2004). Many plant species in temperate climates need prolonged exposure to low non-freezing temperatures to initiate the floral transition, and this vernalization requirement prevents the risk of flowering in fall and winter, but permits flowering the following spring (Glover, 2014; Kim & Sung, 2014). While this plant response is evolutionarily advantageous in a natural environment, it would be beneficial to manipulate vernalization to facilitate shorter generation times in a breeding context.

Various factors determine the effectiveness of the vernalization process. Vernalization response is not immediately triggered by low temperatures; instead, flowering only occurs when the cold stimulus is removed and warm temperatures follow. This indicates that the length of the cold period must be sufficiently long so that the plant avoids flowering after a brief period of cold followed by a short return of warm temperatures in fall (Amasino, 2004). Transitioning from vegetative to reproductive growth in the fall prior to more extreme cold in the winter will reduce the chance for winter survival. The length of cold exposure needed by a plant to transition from the vegetative to reproductive stage varies by species with 1-3 months as typical (Michaels & Amasino, 2000). The temperature that plants experience will also determine the vernalization response. Vernalization must occur in non-freezing temperatures to ensure metabolic activity. The optimal temperature ranges from 1-7 °C although 4° C is the typical temperature. Plants' vernalization response can be facultative or obligate. For plants that have a facultative response, the primary stimulus for floral induction is photoperiod and cold exposure is not required but can accelerate flowering. For plants with an obligate requirement, vernalization is absolutely necessary for flowering (Engelen-Eigles &

Erwin, 1997; Michaels & Amasina, 2000). Most perennials have obligate vernalization requirement (Padhye et al., 2006).

Besides environmental cues such as prolonged exposure to cold and photoperiod, plant age can also determine whether plants may flower after the vernalization treatment. At the juvenile phase, young seedlings of most plants are not competent enough to flower even if inductive environment cues exist (Amasino, 2010). Before being vernalized, juveniles must reach a critical developmental age and grow to a minimum pre-vernalization size before vernalization can occur, which can vary even within populations of a species; a perennial's juvenile phase can be as long as several decades (Amasino, 2010; Reekie et al. 1997; Wesselingh et al. 1997).

S. integrifolium has not been studied for the vernalization requirement, but many members of Asteraceae family require vernalization to induce flowering. For example, *Polymnia canadensis* seedlings can respond to vernalization with only 1 pair of true leaves and will flower under both long and short days (Bender et al., 1999). Safflower (*Carthamus tinctorius*) can be induced to flower faster if seeds are exposed to prolonged cold temperatures (Cullerne et al., 2021). *Rhodanthe floribunda*, an Australian native flower species, has a facultative vernalization requirement—plants were capable of flowering without a cold treatment but cold treatment reduced the time to anthesis. It can also respond to vernalization at a very early stage; older seedlings with longer cold exposure are especially floriferous (Ha and Johnston, 2013). Since other members of the Asteraceae can respond to vernalization at a very early age, it would be useful to determine the effect of age on vernalization response in *Silphium*.

The efficiency of trait selection and genetic improvement in breeding programs is inversely related to the duration of the breeding cycle (Matsoukas, 2014). Late onset of flowering severely hampers the breeding process of trees and many other perennials (Jung & Müller, 2009). Controlling flowering time allows plant breeders to reduce generation time and speed the development of inbreds or breeding populations. Therefore, understanding and investigating *S. integrifolium*'s response to vernalization to

get early flowering has practical significance. The objectives of this study are to 1) determine the length of cold exposure needed to induce flowering and 2) determine the effect of plant age on response to vernalization. Methods that reduce time to flowering will enable shorter breeding cycles and accelerate domestication of Silphium.

Materials and Methods

Two experiments were conducted to characterize the vernalization response in *Silphium*. The first experiment explored the length of cold treatment required for successful vernalization, and the second experiment explored the effect of plant stage development on the response to cold treatment. Successful vernalization was determined by observation of bolting, i.e. a stem emerged from the rosette, signifying a transition from the juvenile phase to the adult phase.

Cold Exposure Time Experiment

Stratified seeds of eight different genotypes of *S. integrifolium* (represented as G1 through G8 in this study) were planted in August of 2020. In this case, each genotype was defined as open pollinated seed from an individual plant from the University of Minnesota *Silphium* Domestication Panel. This panel was comprised of 380 genotypes assembled from plants developed by the Land Institute (Price, 2021). Plants were grown in Sungrow professional potting mix in 5.08 cm wide cone-tainers arranged in 8x8 grids with natural winter lighting (short-day; about 10 hours of light) in a greenhouse at 24°C. Fertilizer was not applied during any part of their growth. When the plants were six months old, they were exposed to 8 different lengths of cold treatment, from 0 weeks (control) to 7 weeks; 8 plants from each genotype were selected, one for each cold treatment length including the control. A total of 56 plants (excluding the 8 control plants) were placed into a lighted cold chamber maintained at 40% humidity and 12-hour light photoperiod at 28.5 $\mu mol.m^{-2}s^{-1}$ light intensity at 4.4 °C. Every week, 8 plants (1 from each genotype) were removed from the cold chamber and repotted into 12.7cm wide square pots in commercial Sungrow professional growing mix and placed into the greenhouse in natural light with long day lengths supplemented by 12+ hours of 1000 $\mu mol.m^{-2}s^{-1}$ light intensity at 24 °C. Each plant was observed every three days to

determine the date of bolting and flowering. Bolting was defined as the emergence of a defined stem from a rosette, and flowering was defined as full yellow coloration of the ray florets on a floral bud. The pots in which the plants were grown were arranged on the greenhouse bench in a completely randomized order.

