

**Propagation and Root Zone Management for Controlled Environment  
Cannabis Production**

by

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# ABSTRACT

## PROPAGATION AND ROOT ZONE MANAGEMENT FOR CONTROLLED ENVIRONMENT CANNABIS PRODUCTION

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Cannabis producers lack reliable information on the horticultural management of their crops. This thesis research was designed to improve horticultural practices for controlled environment cannabis production; topics included propagation, growing substrates, fertilization, and irrigation. To optimize the procedures for taking vegetative stem cuttings in cannabis, several factors were evaluated on how they affect rooting success and quality (Chapter Two). These included number of leaves, leaf tip removal, basal/apical position of cutting on the stock plant, and type of rooting hormone. Removing leaf tips reduced rooting success and cuttings with three fully-expanded leaves had higher rooting success and quality than those with two. Also, a 0.2% indole-3-butyric gel was more effective than a 0.2% willow extract gel to stimulate rooting and cutting position had no effect on rooting. Coir-based substrates with different physical properties were evaluated during the vegetative and flowering stage of cannabis production; optimal organic fertilizer rates were established for each substrate (Chapters Three and Four). During the vegetative stage, cannabis performed well in both tested substrates despite the  $\approx 11\%$  difference in container capacity (CC) between them. During the flowering stage, the substrate with lower CC increased floral dry weight (yield) and the concentration and/or yield of some cannabinoids, including THC, compared to the substrate with higher CC. The optimal organic fertilizer rate

varied by substrate during the flowering stage but not during the vegetative stage; higher fertilizer rate during the flowering stage increased growth and yield but diluted some cannabinoids. Finally, the effects of controlled drought stress timing and frequency during the flowering stage were explored on floral dry weight and secondary metabolism (Chapters Five and Six). When drought was applied during week seven of the flowering stage, through gradual substrate drying over eleven days, floral concentration and content per unit growing area of major cannabinoids were increased. When drought was applied over a period of  $\approx 8$  days during week seven, cannabinoid content was similar to a well-watered control; though, dependent on drought timing, the content of some terpenoids varied. This research provided evidence-based information that can help growers improve the quality and yield of their cannabis crops.

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## CHAPTER ONE:

### Introduction, literature review, and research objectives

#### Cannabis physiology

Cannabis (*Cannabis sativa* L.) is an annual, herbaceous species belonging to the family Cannabaceae, along with hops (*Humulus* spp). It likely originated in central Asia but was introduced into the Americas around 1545 (Small et al., 1975), and is now either cultivated or can be found growing wild across the globe (Clarke and Merlin, 2016).

Cannabis is wind-pollinated and generally dioecious, producing separate male and female plants (Small and Cronquist, 1976). It is usually a short-day plant with changes in its growth cycle governed by the number of daylight hours. The growth cycle is divided into two distinct stages, vegetative and flowering. The vegetative stage lasts as long as the plant receives over 12-hours of daylight and is characterized by high rates of leafy, vegetative growth (Potter, 2014). The flowering stage begins with a short-day photoperiod (~12h) and may last around seven to twelve weeks until senescence, based on the cultivar and growing conditions (Potter, 2014). Day-neutral or 'autoflowering' varieties also exist but are less common, in which growth stages progress regardless of short-day lighting (van Bakel et al., 2011).

During the flowering stage, the inflorescence of the female plant accumulates essential oils, mostly produced and sequestered in glandular trichomes (Dayanandan and Kaufman, 1976). Cannabis essential oils contain a diverse array of secondary metabolites, including a class of meroterpenoid compounds known as phytocannabinoids (Chandra et al., 2017; Potter, 2014). The term phytocannabinoid is used to emphasize the botanical origin of the compounds as they also have animal-derived analogues, termed cannabinoids (Hanuš et al., 2016). Henceforward, the term cannabinoid will be used synonymously with phytocannabinoid.

