EVALUATION OF MASCULINIZATION TREATMENTS TO PRODUCE FEMINIZED HEMP SEED (CANNABIS SATIVA L.)

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ABSTRACT

Evaluation of Masculinization Treatments to Produce
Feminized Hemp Seed (Cannabis sativa L.)

Ted Matthew Fitzgerald

Cannabis sativa L. (hemp) develops plants with either male or female flowers, and growers of hemp greatly prefer female flowers which bear the glandular trichomes that contain cannabinoids. Feminized (all female) seeds are highly desired, which are produced by crossing a female plant with a masculinized female plant. Masculinization is achieved through the inhibition of ethylene and/or addition of gibberellins before flower initiation in female plants. The hemp industry uses silver thiosulfate (STS) to masculinize hemp, but spraying silver poses environmental concerns. This study compared STS to three other ethylene-inhibiting agents: aminoethoxyvinylglycine (AVG), cobalt nitrate (CBN), and 1-methylcyclopropene (1-MCP). Treatments of STS and CBN also included gibberellic acid as a synergist. Plants treated with STS exhibited superior masculinization and pollen dispersal compared to plants treated with AVG, CBN or 1-MCP. Only plants treated with STS or AVG produced pollen in sufficient quantities for collection. This pollen was assayed for germination potential initially and after storage for up to five weeks at 22.2, 7.2, or 1.1°C. Pollen from plants treated with AVG remained viable for four weeks at 1.1°C, whereas STS-treated plants produced pollen that was viable for three weeks at 1.1°C. Due to phytotoxicity problems with AVG, STS remains the best treatment to masculinize female hemp plants when breeding for feminized seeds. In a separate study, flower tissues of hemp had considerably higher total cannabinoid concentrations compared to leaf tissues but significantly lower ratios of cannabidivarin (CBDV) to cannabidiol (CBD). To reduce variability, at least 1 g samples of fresh leaf or flower tissue should be extracted with 10 mL of methanol. Rapid throughput testing of cannabinoids as part of a breeding program should use flower tissue, preferably at the time typical of harvest.

Keywords: Dioecious, Feminized, Masculinization, Ethylene Inhibition, Cannabinoids

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Chapter 1

INTRODUCTION

Hemp (*Cannabis sativa* L.) is a crop that has been cultivated for millennia, yet there is limited scientific understanding of the species. The nutritive content of the seed, medicinal properties of the cannabinoids in the flowers, and the strength of the fibers all make hemp an ideal crop for a multifaceted production model. Breeders of hemp have developed diverse germplasms with many different forms, functions, phenotypes, and purposes. Various methods to breed *Cannabis* have evolved from clandestine practices to applied horticultural techniques as legalization, for both marijuana and hemp, continues.

Recently, there has been an increasing demand for the terpenophenolic substances found predominantly in female flowers of hemp. These chemicals, called cannabinoids, are unique to *Cannabis* and exhibit a wide array of medicinal properties. A cannabinoid of particular interest to hemp plant breeders is cannabidiol (CBD), but many other cannabinoids such as cannabidivarin (CBDV) are generating interest.

Hemp is naturally dioecious. As CBD content is highest in female flowers, hemp breeders have developed techniques to induce masculinized female plants which enable the production of feminized seeds. However, these techniques pose scalability and environmental concerns. It is crucial to investigate the problems associated with feminized seed production so that breeders produce feminized seeds utilizing sustainable practices. The objectives of this research were to 1) evaluate various masculinizing agents, 2) assess pollen viability after storage at various temperatures, and 3) characterize the cannabinoid profiles of leaves and buds of hemp as well as changes in cannabinoid content during prolonged extraction.

Chapter 2

LITERATURE REVIEW

Cannabis sativa L. (hemp) is normally dioecious, with male and female reproductive organs on separate plants (Sakamoto et al., 1995; Mandolino et al., 1999). It is naturally diploid (2n = 20) with homogametic XX chromosomes found in female plants and heterogametic XY chromosomes found in male plants (Sakamoto et al., 1995; Mandolino et al., 1999; Divashuk et al., 2014; Razumova et al., 2016). Sexual expression in hemp is controlled primarily by genetic makeup but can be influenced by both environmental (Heslop-Harrison, 1956) and hormonal factors, as research has shown that plant growth regulators can induce both male and female characteristics in the plant (Galoch, 1978; Mohan Ram and Sett, 1982). In hemp, ethylene biosynthesis increases in meristematic tissues prior to the initiation of female flowers and, conversely, male flowers form when ethylene is suppressed in meristematic tissues (Adal et al., 2021).

Inhibiting ethylene (C_2H_4) synthesis or action, and high levels of gibberellic acid (GA_3), are known to promote masculinization of hemp, whereas auxin, ethylene, and kinetin promote female flower formation (Ram and Jaiswal, 1972; Galoch, 1978). When silver thiosulfate ($Ag(S_2O_3)_2$), an ethylene-action inhibitor, is applied to developing genetically female hemp plants before flowering is initiated, it causes the plants to produce male flowers that contain pollen grains (Mohan Ram and Sett, 1982; Lubell and Brand, 2018). All-female (feminized) seeds are produced when this pollen fertilizes other female flowers. This technique also allows for self-pollination when developing inbreds to produce F1 hybrids, though inbreeding depression becomes a concern when seed is produced through selfing (Kurtz et al. 2020).

Feminized seeds are highly desired by growers of *Cannabis*, both for the psychoactive

and non-psychoactive forms, since the production of cannabinoids in female flower clusters (called "colas" in hemp) is significantly higher than in male clusters (Small et al., 1975; Clarke and Merlin, 2013). Female flowers possess abundant glandular trichomes on their surfaces (Small and Cronquist, 1976). These secretory trichomes serve as biochemical manufacturing sites for numerous resinous cannabinoids, flavonoids, and terpenes that have purported therapeutic effects (Joy et al., 1999; Onaivi et al., 2005). Unpollinated female flowers are preferred in a cannabinoid cropping system, since pistillate flowers reduce the production of secondary metabolites when seeds begin to form (Clarke and Merlin, 2013).

The creation of feminized seeds through the conversion of hemp female to male flowers has not been fully developed for large-scale operations, nor has it been optimized to maximize the production of female seeds. Additionally, current practices for feminized seed production are heavily dependent on silver, which can be toxic to the environment. Silver nitrate – which is the source of silver in silver thiosulfate mixtures – is considered one of the most toxic silver compounds, but silver complexed by thiosulfate has been considered to have low environmental toxicity (Ratte, 1999). Nevertheless, other forms of masculinization are needed to decrease the hemp industry's dependency on silver for breeding purposes.

Botanical Classification

Hemp is an herbaceous, annual plant indigenous to Central Asia and belongs to a small family of flowering plants, Cannabaceae, which also includes *Humulus* (hops) and *Celtis* (hackberry) (Small, 1972). The taxonomic status of the genus *Cannabis* has been debated since it was originally classified by Carl Linnaeus, but the most commonly agreed upon delineation is a monotypic genus (*Cannabis*) with only one representative species (*sativa*) (Rana and Choudary, 2010; Small 2015). However, many other species and subspecies have

been postulated, such as *indica* and *ruderalis*, due to the plant's phenotypic variability and adaptive behavior (Hillig, 2005). The plant's ability to interbreed within the same genus has resulted in a lack of speciation (Small, 1972; Small, 2015). Its adaptive ability to acclimate to a variety of environmental stresses has also resulted in a wide range of physiological and phenotypic characteristics. Although the plant is taxonomically a single species, it has a unique ability to express polymorphic attributes that yield multiple forms with multiple uses.

Flower Expression

The ability to manipulate sexual expression in hemp is an important tool in the development of new cultivars (Moliterni, 2004). Hemp has a malleable, polymorphic sexual phenotype (Galoch, 1978; Small 2015), and many plant growth regulators have been shown to influence its sexual expression (Mohan Ram and Sett, 1982; Lubell and Brand, 2018). It is possible to obtain complete or partial sexual conversion (male to female; female to male) using exogenously-applied compounds. In addition, the appearance of bisexual (or hermaphroditic) flowers can occur in stressful environments where the plant is subjected to unfavorable growing conditions (Bear, 2020), a phenomenon referred to as rodelization.

Pollination is anemophilous and xenogamous, i.e., pollination is by wind and occurs between two distinct individuals (Heslop-Harrison & Heslop-Harrison, 1969; Cruden, 1977; Small and Antle, 2003; Rana and Choudary, 2010). Flowers are typically imperfect, either staminate or pistillate, and incomplete. Staminate flowers consist of loose panicles and have actinomorphic perianths with five tepals (Rana and Choudary, 2010). Within the tepals lie oblong anthers attached by short filaments. Male plants typically grow faster and reach anthesis earlier than female plants.

Female plants typically are shorter, bushier, and reach maturation later than male plants

(Rana and Choudary, 2010). As photoperiod gets shorter, female flowers form and produce dense clusters of racemes at the apical meristem, exposing the style and stigma for pollination. The stigmatic surfaces of female flowers contain single-celled, hair-like filaments which collect airborne pollen.

Photoperiodism

Photoperiodism is defined as the physiological response of plants to night length (period of darkness), although plants are commonly referred to as short- or long-day, or day-neutral in their responses (Jarillo et al., 2008). *Cannabis* is naturally a short-day plant, but some accessions can initiate flowering in continuous light (Borthwick and Scully, 1954; Heslop-Harrison, 1969). Plants that initiate flowering irrespective of daylength (i.e., night length) are known as day-neutral plants, colloquially referred to as "autoflowers" in the hemp industry. Autoflowering lines initiate flowering after a prescribed amount of time (28-35 days in most cases). These day-neutral responses are believed to be adaptions that developed in extreme northern latitudes (to avoid the frost of early winter), or in equatorial regions where fluctuations in daylength are relatively minor (Small, 2015).