Data analysis

A binomial logistic regression was conducted in R Studio (RStudio Team, 2020) with the stats 3.6.2 package to determine whether cold treatment had a significant impact on bolting. Data was analyzed via linear regression analysis in Excel to determine the relationship between cold treatment on time to bolting and time to flowering. One-way ANOVA analysis was conducted in Excel to compare the effects of genotype on time to bolting and flowering.

Age Response to Cold treatment Experiment

In this experiment, 300 seeds were obtained from open-pollinated plants of 7 different genotypes (R1 through R7) from the University of Minnesota's variety trials at the St. Paul field site in the summer of 2020 (Table 2.1).

Table 2.1. Genotypes and experiment names

Experiment name	Genotype name
R1	Rc 19-3
R2	Rc 9-7
R3	Rc 18-8
R4	Rc 14-8
R5	Rc 03504PL20
R6	Rc 4-1
R7	Rc 01307PL20

Seeds were stratified prior to planting by removing them from the inflorescences and placing them onto wet paper towels containing a 1% solution of hydrogen peroxide to reduce pathogen growth. The wet paper towels were placed into a Styrofoam container with another wet paper towel on the top of the seeds to maintain a moist environment. A wet cotton towel was placed on top of the paper towels and another Styrofoam tray was

placed on top of the cotton towel. The Styrofoam trays containing seeds were placed into a cold chamber set at 4.4 °C for 4 weeks. Water was added at 2 weeks to prevent drying out. After 4 weeks, the stratified seeds were removed from the cold chamber, separated into groups of 30 from each genotype, and planted into Sungrow professional potting mix in 12.7cm wide square pots and grown in a naturally lighted greenhouse at 24 °C. They were not fertilized during any part of the experiment. The plants were grown until they reached five different distinct stages of physiological development (Table 2.2):

Table 2.2. *S. intergrifolium* development stages

Stage	# of leaves	Time to reach each stage*
S1	2-4	1-2 weeks
S2	4-6	2-3 weeks
S3	6-8	3-4 weeks
S4	8-10	4-5 weeks
S5	10+	5-6 weeks

*Time needed to reach each stage is genotype dependent.

Each stage had 5 representative individuals from each genotype for a total of 175 plants (7 genotypes x 5 growth stages x 5 reps/stage). As plants reached the required growth stage, they were moved to a lighted cold chamber maintained at 40% humidity and 12-hour day lengths with full-spectrum light at 4.4 °C for 4 weeks and watered once a week. After 4 weeks, the plants were moved from the cold chamber to the greenhouse in natural light with long day lengths supplemented by 12+ hours of 1000 $\mu mol.m^{-2}s^{-1}$ light intensity at 24 °C. A no cold treatment control was not performed for this experiment as the previous vernalization experiment which utilized plant material genetically related to the plants used in this experiment indicated that cold treatment was required for bolting and flowering. Data were collected on the time required for each growth stage to bolt after leaving the cold chamber as well as the time required to flowering after bolting initiated. The experiment was terminated 15 weeks after plants were removed from the cold chamber.

Data Analysis

Data was analyzed via binomial logistic regression performed in R studio to determine if growth stage (plant age) had a significant effect on whether a plant bolted or not. A test of equal proportions was done on growth stages with high percentages of bolting and flowering to determine if the values of bolting and flowering were significantly different from each other. One-way ANOVA analysis was done in Excel to determine if growth stages with high percentages of bolting and flowering were significantly different from each other with respect to time to bolting and flowering. For the ANOVA analysis, growth stage was a fixed effect with days to bolting and flowering as response variables. All significance levels were set at alpha= 0.05.

Results

Effect of cold treatment duration on bolting and flowering

Plants that did not receive a cold treatment (control) did not bolt or flower (Table 2.3). A minimum of three weeks of cold treatment was required to get 100% of plants to bolt. Most of the plants that bolted eventually flowered, but that was not always the case, particularly in the 4-week treatment. Binomial logistic regression analysis (either 1 or 0 if a plant bolted or not) with 0 weeks of cold treatment as the intercept showed that cold treatment had a significant effect (P=7.263e-5) on bolting. The cold treatment of 1-week was not significantly different from the control (P=0.17). Since most plants did not bolt at the 1-week treatment, comparisons of cold treatment effects on time to bolting and flowering will focus on the 2-week to 7-week treatments. The ANOVA revealed a significant effect of cold treatment period on days to bolting and flowering (p= 0.0005 and 0.06, respectively). The number of days required for bolting to initiate after removal from the cold treatment gradually decreased from 24.7 days (2-week) to 15.6 days (7-week). The average time to flower after being removed from the cold gradually decreased from 89.3 days (2-week) to 75.6 days (7 week). There was a significant linear relationship between length of cold treatment and days to bolt and flower after cold treatment (Fig. 2.1 and Fig. 2.2). Although plants vernalized for 1 and 2 weeks had the least days to bolting and flowering since the start of the cold treatment, these plants did not bolt or flower as consistently as plants in other groups. The shortest days to bolting

since the start of cold treatment among the treatments that had consistent bolting (3-7 weeks) was the 3-week treatment at 45.3 days.