Over 100 unique cannabinoids have been identified in cannabis (Ahmed et al., 2008; 2015; ElSohly and Slade, 2005; Radwan et al., 2015); however,  $\Delta^9$ -tetrahydrocannabinol (THC) and cannabidiol (CBD) are the dominant compounds and have been most widely studied for their psychoactive and medicinal properties (Elzinga et al., 2015; Mechoulam et al., 1970; Vemuri and

Makriyannis, 2015). In live plants, cannabinoids exist predominantly as carboxylic acids such as  $\Delta^9$ -tetrahydrocannabinidiolic acid (THCA) and cannabidiolic acid (CBDA; Muntendam et al., 2012). These acids decarboxylate during storage (Ross and ElSohly, 1997; Taschwer and Schmid, 2015) and upon heating (Kimura and Okamoto, 1970) to become neutral cannabinoids, such as THC and CBD.

Plant parts other than inflorescence contain cannabinoids, though in lower quantities. For example, in a female plant upon harvest, THC content of the stems, leaves, and inflorescence were measured at 0.3%, 0.8%, and 15.2%, respectively, and no THC was detected in the dried seeds or roots (Potter, 2004).

Terpenoids are another class of secondary metabolites found in cannabis essential oil (Aizpurua-Olaizola et al., 2016). They are generally present at lower concentrations than THC and CBD, but may have useful biological functions, such as deterrence of herbivores and protection from radiation (Gershenzon and Dudareva, 2007). Terpenoids also have a wide range of useful and desirable properties for human use because of their unique flavors and aromas, as well as antimicrobial properties (Bassolé and Juliani, 2012). While cannabinoids are found predominantly (some argue exclusively) in cannabis (Gertsch et al., 2010), terpenoids are found in many other higher plant species (Bassolé and Juliani, 2012).

Cannabinoids and terpenoids share a similar biochemical pathway. Isopentenyl pyrophosphate (IPP) is the basic building block of all terpenoids and is produced either in the cytosol and mitochondria of the leaves through the mevalonate pathway (Banthorpe et al., 1972), or in the plastids through the mevalonate-independent (DXP) pathway (Eisenreich et al., 1998). The DXP pathway is the source of all mono-, di-, and tetraterpenes, which include many essential oil components (Gershenzon et al., 2000). In cannabinoid synthesis, geranyl pyrophosphate (GPP) from the DXP pathway is combined with olivetolic acid (OA), a product of the polyketide pathway (Flores-Sanchez and Verpoorte, 2008; Hanuš et al., 2016), to produce cannabigerolic acid (CBGA; Fellermeier et al., 2001). CBGA is then converted to more commonly known cannabinoids, such as THCA and CBDA through various synthases (Taura et al., 1996).

Selective breeding has led to diverse physiological and biochemical traits within the species. Varieties selectively bred and cultivated mainly for fiber or seed production are characterized by

low THC and high CBD concentrations, and are generally termed hemp or fiber-type cannabis. Varieties with high THC and low CBD are termed marijuana or drug-type cannabis (van Bakel et al., 2011; Vollner et al., 1986). Fiber-type varieties are often monoecious (Small and Cronquist, 1976) and produce less essential oil compared to a female plant of a drug-type cultivar (Small et al., 1975). A more precise method for differentiating between cannabis varieties is through characterization by cannabinoid ratios and content in their inflorescence, or ‘chemotypes’. Varieties of ‘chemotype I’ have a high THCA:CBDA ratio ( $>1.0$ ) while varieties of ‘chemotype II’ have an intermediate ratio (generally 0.5–2.0). ‘Chemotype III’, or fiber-type cannabis, has mainly CBD, and less than 0.3% THCA (Small et al., 1975). This thesis research focuses on the study of drug-type cannabis (chemotype I and II), which will hereafter be referred to as cannabis.

## **Cannabis usage**

For millennia, cannabis has been a valuable crop for humans. Written accounts of cannabis cultivation for medicinal purposes in ancient Egypt were found in pyramid texts from 2350 BCE (Russo, 2007). Cannabis also proved to be a practical crop in the Roman Empire during which it was cultivated for use as fiber and for medicinal purposes.

Cannabis is presently used as a source of fiber, food, oil, medicine and, as a recreational drug (Chandra et al., 2017); though, its importance as a medicinal and recreational substance will be a topic of further discussion in this thesis research.