Chromosomal Make-up

Research has indicated that sexual determination in *Cannabis* is regulated by an X:autosome equilibrium based on a X chromosome counting system without an active Y chromosome (Faux et al., 2014). Even though hemp is naturally dioecious, monoecious varieties have been cultivated, which has led to research on the mechanisms driving sexual determination (Hall et al., 2012; Razumova et al., 2016). Recent evidence has supported the idea that monoecious cultivars have a quantitative XX chromosomal make-up (Faux et al., 2013). Sexual determination and expression in hemp, however, remains poorly understood

and requires additional investigation.

Chemotypes

A chemotype refers to the chemical phenotype of specific lineages within *C. sativa*. Five chemotypes of *C. sativa* have been described, comprised of undomesticated, landrace, and cultivated varieties (Meier and Mediavilla, 1998). These chemotypes are separated by their accumulation of tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabigerol (CBG) as well as the ratios among those cannabinoids. Chemotype I refers to high-THC, low-CBD accessions; chemotype II refers to high-THC, high-CBD accessions; chemotype III refers to low-THC, high-CBD accessions; chemotype IV refers to low-THC, low-CBD and high-CBG accessions; and chemotype V refers to accessions seldom containing cannabinoids but instead having increased production of terpenes and other volatiles (Mandolino and Caroni, 2004; Pacifico et al., 2008). All hemp varieties are from the chemotype III, IV, or V lineages.

Cannabinoids

Cannabinoid content in hemp is primarily influenced by chemotype but also by variety, growing practices, environmental factors, and density of trichomes on female inflorescences. Over 113 cannabinoids have been identified, although the exact composition and levels of the cannabinoids varies between lineages and varieties (Krizek et al., 2018).

Cannabinoids are present in the plant as acidic molecules and may biosynthesize into other cannabinoids via gene expression or spontaneous decarboxylation when exposed to light or heat (Krizek et al., 2018). The initial cannabinoid in the biosynthetic pathway is cannabigerolic acid (CBGA). CBGA is the precursor to cannabigerol (CBG) – the decarboxylated form – which can then convert to either tetrahydrocannabinolic acid (THCA),

cannabidiolic acid (CBDA), or cannabichromenic acid (CBCA), depending on the presence of specific synthase genes (Reekie et al., 2018; Toth et al., 2020).

To extract cannabinoids from plant materials, various organic solvents – predominantly methanol – have been used. Historically, cannabinoid-extracting solvents included chloroform mixed with methanol, but recently there has been a push to exclude chloroform because of toxicity and oncogenic concerns (Krizek et al., 2017). Explicit protocols for cannabinoid extraction are lacking in the literature, but in a recent study by Patel et al. (2018), 200 mg of dry, ground *Cannabis* flower were extracted in 20 mL of methanol:chloroform (9:1) with homogenization. Dry *Cannabis* samples would be expected to have higher cannabinoid concentrations on a weight-to-weight basis compared to fresh samples.

Pollen Viability

Plant breeders store pollen to facilitate variety development and to preserve germplasm (Choudary et al., 2014). Hemp pollen is very delicate, and its ability to fertilize ovaries rapidly declines after anthesis. Storage must preserve its viability - defined as the ability of a pollen grain to produce an actively lengthening pollen tube (Gaudet et al., 2020).

A variety of techniques to assess pollen viability have been developed, including staining pollen grains with fluorescein diacetate (FDA) to assess enzymatic activity, germinating grains on an agar-sucrose germination medium to observe pollen tube growth, or releasing airborne grains onto live receptive pistillate flowers to confirm seed set (Choudary et al., 2014; Gaudet et al., 2020). Assessing viability using FDA does not necessarily indicate the ability for a pollen grain to germinate. FDA may indicate the presence of enzymatic activity within the grain even when the grain is unable to produce a pollen tube. Therefore, germination on culture medium is a preferred method (Zottini et al, 1997; Engelmann and

Takagi, 2000; Gaudet et al., 2020).

Gaudet et al. (2020) found that *Cannabis* pollen from a genetically male plant can be stored for up to three weeks at 4°C. The pollen had an initial viability of 30-50%, and the viability was 22% after three weeks. The pollen was collected 64 days after seed sowing. This study also found that pollen could remain viable up to 4 months using cryopreservation coupled with vacuum desiccation. After 4 months, pollen viability was 14.6%.

Masculinized pollen, which contain homogametic (XX) chromosomes as opposed to pollen with heterogametic (XY) chromosomes, is invaluable, as it is a necessary precursor to feminized seed production. Optimizing the storage conditions for masculinized pollen is necessary, as these grains have been shown to have numerous irregularities (DiMatteo et al., 2020). It was postulated that the irregularities were due to abnormal meiosis, improper exine formation, or problems with tapetal cells. In a recent experiment by DiMatteo et al., (2020), the germination rate of pollen grains from 5 masculinized hemp genotypes ranged from 0 to 13%. Pollen irregularities greatly affect pollen viability and, as a result, can lead to reduced fertilization, seed set, and seed formation (Baghali et al., 2011). The pollen from masculinized hemp plants is highly valuable, and pollen preservation trials must occur to understand short and long-term usage of storage practices.

Feminized Seed

Growers producing hemp for cannabinoid content desire seeds or other propagules that only produce female flowers. Increased expenditures and decreased efficiency of space utilization can be expected when growing normal, non-feminized seed. Roguing – the process of eliminating male plants – is very time-consuming and labor-intensive, especially at large-scale production sites (Reel, 2019). Additionally, evidence has indicated that having seeds in

the female flowers can lead to lower cannabinoid yields (Meier and Mediavilla, 1998). It is therefore greatly preferable to use feminized seeds - or other forms of female plants, i.e., clones - to avoid unwanted pollination.

Summary

Since the legalization of hemp production for research purposes in the United States in 2014, there has been ongoing breeding by growers and horticulturalists to develop varieties with high cannabinoid (especially CBD) content. A seed production technique which produces all female seed has been developed to meet the demand of hemp growers for maximizing cannabinoid and terpene content as well as the number of smokable flowers. During "feminized" seed production, breeders cross a genetically female hemp plant producing viable pollen to another female plant with pistillate flowers. Techniques to produce feminized seed have been shared among *Cannabis* breeder through both hearsay and published articles, but the methods and procedures have yet to undergo thorough and rigorous scientific testing. Silver thiosulfate readily induces masculinization in female hemp plants and is extensively used by the hemp industry. Additional research is needed to investigate alternatives to silver thiosulfate, an expensive and potentially environmentally-toxic chemical.

Chapter 3

MATERIALS AND METHODS

Hemp plants were grown in an outdoor research field located in Goleta, CA and in a greenhouse located in Sebastopol, CA. The field study consisted of two replications staggered three weeks apart, whereas the subsequent greenhouse trial was a single replicate to further evaluate the most efficacious treatments as indicated by the field study. In all trials, a feminized day-neutral hemp variety, 'Auto Blu' (Beacon Hemp, Santa Rosa, CA), was subjected to ethylene inhibition treatments. Since feminized day-neutral hemp initiates almost exclusively female flowers irrespective of daylength, the first application of the treatments occurred 30 days after seed sowing, with two more applications occurring a week apart on days 37 and 44.

Field Trial

The control consisted of silver thiosulfate (STS) prepared from stock solutions of silver nitrate (Fisher Chemical, Cambridge, MA) and sodium thiosulfate (Science Company, Lakewood, CO) prepared in a 1:4 ratio per Cameron and Reid (1981) and applied at a concentration of 500 ppm, per industry practice (Stromberg, 2020). Additional treatments were 250 or 500 ppm STS in combination with 25 ppm GA_3, 500 ppm cobalt nitrate ($Co(NO_3)_2$), alone, 250 ppm $Co(NO_3)_2$ in combination with 25 ppm GA_3, aminoethoxyvinylglycine (AVG, ReTain®, Valent Biosciences, Libertyville, IL) at 500 or 250 ppm, and 1-methylcyclopropene (1-MCP, Ethyl-Bloc®, Smithers-Oasis, Kent, OH) at 396 ppm (Table 3-1).

The experiment was a factorial randomized complete block design (RCB) with 8 blocks and 8 treatments. Treatment solutions were made using reverse osmosis water and included a

Table 3-1. Treatments used to masculinize female hemp plants in a field trial in Goleta, CA.

Treatment	Rate	# of Applications	N
STS (control)[z]	500 ppm	3	8
STS w/ GA_3 at 25 ppm	500 ppm	3	8
STS ½ rate w/ GA_3 at 25 ppm	250 ppm	3	8
$Co(NO_3)_2$	500 ppm	3	8
$Co(NO_3)_2$ ½ rate w/ GA_3 at 25 ppm	250 ppm	3	8
AVG full rate	500 ppm	3	8
AVG ½ rate	250 ppm	3	8
1-MCP spray	396 ppm	3	8

[z] STS = silver thiosulfate; AVG = aminoethoxyvinylglycine; 1-MCP = 1-methylcyclopropene; $Co(NO_3)_2$ = cobalt nitrate; GA_3 = Gibberellic acid

surfactant spreader, NuFilm P® (Miller Chemical & Fertilizer, Hanover, PA), at 200 µL per 1L solution. Amber-glass spray bottles were used to hold the solutions to reduce possible chemical degradation from UV light. The 1-MCP spray was prepared by dissolving 0.396 g and applying the solution within 5 minutes of preparation as the solution would rapidly release as a fumigant.