Table 2.3. Response of *Silphium integrifolium* to length of cold treatment.

Cold treatment length (weeks)	% of plants bolting	% of plants flowering	Ave. days to bolting after cold	Ave. days to flowering after cold	Total days from cold treatment to bolting	Total days from cold treatment to flowering	Ave. Days from bolting to flowering
0	0.0	0.0					
1	37.5*	25.0	36.3	90.5	43.3	97.5	56.0
2	87.5	75.0	24.7	89.3	38.7	103.3	63.6
3	100.0	87.5	24.3	86.1	45.3	107.1	62.4
4	100.0	37.5	20.1	84.0	48.1	112.0	66.0
5	100.0	75.0	17.4	79.2	52.4	114.2	64.0
6	100.0	100.0	21.9	80.0	63.9	122.0	60.9
7	100.0	100.0	15.6	75.6	64.6	124.6	60.0

** Not significantly different from the control (0 weeks) based on logistic regression.*

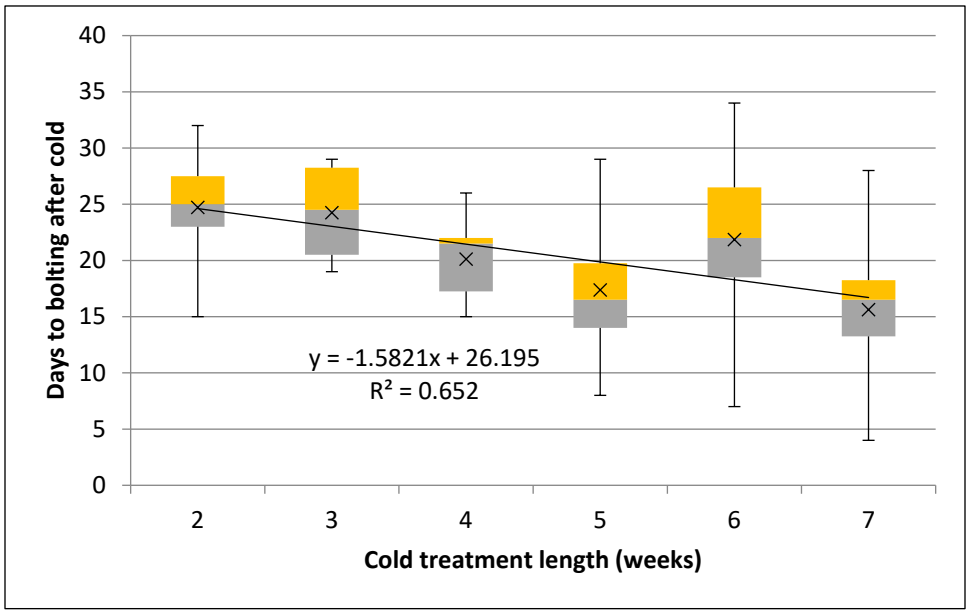

Figure 2.1. Effect of the length of cold treatment on days required to bolt after cold

treatment from 2 to 7 weeks; linear regression analysis gives a multiple R-squared value of 0.652, an adjusted R-squared of 0.5601, and a P-value of 0.053.

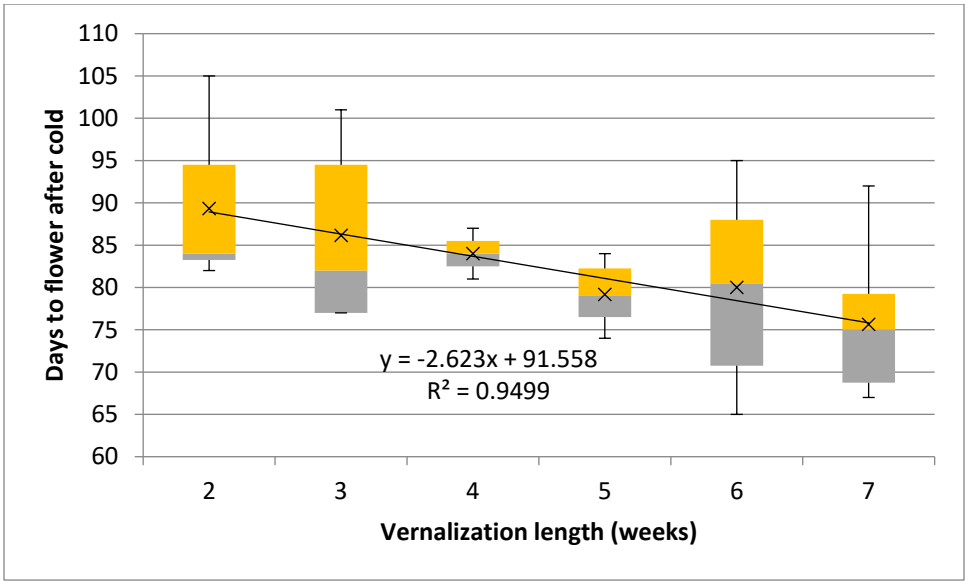

Figure 2.2. Effect of the length of cold treatment on days required to flower after cold treatment from 2 to 7 weeks; linear regression analysis gives an adjusted R-squared of 0.95 and a P-value of 0.0009.