The therapeutic effects of cannabis have been documented throughout history; and, with the discovery of the human endocannabinoids system (Devane et al., 1992), the drug’s unique physiological effects were better understood. The endocannabinoid system includes both cannabinoid receptors (CB1 and CB2) and endogenously produced cannabinoids that interact with them. Cannabinoids found in cannabis, including THC, can bind to cannabinoid receptors to elicit psychoactive or therapeutic effects in humans. Therapeutic effects include analgesic and anti-inflammatory action that are well-documented in animal models (Farquhar-Smith, 2002; Martin and Lichtman, 1998).

The psychoactive properties of cannabis are thought to be mainly due to THC (Wachtel et al., 2002), while CBD is thought to be responsible for muscle relaxant and anti-psychotic effects

(Leggett, 2006). It has been suggested that THC and CBD may have synergistic therapeutic effects with enhanced sedative effects when taken together (McPartland and Russo, 2001). Terpenoids may also have synergistic effects with THC and CBD, though these are less studied (Russo, 2011).

Historically, humans seem to have a conflicting relationship with cannabis and the list of its useful properties is accompanied by a long history of controversy.

## **Cannabis and the law**

For most of history there were no laws regarding cannabis and it was cultivated and used in many countries; in the 1890s cannabis was included in the USA Pharmacopeia for treatment of tetanus, rheumatism, and gout, among other conditions. In the 1930s, the recreational use of cannabis was linked to drug addiction and was associated with the lower tiers of society (Bonnie and Whitebread, 1970). The Marijuana Tax Act, imposed in 1937, made cannabis essentially illegal and it was removed from the Pharmacopeia in 1942 (Martin and Lichtman, 1998).

Similarly, in the United Kingdom, cannabis was prohibited for recreational use in the late 1920s. The production and possession of cannabis for any purpose was outlawed in 1976 under the Misuse of Drugs Act in which cannabis was reclassified as a schedule one drug (Farquhar-Smith, 2002). Likewise, in Canada, the recreational use of cannabis has been illegal since 1923 under the Opium and Drug Act (Graham, 2002).

After most of the world had criminalized cannabis in some way, some countries eventually re-introduced government-regulated access to cannabis for medicinal purposes. In 2002, Canada became the first country to do so. Health Canada announced in 1999 that the medicinal use of cannabis was permitted; however, cannabis use was still illegal under The Controlled Drugs and Substances Act, a contradiction that forced users to choose between health and imprisonment (Graham, 2002). New policies that took effect in 2002 addressed the contradiction by instituting a government-regulated supply of medicinal cannabis for select patients that had been diagnosed with a list of specified illnesses or symptoms (Graham, 2002). The most recent Canadian regulations, from 2015, allow several private, government-licensed entities to produce and distribute cannabis directly to patients. These producers may also conduct cannabis-related

research (*Marihuana for Medical Purposes Regulations*, 2015, SOR/2013-119). Since Canada has legalized access to cannabis for medicinal purposes, other countries have developed similar programs, including The Netherlands, Uruguay, Czech Republic, Israel, and some US States (Chandra et al., 2017; Hazekamp, 2016; Leggett, 2006). Some countries have also initiated efforts to legalize cannabis for recreational purposes, including Canada.

In fact, the legal use of cannabis for medicinal and recreational purposes is becoming increasingly widespread in North America. In 2016, combined spending on medical and recreational cannabis in legal markets was reported at 6.7 billion USD and is projected to reach 22.6 billion USD by 2021 in North America (ArcView Market Research, 2017). Demand has necessitated a similarly fast-growing industry for the legal production of cannabis (ArcView Market Research, 2017).

## **Production of drug-type cannabis**

Horticulture literature relating to cannabis production (Knight et al., 2010; Potter and Duncombe, 2012; Vanhove et al., 2011; 2012) and reviews on global cannabis production (Farag and Kayser, 2015; Leggett, 2006; Potter, 2014) suggest that modern day production occurs primarily in controlled environments (indoors or in a greenhouse) using artificial lighting and either soilless growing substrates or solution culture (hydroponics). Controlled environment production is well-suited for cannabis since environmental parameters can be controlled, allowing year-round production, and the photoperiod can be manipulated to maintain vegetative growth or to trigger flowering (Knight et al., 2010). Year-round production facilitates more frequent cropping than in field production, especially in colder climates such as Canada (Zheng et al., 2011). Further, since cannabis remains a controlled substance in some areas in which it is produced, controlled environments are easier to monitor for security purposes.