Greenhouse Trial

The greenhouse study was a factorial RCB design with 6 blocks and 4 treatments. Based on the results from the field study, only STS at 500 ppm, and AVG at 250 ppm or 500 ppm, were used in this trial (Table 3-2). As before, applications were made every 7 days for three weeks total except for the treatment of 250 ppm AVG, which was applied twice weekly for a total of 6 applications in an attempt to decrease AVG phytotoxicity while maintaining a high

Table 3-1. Treatments used to masculinize female hemp plants in a greenhouse trial in Sebastopol, CA.

Treatment	Rate	# of Applications	N
STS (control)[z]	500 ppm	3	6
AVG full rate	500 ppm	3	6
AVG ½ rate	250 ppm	3	6
AVG ½ rate	250 ppm	6	6

[z] STS = silver thiosulfate; AVG = aminoethoxyvinylglycine

hemp masculinization rate.

Experiment 1: The degree of masculinization (DoM) of treated hemp plants was assessed at one and two weeks after the last application of the treatments. The conversion ratio, staminate induction propensity, and relative pollen abundance – estimated by pollen quantity and output – were determined. A categorical scale (Lubell and Brand, 2018) was used which separates the percentage masculinization into 5 distinct groupings where 1 = 0% masculinized flowers, 2 = 1-30% masculinized flowers, 3 = 31-60% masculinized flowers, 4 = 61-99% masculinized flowers, and 5 = 100% masculinized flowers (Fig. 3-1).

Experiment 2: Once anthers had begun to shed pollen, which occurred on the 60th day after sowing in both the field and greenhouse trials, pollen grains were collected and assessed for viability initially and after subsequent storage for up to five weeks at 1.1°C, 7.2°C, or 22.2°C. Only the hemp plants treated with STS and AVG produced pollen in sufficient quantity to be collectable.

To assess viability, 2,000 to 3,000 pollen grains from each treatment were incubated in a germination solution which consisted of 170 g sucrose, 300 mg $Ca(NO_3)_2$, 100 mg H_3BO_3,

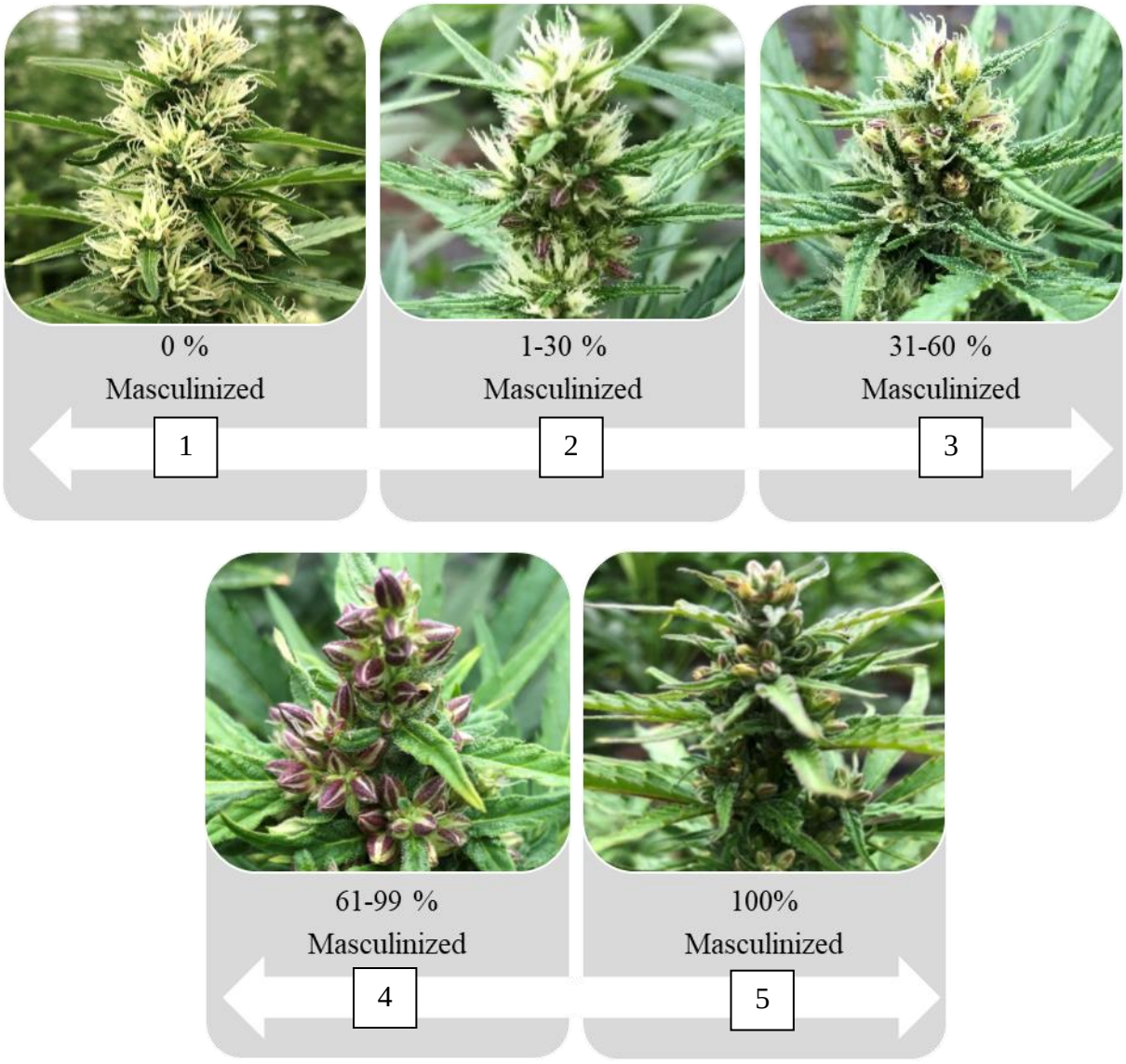

Fig. 3-1. Degree of masculinization scale adapted from Lubell and Brand (2018).

and 7 g agar in 1 liter of deionized water adjusted to pH 6.4 (Zottini et al. 1997). The solution was autoclaved then continuously stirred when subsequently cooling. Testing was initiated when the incubation solution reached 32°C, at which point 60 µl was added to a fresh pollen sample which had been placed in a 1.5 ml vial. The vial was vortexed at high speed for 10 seconds to distribute the pollen in the solution. The solution was poured onto a Petri plate and covered with a glass slip. The Petri plates were then wrapped in parafilm and placed in an

incubation chamber for 18 hours at 24°C. A DM IRBE microscope (Leica, Allendale, NJ) with a 50-W mercury lamp was used to assess pollen viability. A pollen grain was considered viable if a pollen tube had emerged from the pollen exine and had extended to twice the diameter of the grain (Zottini et al., 1997; DiMatteo et al., 2020) (Fig. 3-2). The appearance, shape, and any other notable characteristics of the pollen grains were also recorded. The stored pollen was tested for viability every 7 days.

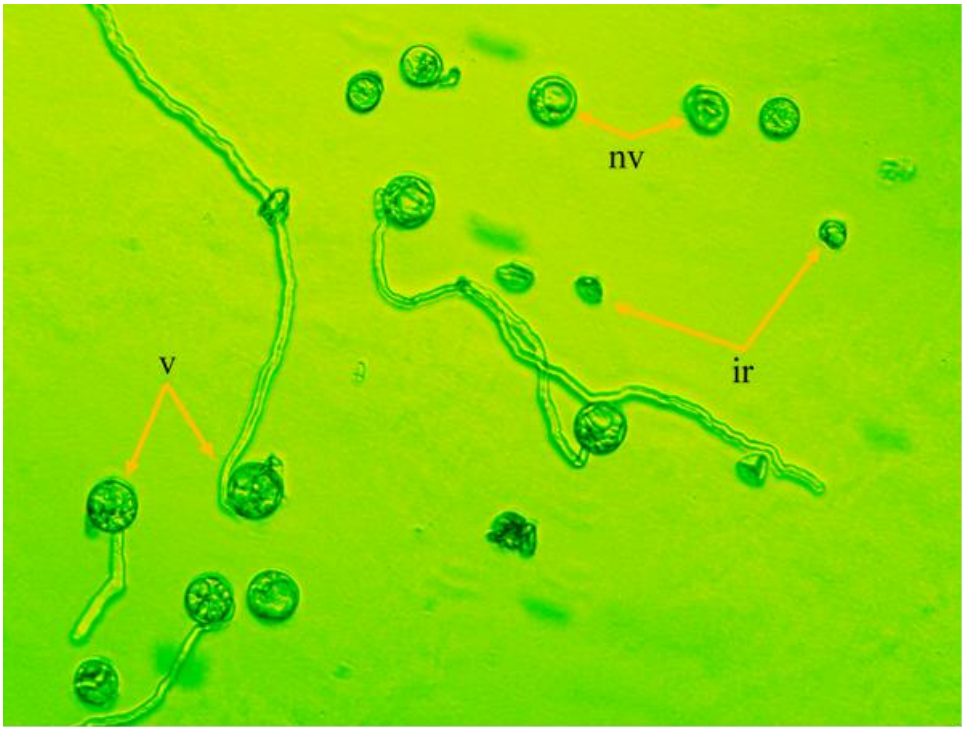

Fig. 3-2. A micrograph showing viable (v), nonviable (nv), and irregular (ir) pollen grains.