Each of the eight replicates in the experiment was a different genotype, so we also analyzed the effect of genotype on days to bolting and flowering across all the cold treatment times when bolting or flowering occurred. There was no significant effect of genotype on days to bolting or days to flowering (Fig. 2.3). However, genotypes did have a significant effect ($P<0.0001$) on days required from bolting to flowering.

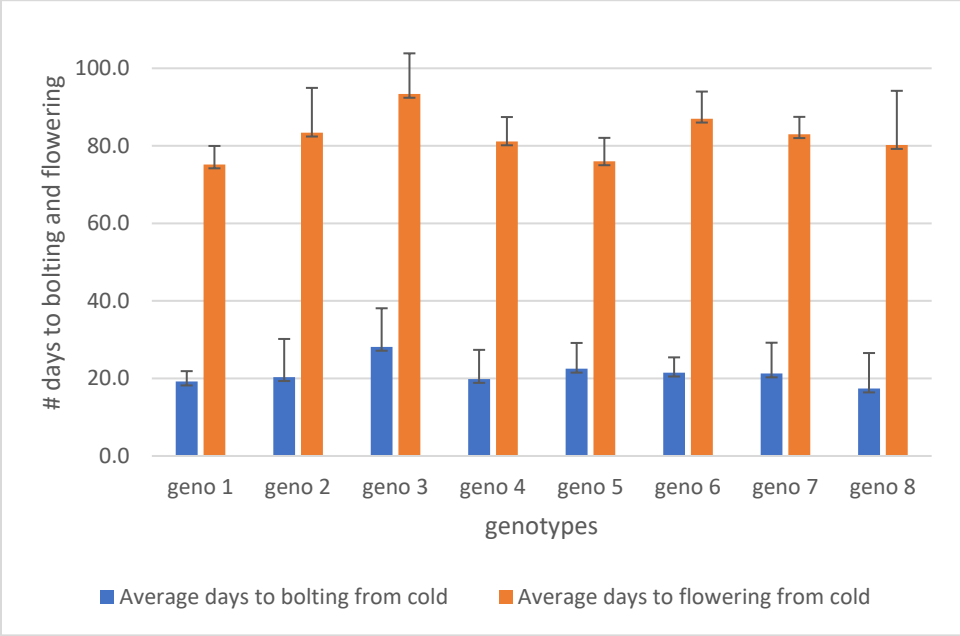

Figure 2.3. Responses of eight genotypes 1 through 8 to cold treatment. Error bars are the standard deviation values.

Effect of plant developmental age on bolting and flowering

Plants did not consistently flower until they reached stages 4 and 5 before entering the cold treatment (Table 2.4). Plants at growth stage 4 required an average of 18 days to bolt after cold treatment, 74.5 days to flower after cold, and 56.8 days from bolting to flowering. Plants at growth stage 5 required an average of 17.9 days to bolt after cold treatment, 71 days to flower after cold, and 53.5 days to flower. These values are slightly less than the values of plants that were vernalized for 4 weeks from the cold treatment length experiment (average of 20.1 days to bolt after cold treatment and 84 days to flower after cold treatment). In the age experiment, 71.4% stage 4 plants bolted and 82.9% stage 5 plants bolted; 51.4% stage 4 plants flowered and 65.7% stage 5 plants flowered. The percent plants bolting in this experiment (71.4%) was less than for the four-week treatment (100%) in the cold treatment length experiment. The equal proportions test indicated that there was no significant difference in number of plants that bolted and flowered between growth stages 4 and 5 (P=0.25 and P=0.22, respectively).

Table 2.4. Response of different growth stages of *Silphium integrifolium* to cold treatment.

Growth stage	% of bolting	% of flowering	Av. days to bolting after cold	Av. days to flowering after cold	Av. days from bolting to flowering
S1	8.6	0			
S2	8.6	0			
S3	14.3	2.9			
S4	71.4*	51.4	18.0	74.5	56.8
S5	82.8*	66.7	17.9	71.0	53.5

** Logistic regression showed stages 4 and 5 have a significant effect on bolting.*

Binomial logistic regression determined that plant age as a whole had a significant effect on bolting (P=4.64e-12). It also indicated that growth stages 1 through 3 did not have a significant effect on bolting (p= 0.457), while stages 4 and 5 were found to have a significant effect on bolting (p= 3.53e-7 and p= 1.59e-7 respectively). There was no significant difference between stage 4 and stage 5 in the number of days required to bolt, flower, or between bolting and flowering (P= 0.881, 0.0895, and 0.103 respectively).