Producing crops in controlled environments requires the understanding of multiple interrelated environmental factors, such as light quality and intensity, air flow, gas exchanges, air temperature, humidity, and how to manipulate these factors to produce a healthy crop (Ishii et al., 2016). Additionally, the environment including and surrounding the roots or ‘the root zone’, has its own set of interrelated factors, such as water relations, nutrient dynamics, gas exchanges, and support for plants (Raviv and Lieth, 2007). Root zone management, specifically for cannabis

grown in soilless growing substrates, will be a major theme of this thesis research. To date, there is limited credible research on this topic.

## **Horticultural research on cannabis**

The legal status of cannabis has stifled research efforts in the past several decades; consequently, the body of peer-reviewed literature on the horticultural management of cannabis is limited (Potter, 2014). The recent relaxation of cannabis regulations in countries such as Canada, The Netherlands, and the UK has facilitated some research in this field.

There are several peer-reviewed studies on cannabis production based on police data from illicit growers (Decorte, 2010). For example, a series of peer-reviewed research papers set out to improve the accuracy of yield estimations for illicit cannabis operations based on horticultural practices. A study in The Netherlands evaluated the effect of plant density on cannabis yield using data from seventy-seven confiscated grow rooms (Toonen et al., 2006). Another, in New Zealand, evaluated the ‘Screen of Green’ method for cannabis cultivation, in which a wire lattice is used to support branches to estimate yields from illicit growth operations using this method (Knight et al., 2010). Finally, two studies in Belgium evaluated the effects of lighting intensity (Vanhove et al., 2011), plant density, and cannabis cultivar (Vanhove et al., 2011; 2012) on cannabis yield. The horticultural designs in most of these studies appear to rely on the anecdotal knowledge of illicit growers without sufficient reference to horticultural research (Potter, 2009); though, they do provide valuable information since other reliable resources are scarce.

The past decade has also produced peer-reviewed research that does not stem from illicit activity with the goals of validating and/or improving upon horticultural management practices. Researchers have begun to elucidate the effects of various horticultural factors on cannabis production. These include: optimum air temperature (Chandra et al., 2011) and carbon dioxide (CO<sub>2</sub>) concentration (Chandra et al., 2008) in the growing environment; the intensity of horticultural lighting (Potter, 2009; Potter and Duncombe, 2012); the effects of a growing substrate microbial biostimulant (Conant et al., 2017); and a comparison between indoor and outdoor cannabis production (Potter, 2009). Additionally, an earlier study evaluated the effects of UV-B radiation on THC concentration in cannabis flowers (Lydon et al., 1987).

These publications provide credible resources for cannabis growers to improve the quality and profitability of their crops. Unfortunately, this pool of resources is lacking compared to resources available to producers of other horticultural commodities that have not been criminalized. Due to a lack of horticultural research, cannabis producers must heavily rely on horticultural methods derived from less credible sources, such as anecdotes and recommendations for other crops. Research on effective, evidence-based cultivation practices, such as those outlined in this thesis research, can improve the quality and profitability of cannabis production.

## **Cannabis propagation**

There are several viable options for propagating cannabis. These include: by seed (Potter 2009; Farag and Kayser 2015), vegetative stem cuttings (Coffman and Gentner, 1979; Potter, 2009), and in-vitro propagation (Lata et al., 2009a; 2009b; 2011).

Genetic uniformity is important to ensure consistent rates of growth and secondary metabolite content. Further, male plants are generally undesirable as they produce much lower quantities of cannabinoids than female plants and may pollinate nearby female plants. In pollinated female plants, THC content can be reduced by over 75% per unit area (Potter, 2004).