Statistical Analysis: Data was analyzed using the PROC MIXED procedure of the Statistical Analysis System (SAS Institute, ver. 9.4). In addition, multiple comparisons for proportions, and Tukey's significant difference test, were utilized to determine differences between treatments within cultivar for the female to male conversion rate. A survival rate

analysis – Cox regression proportional hazards model (JMP, ver. 14, SAS Institute) – was utilized to assess the pollen storage data.

Experiment 3: Breeding lines of hemp were evaluated to determine the overall content of seven cannabinoids within the flower buds and leaf tissues. The cannabinoids evaluated were cannabidiol (CBD), cannabidivarin (CBDV), Δ^9-tetrahydrocannbinol (THC), tetrahydrocannabivarin (THCV), cannabigerol (CBG), cannabigerovarin (CBGV) and cannabichromene (CBC).

Sample Preparation: Autoflowering plants were sampled by collecting a terminal bud on a lower, primary, lateral branch of each plant as the primary apical buds had been treated with silver nitrate, a masculinizing compound. Short-day plants were much slower to flower than the day-neutral plants, so newly-formed leaf tissue, rather than a terminal bud, was sampled from the apex of primary lateral branches. Samples of 0.5 g, 1.0 g, or 1.5 g of flower or 0.25g or 1 g samples of leaf tissue were put into a 15 mL polypropylene tube to which was added 10 mL of HPLC-grade methanol. The extract was filtered through a 0.45 µm nylon syringe filter before injection. All samples, both filtered and unfiltered, were stored at 7.2 °C.

Chromatographic Analysis: Samples were characterized using a Shimadzu GC-2014 gas chromatograph equipped with a flame ionization detector (FID) and an AOCi autosampler. The cannabinoids were separated using an Rxi-35Sil MS, 0.53 ID, 30 m, wide-bore column. The carrier gas was hydrogen held at a flow rate of 3.97 mL/min (Land, 2020).

For analyses, the column temperature was ramped per the following protocol: 190°C for 0.1 min followed by an increase of 10°C per minute up to 225°C for 8 minutes, then 5°C per minute to 250°C followed immediately by 25°C per minute to 315°C. The column was held at 315°C for 1 minute before it was cooled to 190°C (Land, 2020). To avoid degradation of

chromatographic peaks, HPLC-grade acetone was injected after every 10th sample. The injection port septum was replaced every 75 injections, and the injection port liner was replaced every 150 injections, to maintain peak resolution.

The cannabinoid standards CBC, CBD, CBDV, THCV, and CBG were obtained from Millipore Sigma (St. Louis, MO). The Δ^9-THC standard was obtained from Cerilliant (Round Rock, TX) while Cayman Chemicals (Ann Arbor, MI) was the source of the CBGV standard. Calibration curves were developed using the following references concentrations: 500, 250, 100, 50, and 25 ppm (Fig. 3-3, 3-4). Injection volume onto the Rxi-35Sil MS column was 0.6 µL.

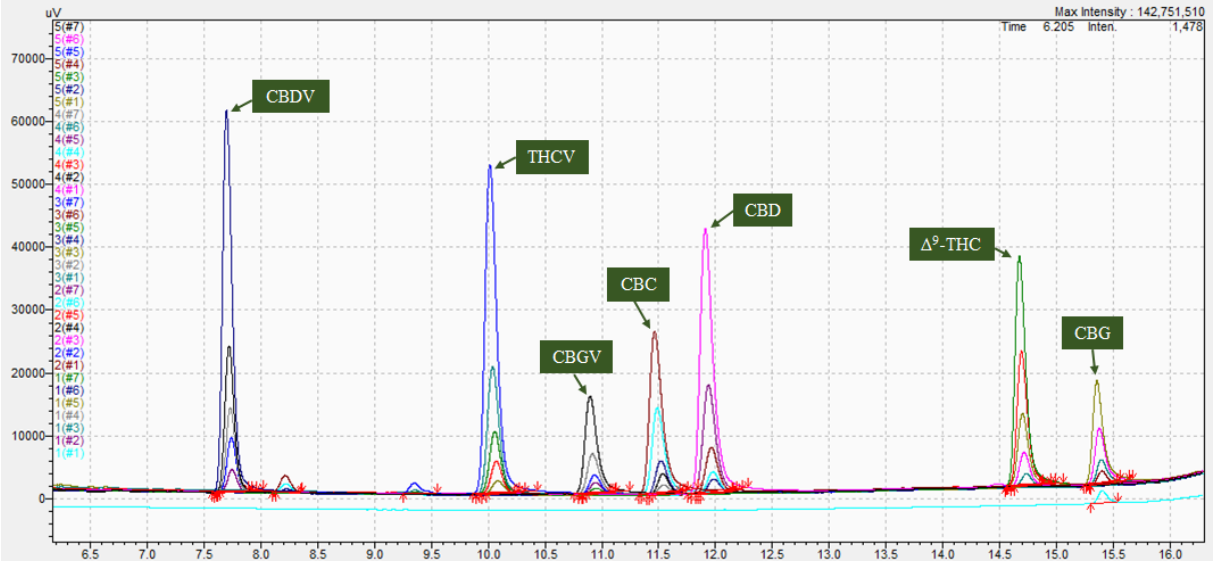

Fig. 3-3. Composite chromatogram indicating the relative elution of 7 cannabinoids and peak size as affected by concentration (500, 250, 100, 50 or 25 ppm). CBDV = cannabidivarin, THCV = tetrahydrocannabivarin, CBGV = cannabigerovarin, CBC = cannabichromene, CBD = cannabidiol, Δ^9-THC = Δ^9-tetrahydrocannabinol, and CBG = cannabigerol.

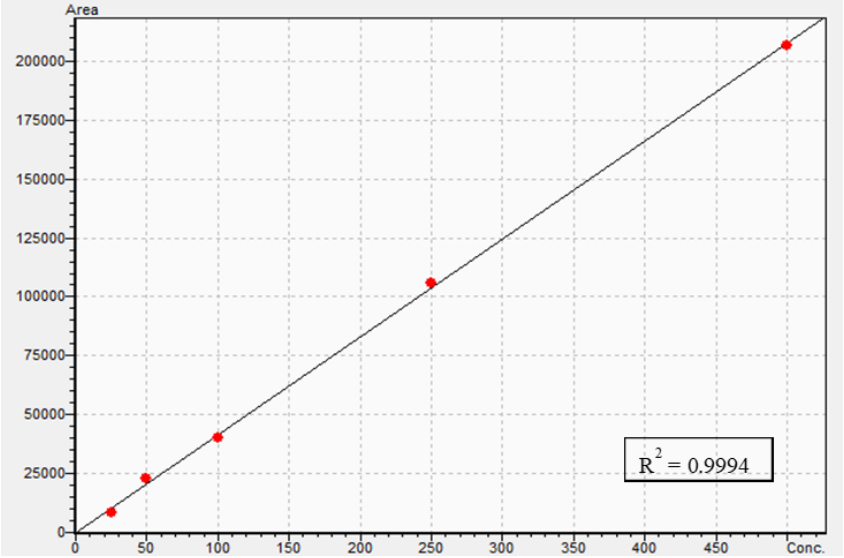

Fig. 3-4. Representative cannabinoid calibration curve: cannabichromene (CBC).

Leaf Extraction Study: Hemp leaves from 2 individual plants were sourced from a greenhouse in Sebastopol, CA and 0.25 or 1 g of tissue was submerged in 10 ml of HPLC-grade methanol for 1, 3, or 5 days. There were three replicates per weight-duration combination. Once the tissue was submerged in the solvent, all samples were stored at 7.2°C. Extracts were filtered and analyzed per the protocol previously described.

Only two cannabinoids, cannabidiol (CBD) and cannabidivarin (CBDV), were used in the final data analysis. Data was subjected to ANOVA and Tukey Kramer's means comparison to determine differences in total cannabinoid content as affected by extraction duration, as well as differences in CBDV:CBD ratio.

Flower Extraction Study: Hemp flowers from 5 individual plants were sourced from a greenhouse in Sebastopol, CA and 0.5, 1, or 1.5 g of tissue was submerged in 10 ml of HPLC-grade methanol for 2, 3, 5, 7, or 10 days. There were five replicates per weight-duration combination. Once the tissue was submerged in the solvent, all samples were stored at 7.2°C. Extracts were filtered and analyzed per the protocol previously described.

The quantities of seven cannabinoids (CBD, CBDV, THC, THCV, CBG, CBGV, and CBC) in the tissues were used in the data analysis. Data was subjected to Tukey Kramer's means comparison to determine differences in total cannabinoid content as affected by extraction duration and tissue quantity. Additionally, data was analyzed for differences in CBDV:CBD ratio.

Chapter 4

RESULTS

Phytotoxicity Overall

Noticeable phytotoxic responses were observed 24 hr after masculinization treatments were first applied to the hemp plants, especially for plants treated with AVG. Plants treated with 250 or 500 ppm AVG exhibited phytotoxicity, which was characterized by chlorosis of the apical meristem and associated newly-expanding leaves (Fig. 4-1). Plants treated with 500 ppm AVG tended to have a greater phytotoxic response and recovered less quickly from the phytotoxicity than plants treated with 250 ppm AVG. For plants sprayed with 250 ppm AVG, the phytotoxicity often diminished within 3 to 4 days after spraying, although yellowing of leaf tips could be present even after flower initiation. Severe AVG phytotoxicity at flowering was observed as yellow banding on older leaves and dried, distorted inflorescences (Fig. 4-2).