Discussion

Requirement for vernalization and length of cold treatment period

The vernalization response can be facultative or obligate. For plants with facultative vernalization requirement, photoperiod is the primary stimulus for floral induction and cold exposure is not required but can accelerate flowering. In contrast, for plants with an obligate requirement, plants cannot flower without prior cold exposure (Engelen-Eigles & Erwin, 1997; Michaels & Amasino, 2000). Based on the findings of the cold treatment length experiment in which none of the control plants bolted or flowered, we conclude that *S. integrifolium* has an obligate vernalization requirement. This is in accordance with the finding that most commercially-grown perennials have obligate vernalization

requirement for flowering (Padhye et al., 2006). We found that the length of the cold treatment decreases time to flower by decreasing time to bolting; as cold treatment time increased, the days required to flower after cold treatment decreased. Similar findings were also reported in other species. For example, in peaches, increased chilling exposure decreased the time to bud-break (Okie & Blackburn, 2011); in *Brassica napus*, a crop that can be both annual or biennial, increased vernalization length was found to decrease time required to flower even in annual lines (Ferreira et al., 1994). Plants vernalized for longer periods also have higher percentage of flowering; this is similar to the response of *Chrysanthemum superbum* (a member of the *Asteraceae*) to cold treatment when flowering was most frequent with longer periods of chilling of up to 16 weeks of cold (Damann & Lyons, 1996). In addition, in *Coreposis grandifolia*, another member of the *Asteraceae*, the number of plants that produced inflorescences (flowered) was more than doubled on plants vernalized for 8 weeks compared to those exposed to cold for 6 weeks (Niu et al., 2002).

Vernalizing plants for 3 weeks can greatly reduce breeding cycle time

Increasing cold treatment time did not significantly decrease total time (since the start of cold treatment) to flower. Plants vernalizing for 3 weeks yielded the least total time needed to induce flowering. Based on the regression analysis, each additional week of vernalization beyond 3 weeks resulted in 2.6 days less to flower. So in practical terms, three weeks of vernalization is the fasted path to flowering. The estimated 107 days from the start of cold treatment to flowering using a greenhouse and cold chamber would be considerably shorter than planting in the field in the fall and reaching flowering by the following June or July. One may choose to vernalize *S. integrifolium* for two different reasons: one would be to induce flowering while the other to induce bolting. Inducing flowering is useful for generation of seed, phenotyping floral structure, and carrying out hybridization. Inducing bolting is useful for generating more aboveground biomass for analysis and phenotyping or tissue culture using axillary buds. Vernalizing for 3 weeks will provide the shortest time for both bolting and flowering.

Flowering response to cold treatment is size-dependent

The effect of cold treatment may be dependent on the physical size of the plant; the size at which a plant may respond to vernalization varies greatly from species to species (Prins et al., 1990). *Crisium vulgare* plants, a member of Asteraceae, were found to have a better chance of flowering at larger rosette diameters and larger plant weights (Prins et al., 1990). It was also found that rosette diameter of *Crisium* and many other *Asteraceae* species before or just after winter (or cold treatment period) was a good predictor of flowering. In other plants, individuals must achieve a certain post-cold treatment physiological size (different depending on species) in order to flower (Bender et al., 2002). In this study, plants in growth stages 1 through 3 did not reach a size sufficient to respond to cold treatment, and those from stages 4 and 5 were found to be able to respond to cold treatment, although they did not bolt or flower at a 100 percent rate. In practical terms, growing plants to stage 4 or 5 for inducing flower is desirable, and it is possible that if plants were exposed to cold at developmental stages beyond stage 5, bolting and flowering percentages may increase further. Size-dependent vernalization need in *Silphium* indicates that *Silphium* also must reach a certain size before being able to respond to vernalization and subsequently flower.

On the other hand, in some Asteraceae species, vernalization for flowering can be achieved at very young ages. For example, *Polymnia canadensis* plants can be vernalized for flowering with just 1 pair of true leaves. In Ha and Johnson's 2013 research on *Rhodanthe floribunda*, seedlings of four age groups ranging from 1 to 28 days old were exposed to different cold periods from 0 to 3 weeks at 20/10 °C. This study determined that *R. floribunda* has a facultative requirement for flowering in response to vernalization and can respond to cold treatment at a very early stage (as early as 2 weeks old), indicating a short juvenile phase of this species (Ha and Johnson, 2013). Additionally, the longest cold exposure and the oldest seedlings prior to chilling had faster development rate and were more floriferous (Ha and Johnston, 2013). In the present study, however, *S. integrifolium* plants with only 1 pair of true leaves (growth stage 1) were not successfully vernalized for flowering. The existence of an outlier flowering in stage 3 may be due to genetic differences in this particular genotype, as there have been reports of young *S.*

integrifolium plants bolting when planted as seed in the fall and were exposed to brief cold spells before the killing frost as observed in some trials at The Land Institute. Sufficient stores of carbohydrates allow metabolic processes relating to vernalization to occur (Niu et al., 2002). It is possible that plants at stages 4 and 5 are capable of responding to cold treatment and flowering because they have sufficient stores of carbohydrates.