Propagation by seed, while simple and inexpensive, does not ensure genetic uniformity and may, therefore, be unsuitable for controlled environment cannabis production, especially for large scale operations. In-vitro propagation can be useful for the preservation of germplasm (Lata et al., 2009a; 2009b; 2011), but may be restively expensive for medium and small-scale producers. Propagation using vegetative stem cuttings is often preferred by cannabis growers. It is a low-cost method that delivers genetically uniform plants with consistent rates of growth and cannabinoid production when compared to propagation from seed (Coffman and Gentner, 1979; Potter, 2009).

Researchers have compared the efficacy of propagation through stem cuttings for cannabis to other methods, such as propagation from seed (Coffman and Gentner, 1979; Potter, 2009); however, there is scant research on how to improve the procedure. Optimized procedures for propagation through stem cutting in cannabis can help growers improve their propagation success rates and reduce both waste and costs.

## **Integrated root zone management**

There are various methods that can be successfully employed to produce plants under controlled environments. Modern day horticultural production systems generally do not involve the use of soil as its physical properties do not allow for sufficient drainage that is required for container-production; these systems are termed ‘soilless culture’ (Raviv and Lieth, 2007). Systems in which plants are suspended in a circulating nutrient solution, without a solid growing substrate are termed ‘solution culture’. Those in which plant roots are sprayed or misted with nutrient solution are termed ‘aeroponics’. Other methods employ solid soilless substrates that are held in a container. This last category will be discussed in further detail in this thesis.

The root zone of a container-grown plant is the region of the growing substrate, including gas and water, which directly interacts with the roots. Integrated root zone management (IRM) is a production strategy in which the interactions between the water, nutrients, gases, and growing substrate that surround the roots of a plant are considered when making horticultural management decisions (Zheng, 2016). Each of these factors: water, nutrients, gases, and growing substrate, play an integral role in container production, but they must be considered collectively because variations in one factor generally impact the others. For instance, appropriate choice of growing substrate is crucial for container production because the substrate acts as a reservoir for water and nutrients, and the solid fraction of the substrate provides support for the roots (Marfà et al., 2002). Different substrates have diverse physical and chemical properties; therefore, to ensure a healthy root zone environment it is essential to irrigate and fertilize plants accordingly (Zheng, 2016).

Fertilization is another important consideration for controlled environment cannabis production; over-fertilization can lead to salt accumulation in the root zone, whereas under-fertilization can cause nutrient deficiency and lower yields (Bar-Yosef, 1999); however, no recommended fertilizer application rates have been published for cannabis production in scientific literature. For field hemp production, the suggested fertilization rate is around 100 - 200 kg N·ha<sup>-1</sup> (Aubin et al., 2015; Vera et al., 2004), which is similar to other high-yielding field crops such as wheat (Baxter and Scheifele, 2008). It is difficult however, to estimate fertilizer requirements of drug-

type cannabis based on hemp, or other crops due to the differences in species and growing conditions (Wright and Niemiera, 1987).

Root zone water and irrigation is another important consideration in IRM. Water facilitates evapotranspiration, maintains cell/plant turgor, and transports vital nutrients through the plant's vascular system (Bar-Yosef, 1999). Factors such as irrigation frequency, irrigation volume, and irrigation water quality must all be considered in the IRM approach (Zheng, 2016). For instance, high irrigation frequency is known to increase growth in some plant species (Katsoulas et al., 2006; Silber et al., 2003; 2005). In container-grown roses (*Rosa hybrid* L., 'First Red'), doubling irrigation frequency while maintaining constant total irrigation volume increased the dry weight of cut flower shoots by approximately 30% compared to a lower frequency irrigation (Katsoulas et al., 2006). The increased growth from frequent irrigation has been attributed to enhanced nutrient uptake; specifically, improved phosphorus mobilization and uptake (Silber et al., 2003; 2005). Over-irrigation or under-irrigation may become a concern if growing substrate and irrigation interactions are not considered. If the growing substrate has a relatively high water holding capacity, frequent irrigation may lead to waterlogging, and reduce availability of oxygen in the root zone. A well-oxygenated root zone is vital for good nutrient uptake, root growth, and prevention of root-borne disease (Jackson and Colmer, 2005; Zheng et al., 2007). Under-irrigation can lead to toxic salt build-up in the root zone and reduced plant growth from restricted water availability (de Kreij and Straver, 1988). Further, the optimum range of root zone water content varies among plants, so species-specific evaluations are often required to determine appropriate irrigation and/or substrates for a crop.