In the present study, there appears to be an association between the use of 25 ppm GA_3 and plant death. For both field trials, 12 out of the 13 plants that collapsed and died were treated either with 500 ppm STS + 25 ppm GA_3 (5 plants dead) or 250 ppm STS + 25 ppm GA_3 (7 plants dead). Only one plant that died was treated with 500 ppm STS. Plants treated with AVG developed phytotoxicity, but not to the lethal degree of plants treated with GA_3 (Fig. 4-2). Plants treat with cobalt nitrate developed slight tissue damage, and there was an incomplete conversion of female to male flowers. 1-methylcyclopropene treatments, applied as both a fumigant and spray, did not cause any phytotoxicity but led to zero masculinization (no fumigation results shown).

Fig. 4-1. Hemp phytotoxic responses to 500 or 250 ppm AVG, or 500 ppm STS, 24 hours after first application.

Field Study: Masculinization

Male flowers began to appear during the week after the 2nd set of sprays, especially for plants treated with STS. After 3 applications, the industry standard of 500 ppm STS resulted in superior masculinization (Table 4-1) and pollen dispersal, compared to the other masculinization agents. Plants treated with AVG tended to be more masculinized than plants treated with STS + GA_3. Treatments of CBN and 1-MCP resulted in inferior masculinization and were effectively eliminated from subsequent experimentation. Pollen dispersal was

Fig. 4-2. Hemp responses to masculinization treatments: a. stem elongation in response to 500 ppm STS + 25 ppm GA_3; b. phytotoxic response to 500 ppm cobalt nitrate; c. phytotoxic response to 500 ppm AVG.

observably higher in plants treated with 500 ppm STS compared to plants from the other treatments. Plants treated with AVG at either the 250 or 500 ppm levels were able to produce pollen in substantial quanties, but the quantities still tended to be less than that from plants treated with 500 ppm STS. Incomplete masculinization – female bracts and trichromes still present on the flowers – was observed in some plants treated with either level of AVG. However, approximately 10% of plants treated with 250 or 500 ppm AVG were induced to masculinize to the same extent as plants treated with 500 ppm STS (Fig. 4-3).

Table 4-1. Degree of masculinization of hemp plants as affected by masculinization sprays.

Treatment	Masculinization Rating		
	Field	Greenhouse	Average [z]
500 ppm silver thiosulfate (STS)	4.67 a[y]	4.33 a	4.57 a
500 ppm aminoethyoxyvinylglycine (AVG)	3.88 b	4.33 a	4.00 b
250 ppm aminoethyoxyvinylglycine, once weekly	3.81 b	3.23 b	3.65 b
250 ppm aminoethyoxyvinylglycine, twice weekly		4.17 a	
500 ppm silver thiosulfate + 25 ppm GA_3	3.45 b		
250 ppm silver thiosulfate + 25 ppm GA_3	3.55 b		
250 cobalt nitrate (CBN) + 25 ppm GA_3	2.25 c		
500 ppm cobalt nitrate	1.50 d		
396 ppm 1-methylcyclopropene (1-MCP)	1.00 d		

[z] Masculinization rating for the combined data from the field and greenhouse studies.
[y] Means within a column with the same letter are not significantly different at the 95% confidence level according to the Tukey-Kramer HSD test.

Fig. 4-3. Masculinized female plants shortly before anthesis, one week after final application of masculinization sprays: a. masculinized flowers induced by AVG at 250 ppm; b. masculinized flowers induced by STS at 500 ppm.

Greenhouse Study: Masculinization

The three most efficacious treatments from the field study – weekly applications of 500 ppm STS, 250 or 500 ppm AVG – and a twice-weekly application of 250 ppm AVG, were used in a subsequent greenhouse study. All plants successfully masculinized and produced pollen in sufficient quantities for collection. Treatments of 500 ppm STS or 500 ppm AVG applied weekly or 250 ppm AVG applied twice weekly masculinized plants more than weekly applications of 250 ppm AVG (Table 4-1).

Masculinization Overall

When comparing the three treatments used in both the field and greenhouse, weekly applications of 500 ppm STS induced superior masculinization as opposed to 250 or 500 ppm AVG applied weekly or twice weekly (Table 4-1).

Field Study: Pollen Storage

Pollen from field-grown masculinized plants was collected at anthesis, germinated, and the viability data was subjected to survival analysis. There was no effect of masculinization treatment ($p < 0.5102$) on the germinability of pollen initially or after storage at 1.1, 7.2, or 22.2°C, nor was there an effect of temperature on the storability of the pollen from the field ($p < 0.2272$). The pollen had an initial germinability of 2.2% and, after one week at 1.1 or 7.2°C, germinability increased to 3.3% or 5.8%, respectively (Fig. 4-4). Pollen remained viable for three weeks at 1.1 and 7.2°C.

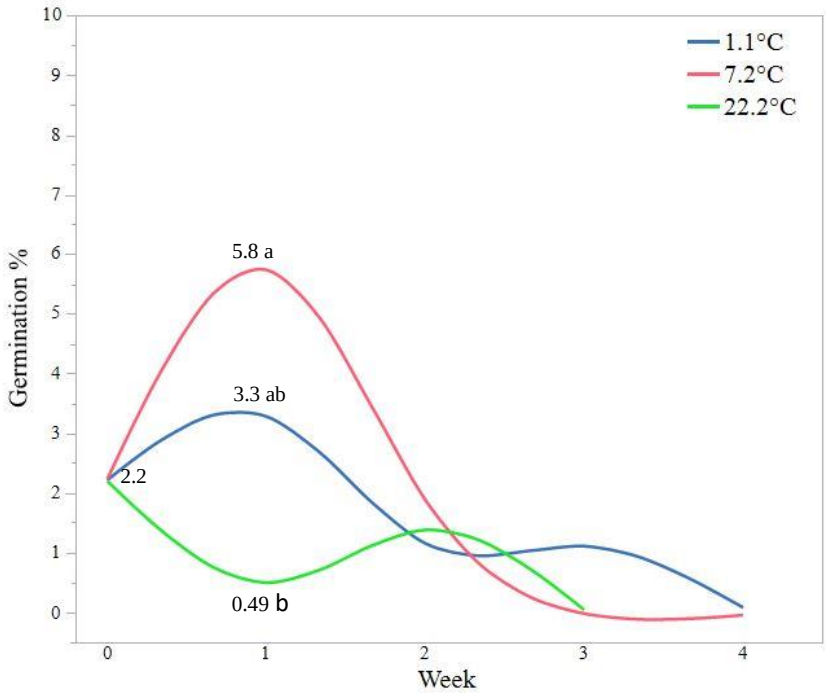

Fig. 4-4. Germination rates of pollen from the Goleta field study, stored at 1.1°C, 7.2°C, or 22.2°C. Week 1 germinability means separated by the Tukey-Kramer HSD test.

Greenhouse Study: Pollen Storage

The germinability of pollen collected from hemp plants grown in a greenhouse was similar to that of pollen from field-grown plants ($p < 0.0001$). The initial germination rate of the pollen was 3.3%. After one week, pollen stored at 1.1°C had a germination rate of 4.9% whereas the pollen stored at 7.2°C had a germination rate of 5.8%. Masculinizing treatment in the greenhouse had no effect on pollen germinability ($p < 0.9355$), but storage temperature had an effect on pollen viability ($p < 0.0045$). Pollen stored at 1.1°C remained viable for four weeks, whereas pollen stored at 7.2°C was viable for three weeks (Fig. 4-5). Pollen stored at 22.2°C remained viable for only one week.

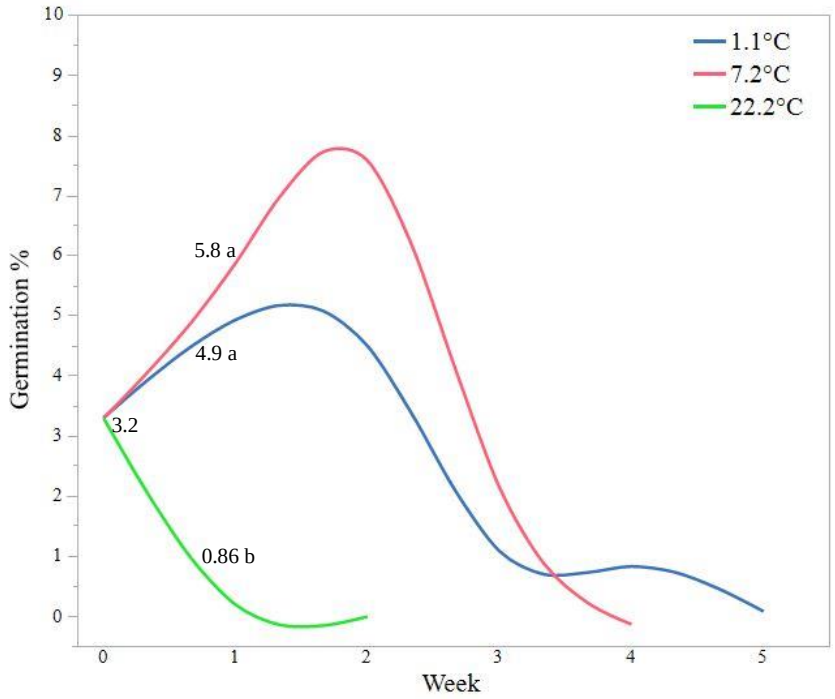

Fig. 4-5. Germination plot of pollen from the Sebastopol greenhouse study stored at 1.1°C, 7.2°C, and 22.2°C with pollen. Week one germinability means separated by Tukey-Kramer HSD.