It is interesting to note that the average days to flowering after cold treatment for the 4-week treatment in the cold treatment length experiment (84 days) was slightly longer than the stage 4 and 5 age experiment (71.0 and 74.5, respectively) despite the fact that these plants were much older. This may be because of a genotype difference as well as a difference in plant age between the two experiments. There are reports of *S. integrifolium* seedlings capable of bolting and flowering without any cold treatment; it is possible that the plants used in the cold treatment age experiment are capable of bolting and flowering sooner than the plants used in the cold treatment length experiment.

Light, temperature, and other factors' role in successful cold treatment

All cold treatments in this experiment were conducted in a lighted growth chamber; it is possible that if the plants were vernalized in complete darkness, they may not have been able to respond to cold treatment due to insufficient carbohydrates caused by lack of light. Niu et al., (2002) reported that complete darkness coupled with a very low temperature (-2.5°C) caused poor plant performance and even plant death. The cold treatment done in this experiment was at 4.4°C and plants were not hardened off prior to low-temperature treatment; it is possible that hardening/acclimating plants at a slightly higher temperature (such as 5°C) can allow plants to withstand a lower cold treatment temperature (Niu et al., 2002), which may result in a better vernalization response. Light quality (such as ratio of red to far-red light) may also play a role in the vernalization response (Niu et al., 2002) which may be investigated in future *S. integrifolium* research.

Conclusion

In terms of increasing the speed and efficiency of breeding of *S. integrifolium* and facilitating the domestication process, these experiments provide valuable insight. I propose a possible "speed breeding" scheme in which *S. integrifolium* seed is treated with 200 µL of ethephon diluted in 500ml of deionized water overnight (Reinert et al., 2018) to break dormancy, then planted and grown to growth stage 5 (with 10 or more leaves) which will require around 1 to 2 months. They will then be moved into a cold chamber and vernalized for 3 weeks in 4.4 centigrade, then moved back to the greenhouse for bolting and flowering. This process will require 2 months for seeds to germinate and grow to sufficient size, one month for cold treatment in a cold chamber, and around 2 months to bolt and flower for a total of approximately 5 months from seed to flower, at which point pollinations/breeding and floral phenotyping may be conducted.

Chapter 3 Establishing a *S. integrifolium* Sterilization Protocol for Mircropropagation

Introduction

Found in 21 U.S. states, *S. integrifolium integrifolium* Michx. (rosinweed) is a native herbaceous perennial plant closely related to sunflower (Van Tassel et al., 2017). Because of its long-lived nature, high quality forage biomass yield comparable to corn, along with edible seeds with high quality oil akin to domesticated oilseed sunflower, this species has piqued interest in the plant science community for its potential to become a domesticated perennial oilseed/forage crop (Vilela et al., 2018). In addition, *S. integrifolium* possesses a complex and deep root system that allows it to improve soil structure and health as well as provide food to a wide range of native pollinating insects (Vilela et. al, 2018). Domestication efforts have been ongoing for longer than 15 years with experimental trials occurring primarily in Kansas. Currently, *S. integrifolium* is propagated via seed, cuttings, and divisions. As an obligate outcrossing species, growing from seed cannot achieve true-to-type propagation; propagation through cuttings and divisions however is slow, and cannot ensure disease-free production. Previously, there did not exist a sterilization and micropropagation protocol suitable to achieve rapid true-to-type multiplication of *S. integrifolium*. Obtaining significant numbers of elite genotypes developed from breeding and selection efforts thus has been difficult since clonal propagation for *S. integrifolium* has been limited to cuttings and division.

Micropropagation is a tissue culture-based technique for the *in vitro* regeneration of whole plants from organs, tissues, cells or protoplasts (Beversdorf, 1990). Micropropagation is dependent on the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions in order to produce true-to-type clones of pathogen-free plants (George, 2008; Loyola-Vargas & Ochoa-Alejo, 2018). Perhaps the most important step for culture establishment is the sterilization of the explants. An effective way of preventing bacterial contamination *in vitro* requires the elimination of contaminating microorganisms from the initial plant explants that are introduced into the culture (Mihaljević et al., 2013). The methods for reducing

contaminations include the use of explants from donor plants maintained under a strictly sanitary regime, reduction of the size of the initial explants to just the apical meristem and eliminating microbial contaminants by pretreatment with one or more chemical antiseptics as well as using antibiotics when explants are cultured on a nutrient medium (George, 2008; Mihaljević et al., 2013).

Perennial crops can have endophytes in specific tissues or can be infected systemically. Endophytes are mainly bacteria entering through natural openings on the plant surface, or through wounds (Cassells, 2012) although fungal endophytes are also known (Waqas et al., 2015). Surface sterilization is not always sufficient for field-grown plants if endophytic contamination is detected, and antibiotics and/or fungicides may be added to the culture medium as an attempt to eliminate the contaminant (Cassells, 2012; Shields et al., 1984). Many factors can determine the success of sterilization, including the types of sterilizing agent(s) used and their concentrations, the period of exposure of the plant tissue to the sterilization agent(s), and the plant organ type used (crown buds, stems, leaves, etc.). Some sterilizing agents include ethanol, sodium hypochlorite (bleach), hydrogen peroxide, and Plant Preservative Mixture PPM, a proprietary mixture of two broad-spectrum isothiazolone biocides. Although it is difficult to eradicate endophytic bacteria with PPM because of the time required for uptake, greater absorption of PPM can be achieved through vacuum infiltration, which allows the sterilizing compound to penetrate deeper into tissues than occurs by simply soaking tissues in the sterilizing compound (Tague et al., 2006). PPM was also found to infiltrate younger axillary buds more effectively, potentially because younger plant tissues are both thinner and less dense (Miyazaki et al., 2010).