Research evaluating specific growing substrates or irrigation methods, for example, are impractical without considering the interactions with other root zone factors. Conversely, an IRM approach to horticultural research considers all the interacting root zone factors, and its use is important in the design of horticultural experiments. To our knowledge, there is no peer-reviewed research on growing substrates, fertilizer management, or irrigation for controlled environment cannabis production. Improving these aspects of horticultural management through systematic research using IRM can provide growers evidence-based resources to grow safe and high-quality cannabis.

## Thesis research goal and objectives

Considering the limited scientific research on cannabis production, the medical significance of this crop, and its economic importance, the goal of this thesis research was to improve the horticultural methods for controlled environment cannabis production, focusing on irrigation, growing substrates, fertilization, and propagation, using the IRM approach.

Specific objectives were as follows:

1. Evaluate the influence of the following factors and their interactive effects on propagation using stem cuttings in cannabis: (i) number of leaves, (ii) leaf tip removal, (iii) basal/apical position of stem cutting, and (iv) type of rooting hormone.
2. Evaluate coir-based organic growing substrates for vegetative- and flowering-stage cannabis in a controlled environment and determine optimal organic fertilizer rates for these substrates.
3. Evaluate the effects of drought stress on floral yield and cannabinoid content in cannabis.
4. Evaluate the effects of drought stress timing and frequency on cannabinoid and terpenoid content in cannabis.

Each chapter in this thesis is an independent manuscript which has either been published (Chapters 2, 3, and 4) or is being prepared for publication in a peer-reviewed horticultural journal (Chapters 5 and 6). The papers that are currently published are the following:

Caplan, D., M. Dixon, and Y. Zheng. 2017a. Optimal rate of organic fertilizer during the flowering stage for cannabis grown in two coir-based substrates. *HortScience* 52:1796–1803.

Caplan, D., M. Dixon, and Y. Zheng. 2017b. Optimal rate of organic fertilizer during the vegetative-stage for cannabis grown in two coir-based substrates. *HortScience* 52:1307–1312.

Caplan, D., J. Stemeroff, M. Dixon, and Y. Zheng. 2018. Vegetative propagation of cannabis by stem cuttings: effects of leaf number, cutting position, rooting hormone and leaf tip removal. *Can. J. Plant Sci.* <https://doi.org/10.1139/CJPS-2018-0038>.

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## CHAPTER TWO:

# Vegetative propagation of cannabis by stem cuttings: effects of leaf number, cutting position, rooting hormone and leaf tip removal<sup>1</sup>

### Abstract

This study evaluated the influence of the several factors and their interactive effects on propagation success using stem cuttings of cannabis (*Cannabis sativa* L.). Factors included: (i) Leaf number (two or three) (ii) leaf tip removal (1/3 of leaf tips removed) (iii) basal/apical position of stem cutting on the stock plant, and (iv) rooting hormone (0.2% indole-3-butyric (IBA) acid gel or 0.2% willow (*Salix alba* L.) extract gel). Cuttings were placed in a growth chamber for twelve days then assessed on their rooting success rate and root quality using a relative root quality scale. The IBA gel delivered a 2.1 times higher rooting success rate and 1.6 times higher root quality than the willow extract. Removing leaf tips reduced rooting success rate from 71% to 53% without influencing root quality. Cuttings with three leaves had 15% higher root quality compared to those with two, but leaf number did not influence rooting success rate. Position of cutting had little effect on rooting success or quality. To achieve maximum rooting success and root quality, cuttings from either apical or basal positions should have at least three fully expanded, uncut leaves and the tested IBA rooting hormone is preferential to the willow-based product.

**Key words:** *Cannabis sativa*, IBA, marijuana, rooting success rate, willow extract

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## Introduction

Cannabis (*Cannabis sativa* L.) production for legal markets in North America, including both medical and recreational, is quickly becoming a profitable industry. North American spending on legal cannabis was estimated at 6.7 billion USD in 2016 and is projected to reach 21.6 billion by 2021 (ArcView Market Research 2017).