Survival Analysis

A survival analysis was conducted on the combined pollen viability data from both field- and greenhouse-grown plants. Temperature had a significant effect on the survivability of stored pollen ($p = 0.0021$). The survivability of pollen tended to be higher in the pollen samples stored at 1.1°C with the pollen having the capacity to germinate until week five. These results were followed closely by pollen stored at 7.2°C, where pollen retained the ability to germinate until week four. When pollen was stored at 22.2°C, its capacity to germinate ended by week three (Fig. 4-6). A steady weekly decline in germinability was noticeable for pollen stored at any of the temperatures (Fig. 4-6, 4-7).

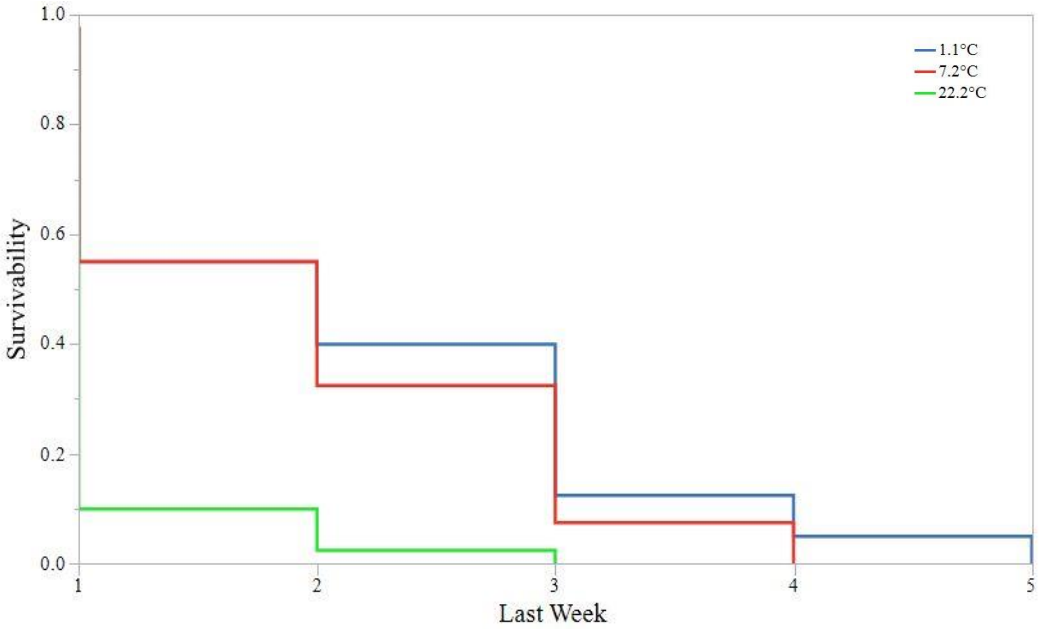

Fig. 4-6. Survivability of hemp pollen as affected by storage temperature. Differences assessed by the Wald's Effect test.

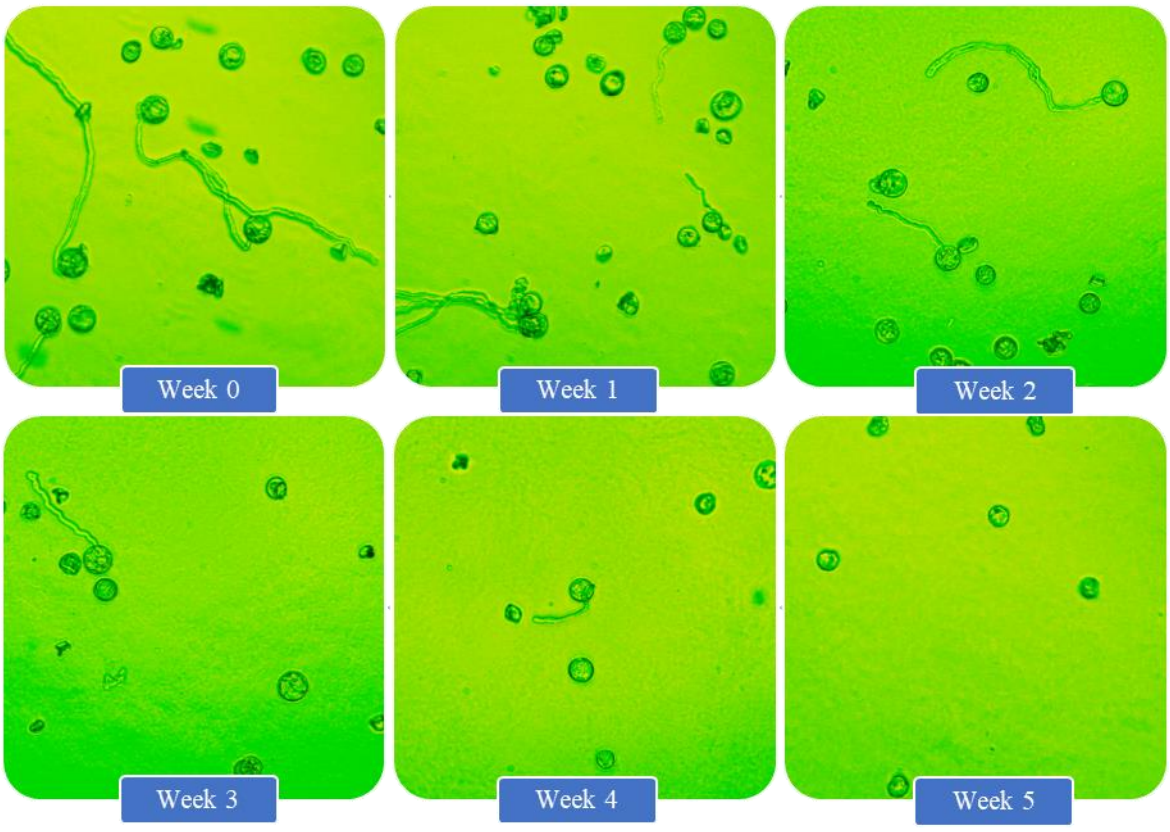

Fig. 4-7. Micrographs of pollen grains from STS-induced masculinized plants, stored at 1.1°C for up to five weeks. If viable, the grains produced pollen tubes.

Pollen Irregularity

There was a slight correlation between the percentage of irregularly-shaped pollen grains and pollen germination rates across all samples during the storage studies. Though the regression model was significant ($p < 0.0001$), the R^2 was very low at 0.1117, indicating that little of the variation in germination could be explained by pollen shape itself. Irregularities in grain shape can influence the viability of pollen (Niklas, 1985; Roeckel-Drevet and Digonner, 1995; Fonseca and Westgate, 2005; DiMatteo et al., 2020).

Plant Height

Field-grown hemp varied in growth much more than greenhouse-grown plants ($p < 0.0001$). In the field, plants treated with a masculinizing mixture incorporating GA_3 grew taller than plants treated with a GA_3-free mixture, as GA_3 promotes elongation of stems and branches. Plants treated with 250 or 500 ppm AVG or 500 ppm STS tended to be stunted in size, but the height of AVG treated plants, overall, was the same as plants treated with 500 ppm cobalt nitrate or 396 ppm 1-MCP (Table 4-2). In contrast, when hemp was grown in a greenhouse, treatment had no effect on height (Table 4-3). Plants grown in fields to serve as pollen donors can be expected to have stunted, more variable height compared to similar plants grown in a greenhouse.

Table 4-2. Height measurements of field-grown ‘Auto Blu’ hemp plants in Goleta, CA.

	Height Measurement (cm)			
Treatment	Day 30	Day 37	Day 44	Day 51
500 ppm STS[z]	14.72 b[y]	31.97 c	51.38 c	66.09 c
500 ppm AVG	15.53 b	32.28 c	52.72 c	68.25 bc
250 ppm AVG	15.78 b	33.31 c	53.50 c	69.28 bc
500 ppm STS + 25 ppm GA_3	19.84 a	47.47 a	74.19 a	97.38 a
250 ppm STS + 25 ppm GA_3	17.03 ab	45.10 ab	72.45 ab	94.95 a
250 CBN + 25 ppm GA_3	18.25 ab	47.28 a	75.56 a	96.06 a
500 ppm CBN	16.22 b	34.78 c	57.09 c	73.31 bc
396 ppm 1-MCP	15.91 b	37.63 bc	61.38 bc	77.28 b

[z] STS = silver thiosulfate, AVG = aminoethyoxyvinylglycine, GA_3= gibberellic acid, CBN = cobalt nitrate, 1-MCP = 1-methylcyclopropene

[y] Means within a column with the same letter are not significantly different at the 95% confidence level according to the Tukey-Kramer HSD test.

Table 4-3. Height measurements of greenhouse-grown ‘Auto Blu’ hemp plants in Sebastopol, CA.

	Height Measurement (cm)			
Treatment	Day 30	Day 37	Day 44	Day 51
500 ppm STS[z]	29.25 a[y]	52.42 a	70.67 a	74.75 a
500 ppm AVG, once weekly	32.92 a	52.75 a	73.67 a	79.50 a
250 ppm AVG, once weekly	33.42 a	57.08 a	79.67 a	85.92 a
250 ppm AVG, twice weekly	31.83 a	54.15 a	74.92 a	80.17 a

[z] STS = silver thiosulfate, AVG = aminoethyoxyvinylglycine,

[y] Means within a column with the same letter are not significantly different at the 95% confidence level according to the Tukey-Kramer HSD test.