Because of the convenience and the potential of decreasing the breeding cycle, i*n vitro* techniques are now applied extensively in many breeding programs; however, the lack of well-established *in vitro* techniques often limits more extensive practical uses especially with newer species (Zulkarnain et al., 2015). At present, another species in the *Silphium* genus, *Silphium perfoliatum* L. (cup plant), has been studied regarding the processes required for seed sterilization and micropropagation from apical explants of seedlings in

in vitro cultures (Tomaszewska-Sowa & Figas, 2011). But, in contrast, there does not exist a *S. integrifolium* micropropagation protocol. This current research aims to establish a *S. integrifolium* tissue sterilization protocol for stems (for clonal propagation), seeds (for embryo rescue or callus culture), and anthers (for anther culture), which would be useful for future research on *S. integrifolium* micropropagation and related tissue culture methods.

Materials and methods

S. integrifolium's stems, seed, and anthers were utilized in this work. In July 2021, three-year-old field-grown plants were dug out of the soil from field plots located in the St. Paul campus of the University of Minnesota, MN and immediately, washed, the top 25 percent of their stems were trimmed off, and they were planted in 12-in. pots with Sungrow professional sterile potting mix and set to grow in the greenhouse at 32.2°C. After five weeks, the new stems that were produced were cut into 30 pieces with two nodes that were 4 cm long and utilized for sterilization protocols. Seed and anther tissues were also collected from other greenhouse-grown plants grown in the same conditions. MS medium (Murashige and Skoog, 1962), with 15 g of sucrose and 2 ml of PPM per liter was used as the growth media. Solutions for sterilization contained plant preservative media (PPM) which is composed of 5-chloro-2-methyl- 3(2H)-isothiazolone and 2-methyl-3(2H)- isothiazolone (Plant Cell Technology, Washington, DC) ethanol, Softsoap antibacterial soap (Colgate-Palmolive, New York City, NY), commercial bleach (6% NaClO, Clorox, Oakland, CA) 20%, hydrogen peroxide (H_2O_2), and 10%, Polysorbate 80 (Tween 80, a nonionic surfactant wetting agent to decrease surface tension). All plant tissues were grown in petri dishes with sterile MS medium after sterilization for one month in growth chambers at 25°C with a 12-hour light photoperiod at 28.5 $\mu mol.m^{-2}s^{-1}$of light intensity supplied by cool white florescent tubes to promote growth and development.

Procedure for sterilizing stems

1. Removed all leaves from stems except leaf primordia near the meristem of each stem.

2. Washed tissues with antibacterial soap and 10% alcohol for 5 minutes.
3. [Alternative step] Vacuum infiltrated stems with 2ml/L PPM for 3 minutes at 600 Torr. This step can be skipped if choosing to do step 9.
4. Washed stems with 2ml/L of PPM in double-distilled water and gently shaken by hand for 1 minute.
5. Washed with 1% antibacterial soap for 1 minute; rinsed twice with 2 ml/L PPM.
6. Poured 70% ethanol over stems for 5 seconds in sterile sieve; rinsed again with 2 ml/L PPM.
7. Placed stems into 20% bleach with 1 added drop of Tween 80, and gently shook periodically for 20 minutes.
8. Rinsed with 2 ml/L PPM.
9. [Alternative step] Placed into 10% H2O2 for 10 minutes followed with a final wash with 2 ml/L PPM. This step can be skipped if step 3 (vacuum infiltration) was performed.
10. Placed onto sterile MS culture medium.

Procedure for sterilizing seeds

This procedure was essentially the same as the above procedure for sterilizing stems but without the vacuum infiltration nor H_2O_2 treatments. The seed coat was removed before sterilization.

Procedure for sterilizing anthers

1. Using sterile forceps, removed anthers from an unopened or slightly opened *S. integrifolium* flower bud.
2. Washed with 2 ml/L PPM for 1 minute, and then washed with 1% antibacterial soap for 1 minute.
3. Placed into 20% bleach solution with 1 drop (~20 µl) of Tween 80 added and gently swirled periodically for 20 minutes.
4. Washed with 2 ml/L PPM for 10 seconds, dipped into 70% ethanol for 5 seconds, and followed with a final wash with 2ml/L PPM for 10 seconds.
5. Placed onto sterile MS culture medium.