Cannabis is an annual herbaceous species which has been widely cultivated and used as a medicinal plant since ~ 2800 BCE (Russo 2007). Its medicinal value is attributed mainly to a group of secondary metabolites called cannabinoids which are concentrated mostly in the essential oils of unfertilized female cannabis flowers (Potter 2014). Cannabis cultivation and possession were outlawed in the United States in 1971 and much of the world followed suit soon after (Potter 2009). Since then, some countries including Canada and The Netherlands have relaxed their regulations and implemented programs allowing access to cannabis for medicinal purposes. In these programs, strict safety standards are enforced to control the quality of cannabis being distributed to patients; however, there is little guidance for growers regarding horticultural management. For growers, horticultural guides and online resources are available but few are based on peer-reviewed scientific research (Potter 2009; Caplan et al. 2017a; Caplan et al. 2017b)

Based on our communications with Canadian medicinal cannabis producers and recent reviews on the state of global cannabis production (Leggett 2006; Potter 2014; Farag and Kayser 2015) modern day cannabis production occurs primarily in controlled environments using artificial lighting and either soilless growing substrates (Caplan et al. 2017a; Caplan et al. 2017b) or solution culture. Further, some medical cannabis growers favor organic production practices since consumers and regulating bodies often demand pesticide-free cannabis. Cannabis is propagated by seed (Potter 2009; Farag and Kayser 2015), vegetative stem cuttings (Coffman and Gentner 1979; Potter 2009) and in-vitro propagation (Lata et al. 2009a, 2009b, 2011). Propagation using vegetative stem cuttings is often preferred by cannabis growers. It is a low-cost method that delivers genetically uniform plants with consistent rates of growth and cannabinoid production when compared to propagation from seed (Coffman and Gentner 1979; Potter 2009).

To our knowledge, no peer-reviewed research exists on optimizing propagation by stem cuttings in cannabis; however, this method has been investigated in other economically important species such as *Pisum sativum* L. (Eliasson, 1978), *Lippia javanica* L. (Soundy et al. 2008), some timber crops (LeBude et al., 2004; Ofori et al., 1996) and some ornamental nursery crops (Grange and Loach 1985). The primary goal of propagation by stem cuttings is to facilitate the formation of adventitious roots. Several factors have been identified which support adventitious rooting in vegetative stem cuttings (Hartmann et al. 2002). Some of these include: leaf area (or leaf number), cutting position on the stock plant, the use of rooting hormones, lighting, rooting medium, water status, and mineral nutrition. The present study focused on the first three of these factors.

Leaves act as sources of photosynthate for cuttings which is important for successful rooting. Increased leaf area and/or number may improve rooting success rate and formation of adventitious roots in cuttings (Leakey and Coutts, 1989; Ofori et al., 1996). Leaves also stimulate rooting as sources of rooting co-factors and endogenous auxin (Haissig 1974). Conversely, greater leaf area and/or number provide a larger surface area for evapotranspiration and evapotranspirative water loss which may negatively affect rooting success rate (Davis and Potter 1989). A reduction in leaf area may reduce evapotranspiration-induced stress (Leakey and Coutts 1989; Ofori et al. 1996) or avoid crowding in the propagation environment (Aminah et al. 1997). A common practice in modern day cannabis productions, based on our communications with Canadian medicinal cannabis producers and gray resources (Cervantes 2006), is to keep 2 to 3 leaves on each cutting and to remove about 1/3 of the leaf tips. The optimal leaf number on stem cuttings varies between species (Machida et al. 1977; Aminah et al. 1997; Alves et al. 2016) so species-specific evaluations are necessary.