Cannabinoids in Leaves

The total content of cannabinoids (CBD + CBDV) in 1 g samples was slightly over twice the content in 0.25 g samples regardless of extraction period (Fig 4-8). When 0.25 g samples of leaf tissue were extracted, the total content of CBD and CBDV tended to decrease over the 5 days of the study (Fig. 4-8). In contrast, the total cannabinoids in extracts of 1 g samples of leaf tissue tended to increase over 5 days extraction, indicating that a longer extraction period was needed to fully leach the cannabinoids from this amount of leaf tissue.

The ratio of CBDV to CBD for 0.25 g samples was over double that of the ratio for 1 g samples (Table 4-4). Since leaf tissue was sourced from the same plants, the samples were expected to yield very similar CBDV:CBD ratios. The results suggest a lack of reproducible quantification when assessing cannabinoid content using a relatively small amount of fresh hemp tissue.

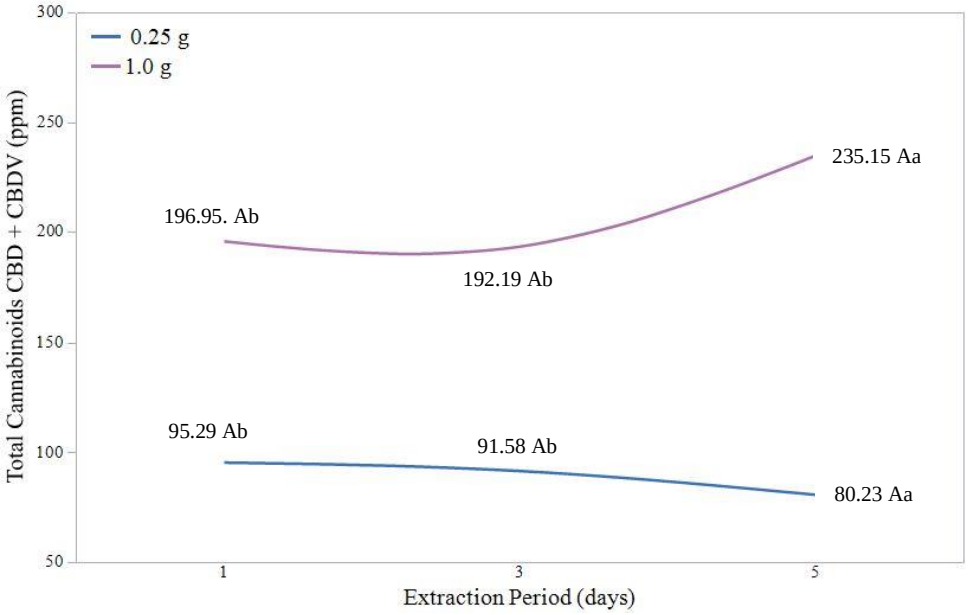

Fig. 4-8. Changes in cannabinoid content (CBD + CBDV, only) from fresh hemp leaf tissue over 5 days of extraction. Means with the same upper-case letter within a sample weight, or lower-case letter within an extraction period, are not significantly different at the 95% confidence level according to the Tukey-Kramer HSD test.

Table 4-4. Cannabidivarin (CBDV) to cannabidiol (CBD) ratio of hemp leaf tissue.

	Extraction Time								
Weight (g)	Day 1			Day 3			Day 5		
	CBDV	CBD	Ratio[y]	CBDV	CBD	Ratio	CBDV	CBD	Ratio
0.25	65.72[z]	29.52	2.23 a[x]	64.44	27.10	2.38 a	60.32	20.25	2.98 a
1.0	102.67	93.44	1.09 b	101.72	91.17	1.12 b	116.51	118.64	0.98 b

[z] Values are in ppm.
[y] Ratio of CBDV to CBD.
[x] Means with same letter within a column are not significantly different at the 95% confidence level according to the Tukey-Kramer HSD test.

Cannabinoids in Buds

Flower tissues had considerably higher total cannabinoid concentrations compared to leaf tissues (Tables 4-4, 4-5). In contrast, the ratio of CBDV:CBD in flower samples was substantially less than in leaf samples. Cannabinoid levels were affected by the amount of tissue that was extracted, as was expected. The highest level of cannabinoids was extracted from 1.5 g of flower tissue after 7 days. When 1.5 or 1 g of tissues was extracted, total cannabinoid levels tended to decrease with 10 days extraction. Though at each sampling date there were differences in total cannabinoid content as affected by sample weight, due to high variability, there was no effect of extraction time on the amount of cannabinoids leached from any weight of tissue. The results suggest that for the 1.0 and 0.5 g samples, an extraction time of less than 2 days could be acceptable when dealing with fresh flower tissue.

Overall, there was a higher level of CBDV than CBD in extracts from 0.5 g of flower tissue compared to extracts from 1.0 or 1.5 g of flower tissue, regardless of extraction time (Table 4-6). The 1.0 g and 1.5 g sample extracts had comparable CBDV to CBD ratios.

Table 4-5. Total cannabinoid (CBD + CBDV + THC + THCV + CBG + CBGV + CBC) concentration (ppm) in hemp flower samples.

	Extraction Time					
Weight	Day 2	Day 3	Day 5	Day 7	Day 10	Average
0.5 g	1034.1 b[z]	1067.7 b	1015.8 b	994.0 c	1036.7 b	1029.7 c
1.0 g	1910.8 ab	1851.3 ab	1836.6 ab	2101.7 b	1872.0 ab	1914.5 b
1.5 g	2449.7 a	2677.7 a	2556.8 a	3579.3 a	2796.2 a	2812.0 a

[z] Means with same letter within a breeding line are not significantly different at the 95% confidence level according to the Tukey-Kramer HSD test.

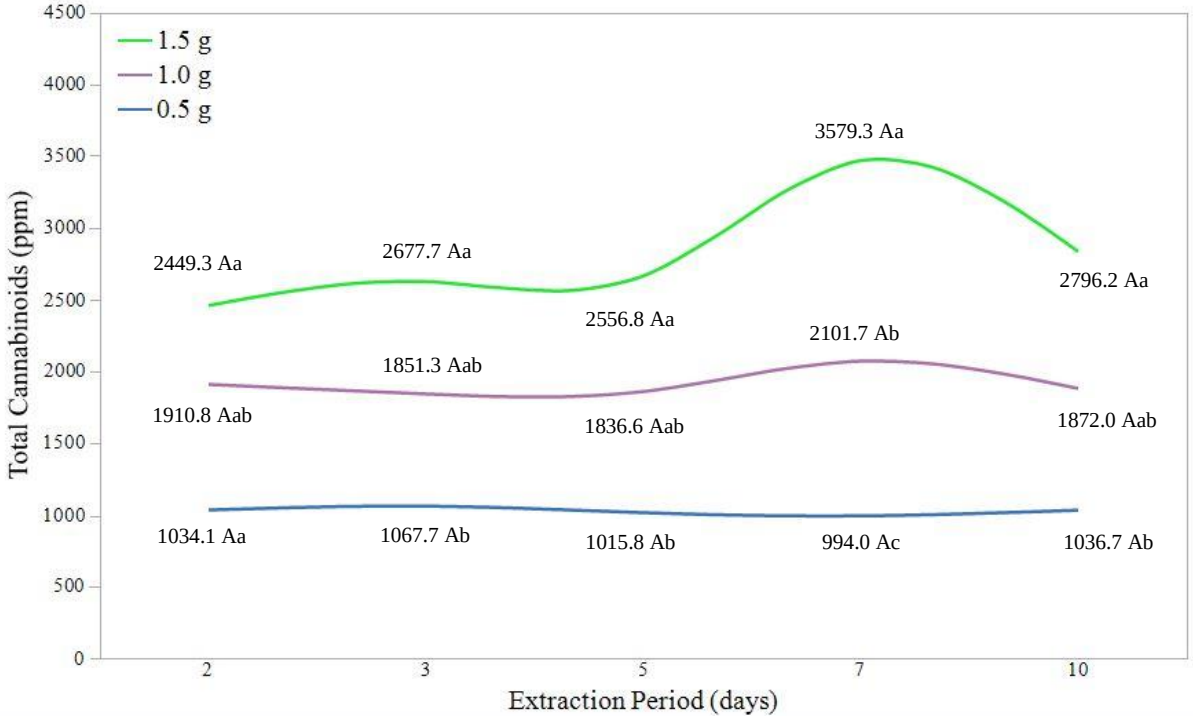

Fig. 4-9. Changes in total cannabinoid content (CBD + CBDV + THC + THCV + CBG, CBGV + CBC) in flower tissue over 10 days of extraction. Means with upper-case letters within a sample weight, and lower-case letters within an extraction period, are not significantly different at the 95% confidence level according to the Tukey-Kramer HSD test.

Table 4-6. Cannabidivarin (CBDV) to cannabidiol (CBD) ratio of hemp flower tissue.