Results

After being grown in the growth chamber for 5 weeks, all 30 stems sterilized with this protocol were still clean and showed no visible signs of microbial contamination, and 20 had begun growing primordial leaves with the stems displaying phototropic bending and rooted in the media, indicating that the sterilization procedure was effective (see Fig. 3.1). However, a small amount of necrosis was present on some stems which possibly occurred as a result of the 10% H_2O_2 treatment. To test this possibility, the stem sterilization experiment was repeated using the stem materials from the same plants, and the H_2O_2 was replaced with vacuum infiltration of 2% PPM in the beginning of the protocol; stem tissues resulting from vacuum infiltration exhibited a 100% success (no colonies of bacteria/fungi visible on all 30 stems used) without tissue damage. Seeds and anthers sterilized with the described protocols remained without observable contamination, with seeds germinating on their plates after stratification for 4 weeks (see Fig. 3.2) and anthers remaining viable (not desiccated nor necrotic) that could be transferred onto growth media containing hormones such as auxins and cytokinins.

Figure 3.1. Sterilized stems growing in vitro

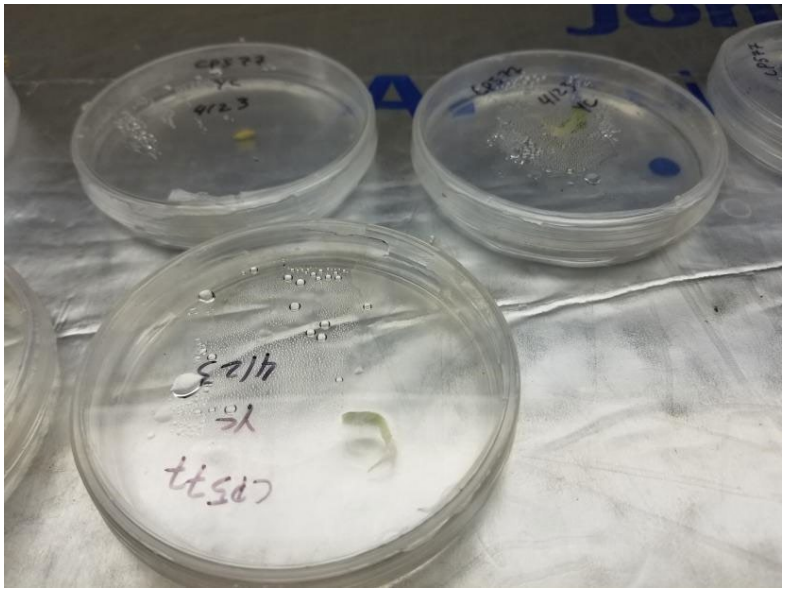

Figure 3.2**.** Sterilized seeds germinating *in vitro*

Discussion

The protocols to obtain microbe-free stem, seed, and anther tissue in *S. integrifolium* will facilitate future research on micropropagation and *in vitro* mutagenesis using these tissues. They are not dissimilar from the micropropagation protocol proposed for a related species, *S. perfoliatum*, which achieved good results with 30% bleach and 70% ethanol as the main agents in the seed sterilization process (Tomaszewska-Sowa & Figas, 2011); however, that procedure was limited to seeds which are of limited use in the replication of elite germplasm lines as seeds cannot ensure true-to-type propagation, whereas this current protocol was shown to be effective with stems and anther tissues as explants, which would thus allow for clonal propagation as opposed to the seed-based protocol used in the *S. perfoliatum* experiments. Any sterilization protocol potentially may need to be adapted for plants grown in other environments or for genotypes with quite different morphologies. Such difference can influence physical behavior during sterilization or, for plants grown under quite different conditions; it may affect external or endophytic microbial populations. Plant tissues used in these experiments were obtained from plants grown initially in fields in the upper Midwest in North America and are thus from a more or less uniform source. Although genetic and extrinsic difference can vary (i.e. characteristics such as stem size, presence, and size of trichomes on the stem), these

protocols provide useful procedures that can be optimized for individual differences should changes prove necessary.

The stem sterilization protocol we utilized included taking plants from the field and greenhouse acclimating the plants that produced shoots in the greenhouse over a five-week period. However, the field-grown plants in this experiment produce new shoots within a week in the greenhouse. Thus, it is possible that the shoots produced within the period one week of transplant or longer may be suitable should a more rapid field to culture time be desired. Similarly, although the sterilized seeds required stratification for germination after being sterilized, the dormancy requirement may be overcome by incorporating a growth regulator such as Ethephon (an ethylene-release agent) into the sterile growing media (Reinert et al., 2018). By combining methods such as reducing seed dormancy periods as well as tissue culture micropropagation utilizing this protocol, the breeding process of *Silphium* may be shortened considerably.

Conclusion

The results of the *S. integrifolium* sterilization protocol can be applied in any *in vitro* procedures such as development of a full micropropagation protocol, mutagenesis experiments, callus formation/transformation experiments, and many more. The sterilization protocol serves as the first step in obtaining more disease-free, true-to-type *S. integrifolium* tissues to be used in subsequent *in vitro* experiments or for propagation purposes. Obtaining healthy and viable *S. integrifolium* plants can speed up the breeding process even more as a cutting propagated *in vitro* with effective ratios of growth hormones and then acclimated into a greenhouse environment may flower even faster than a seed-propagated plant which will require time to germinate and does not have the head start a cutting may have. In short, establishing sterilizing protocols for micropropagation will help to speed up the breeding and domestication process by allowing for the mass production of disease free, true-to-type desirable genotypes.

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