	Extraction Time														
Weight (g)	Day 2			Day 3			Day 5			Day 7			Day 10		
	CBDV[z]	CBD	Ratio[y]	CBDV	CBD	Ratio	CBDV	CBD	Ratio	CBDV	CBD	Ratio	CBDV	CBD	Ratio
0.5	60.1	765.1	0.078 a[x]	61.5	791.8	0.078 a	57.8	749.8	0.077 a	58.0	732.3	0.079 a	59.6	755.9	0.079 a
1.0	79.4	1517.7	0.052 b	80.2	1458.3	0.055 ab	80.1	1456.1	0.055 a	84.6	1683.0	0.050 b	77.5	1487.2	0.052 b
1.5	88.2	1964.0	0.045 b	95.4	2166.5	0.044 b	94.6	2078.8	0.045 a	111.7	2926.5	0.038 a	100.5	2282.4	0.044 b

[z] Values are in ppm.
[y] Ratio of CBDV to CBD.
[x] Means with same letter within a column are not significantly different at the 95% confidence level according to the Tukey-Kramer HSD test.

Chapter 5
DISCUSSION

Masculinization

In the present study, STS was the best treatment to masculinize female hemp plants when breeding for feminized seeds due to phytotoxicity problems and incomplete masculinization with AVG. Lubell and Brand (2018) found that STS at 3 mM (equivalent to 983.6 ppm) applied weekly for a total of 3 sprays was the most effective concentration to masculinize female hemp plants. This study also noted that silver thiosulfate at 0.3 mM (98.4 ppm) was able to partially convert female plants but resulted in negligible masculinization. In contrast, Green (2015) found that a single foliar spray of 0.3 mM STS was able to convert a female hemp plant into a pollen-producing male. In the present study, 500 ppm STS applied 3 times (once per week) was found to fully masculinize female 'Auto Blu' hemp plants (Table 4-1). The work of Adal et al. (2021) suggests that masculinization is dependent not only on the masculinizing treatment but also on the genes related to sex expression within a genotype. Further work is therefore necessary to determine the interaction of STS with hemp genotype, and consequently, to determine the level of STS that is most effective over a wide range of breeding lines.

In the present study, phytotoxicity was most evident for plants treated with STS or AVG (Fig. 4-1). Phytotoxicity of plants treated with STS was characterized by slight necrosis of the developing leaves and buds, whereas the phytotoxicity of plants treated with AVG was characterized by yellowing at the base of young leaves which advanced towards the leaf tips as the tissues matured. Lubell and Brand (2018) did not observe any hemp phytotoxicity

when applying 3 mM of STS, whereas Mohan Ram and Sett (1982) observed severe necrosis of plant tissues when applying 25 to 100 µg of STS directly to apical meristems. Mohan Ram and Sett (1982) also noted a delay of lateral budbreak and staminate flower formation when directly treating the shoot tips with concentrated droplets of STS. Mohan Ram and Sett (1981) observed a strong inhibition of growth when using AVG as a masculinizing agent. In the present study, there was some inhibition of vertical growth after application of 250 or 500 ppm AVG or 500 ppm STS, but this stunting did not render the converted females unable to masculinize or disperse pollen.

Cobalt nitrate treatments were phytotoxic to hemp tissues and did not result in the formation of pollen-producing male flowers in the present study. In contrast, Mohan Ram and Sett (1979) were able to cause masculinization with cobalt chloride, although they also observed leaf yellowing and deformation. Regardless, cobalt nitrate was not considered an effective masculinization treatment in the present study.

Foliar spray and fumigation treatments of 1-MCP did not induce any masculinization of female hemp plants. Should 1-MCP be further studied as an alternative to STS, a recommendation would be to treat plants with LandSpring™ (AgroFresh, Philadelphia, PA), a new wettable powder formulation of 1-MCP, because the ethylene-inhibiting molecule releases inside of the plant tissue upon penetration.

Galoch (1972) and Mohan Ram and Jaiswal (1972) noted that female hemp plants treated with GA_3 were able to produce male flowers which were promoted by the inhibition of auxin action. In the present study, a low level of GA_3 was applied as a potential synergist with STS or CBN to determine if STS or CBN levels could be reduced to reduce tissue damage while maintaining acceptable masculinization. When part of an STS or CBN treatment, the GA_3 was

included in each of the three applications. Though no GA_3 phytotoxicity on hemp has been reported in the literature, in the present study, plants treated with GA_3 became excessively elongated which commonly caused the plants to collapse and die. Since 500 ppm STS by itself was the most efficacious masculinization treatment but was slightly phytotoxic, further work is needed to determine if lower levels of both STS and GA_3 in a combined spray could enhance masculinization without causing excessive vertical plant growth.

Pollen Storage

In the present study, pollen from masculinized female hemp plants had a minimal germination rate, initially and after storage, in contrast to the germination rates of pollen from genetically male plants (Choudary et al., 2014; Gaudet et al., 2020). Additionally, DiMatteo et al. (2020) also found pollen from masculinized hemp plants to be less viable initially compared to pollen from genetically male plants. Though the germination rates in the present study coincided with the rates observed by DiMatteo et al. (2020), different genotypes were used in these studies. Gaudet et al. (2020) also used a different germination medium and observed higher germinability. Regardless, further research must be conducted to investigate how the viability of hemp pollen may be affected by genotype.

Irregularly shaped pollen grains are commonly produced by masculinized hemp plants (DiMatteo et al., 2020) and, as a result, may negatively influence germination rate. Dehydration of pollen grains could be the primary cause of irregular shape (Niklas, 1985). Pollen from *Zea mays* L. (maize) had diminishing viability as pollen moisture content decreased over time (Fonseca and Westgate, 2005). After anthesis and programmed dehydration, maize pollen had disrupted ATP formation, leading to a loss in viability (Roeckel-Drevet and Digonner, 1995). In the present study, observations of dehydrating

pollen grains – characterized by desiccation and collapse of the cell wall – occurred within 1 hr at room temperature (22°C), causing irregular grain formations. In addition to the inherent irregularity in pollen from masculinized hemp plants, transportation times from Goleta (2 hours) and Sebastopol (6 hours) under ambient conditions could have contributed to the low germination rates observed in this study. The pollen was transported at ambient temperatures (17-18°C) to avoid condensation within the transport vials.

Dehydration of pollen grains is a natural process upon anthesis. Dehisced pollen readily releases water – dehydrating the grain – allowing for atmospheric dispersal especially in anemophilous species (Niklas, 1985). Research on the optimal vapor pressure deficit for collected pollen grains could provide insight on the best temperature and relative humidity for germination and storage of hemp pollen. Gaudet et al. (2020) found that vacuum desiccation using baked whole wheat flour of hemp pollen grains prior to cryopreservation maintained germinability up to 4 months when the grains were stored in liquid nitrogen. They noted that pollen grains must be sufficiently desiccated to avoid freezing injury but also hydrated enough to maintain viability.

Pollen stored at 1.1 or 7.2°C had higher germinability after one week of storage compared to the initial germination (Fig. 4-4, 4-5). This implies that some of the pollen was immature at collection and further matured during storage at these lower temperatures. Additionally, pollen coming from the field could have had a lower water content upon collection and, as a result, decreased viability for the initial germination assessments (Fonseca and Westgate, 2005; Roeckel-Drevet and Digonner, 1995). During one week of storage at 1.1 or 7.2°C, the pollen grain may have rehydrated in the low temperature, higher humidity atmosphere which could have contributed to higher germination rates. Future research should

investigate the optimal moisture content of hemp pollen prior to germination assessment and for short- and long-term storage.

Cannabinoid Extraction

In the present study, total cannabinoid content varied between leaf and flower samples and the amount of tissue that was extracted influenced the ratio of CBDV:CBD (Tables 4-4, 4-6). Flower samples with 0.5, 1, or 1.5 g were extracted in 10 mL of methanol for 2-, 3-, 5-, 7- or 10-days whereas leaf samples with 0.25 or 1 g were extracted in 10 mL of methanol for 1-, 3-, or 5-days. Leaf samples of 0.25 g or flower samples of 0.5 g yielded high CBDV:CBD ratios across all extraction periods, which may have inadvertently led to high values for the relative differences between CBDV and CBD. With the lower amounts of plant tissue, minimal quantification of cannabinoids caused by truncated chromatographic peaks, especially for CBD, led to higher ratios of CBDV:CBD.

The data from the present study suggests when preparing leaf or flower samples for chromatographic assessment, it is recommended to extract at least 1 g of either leaf or flower fresh tissue with 10 ml of methanol for at least 1-day. Further research should investigate the minimum extraction time for both leaf and flower samples to maintain accurate quantifications and relative differences between cannabinoids.

Quantifying cannabinoids in fresh hemp tissue is under-researched, since most studies focus on dry samples. Cannabinoid extraction was affected by tissue type, the amount of tissue extracted, and extraction time (Tables 4-4, 4-6; Fig. 4-8, 4-9). Data indicates that the greater the amount of tissue and the longer the tissue soaks in solvent prior to filtration, the higher the potential variability (Fig. 4-8). Further investigation is needed to optimize fresh

hemp-tissue extraction protocols as well as to determine optimal sample collection times as hemp matures.

Chapter 6

CONCLUSIONS

STS at 500 ppm applied once per week for 3 weeks was found to be the most efficacious masculinization treatment in the present study. AVG could be an alternative to STS, as it was at least able to partially masculinize female plants and, in some cases, fully masculinize the plants. If breeders of hemp wish to preserve masculinized pollen for short-term storage, it is recommended that it be stored between 1.1 – 7.2°C and used within three weeks.

Breeders who wish to employ chromatographic analyses on fresh tissue of hemp should extract at least 1 g samples of fresh leaf or flower tissue into 10 mL of methanol. Since a short extraction period would allow for rapid throughput testing of cannabinoids, further work is necessary to optimize the tissue extraction process. Additionally, further work is needed to characterize changes in cannabinoids as affected by hemp maturation stage and tissue type.

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