The ability of stem cuttings to form adventitious roots often depends on the maturity of the stock plant. Cuttings from juvenile plants generally have improved rooting over those from mature plants (Altamura 1996). Juvenile plant material sometimes has a higher content of endogenous auxins and other rooting promoters compared to mature material (Husen and Pal 2006). This difference is evident in a number hardwood species such as oak (Morgan and McWilliams 1976), teak (Husen and Pal 2006), and American elm (Schreiber and Kawase 1975). Further, in hardwood cuttings, maturity often varies by cutting position on the stock plant; stems from more

basal regions often retain juvenile characteristics and have improved capacity to form adventitious roots (Hackett 1970). There is limited information and mixed findings on the effects of cutting position on adventitious rooting in softwood and herbaceous plants. In *Schefflera arboricola*, softwood cuttings from more basal regions had lower rooting success rate and number of roots (Hansen 1986) while in fever tea (*Lippia javanica* L.), cutting position had no effect on rooting success (Soundy et al. 2008).

It is well documented that treating the basal portions of stem cuttings in synthetic auxins such as indole-3-butyric acid (IBA) can improve rooting success rate, increase the speed of rooting and increase the quantity of adventitious roots (Hartmann et al. 2002). In organic production, synthetic auxins such as IBA are often not permitted; thus, alternatives hormones or techniques are used to improve rooting success rate and quality. Willow (*Salix alba* L.) shoot extract is a naturally-derived alternative to synthetic auxins and has been used successfully as natural rooting hormone for mung bean (*Vigna radiata* L.) cuttings (Arena et al. 1997) but without effect on olive (*Olea europaea* L.) cuttings (Al-Amad and Qrunfleh 2016) or willow (Kawase 1964) cuttings. Currently there is no peer-reviewed literature on any of the factors described above on cannabis.

The objective of the present study was to evaluate the influence of the following factors and their interactive effects on propagation using stem cuttings in cannabis: (i) number of leaves, (ii) leaf tip removal, (iii) basal/apical position of stem cutting, and (iv) type of rooting hormone.

## **Materials and Methods**

### **Stock plant conditions**

Stock (mother) plants were maintained under 18-hour photoperiod with a mean canopy-level light intensity at  $105 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (s.d.  $\pm 61.2 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) using ceramic metal halide 3100K lamps (Philips Lighting, Markham, ON, Canada). Temperature (day/night) was maintained at  $20^\circ\text{C}$  (s.d.  $\pm 0.03^\circ\text{C}$ ), air relative humidity (RH; day/night) was maintained at 63% (s.d.  $\pm 2.3$ ) and carbon dioxide ( $\text{CO}_2$ ) concentration (day/night) was maintained at 646 ppm (s.d.  $\pm 59.7$ ).

Stock plants were potted in 12.5 L air pruning pots (306 mm diameter  $\times$  275 mm height; Caledonian Tree Company Ltd., Pathhead, United Kingdom) containing a custom blended organic growing substrate (60% sphagnum peatmoss and 40% bulk coconut coir; Premier Tech, Rivière-du-Loup, QC, Canada). The stock plants were 10 months-old and had between 20 and 25 nodes on their main stems. The plants were fertigated as needed using Nutri Plus Organic Grow liquid organic fertilizer (4.0N–1.3P–1.7K; Nutri Plus; EZ-GRO Inc., Kingston, ON, Canada) at a rate of 68 mg N·L<sup>-1</sup> amended with 2 mL·L<sup>-1</sup> of Calcium-Magnesium supplement (0.0N–0.0P–0.0K–3.0Ca–1.6Mg; EZ-GRO Inc.) and 22.9 mg N·L<sup>-1</sup> of Organa ADD micronutrient supplement (2.0N–0.0P–0.0K; EZ-GRO Inc.), with a 20% leaching fraction. Other nutrient element concentrations in Organa ADD were (in mg L<sup>-1</sup>): 100.0 Ca, 29851 Zn, 4892 Mn, 1239 B, 12.7 Mo, 2419 Cu, and 2917 Fe.

### **Plant culture and treatments**

*Cannabis* [*Cannabis sativa* L. ‘WP:Med (Wappa)’] cuttings were taken at a length of  $\approx$ 13 cm and with three fully-expanded leaves from stock plants. Cuttings were taken from the ends of axial limbs and cut at a 45° angle. Each cutting was rooted in a 5.7 cm wide, 5.7 cm tall peat-based pot (Jiffy Products N.B. Ltd., NB, Canada) containing Pro-Mix PG Organic growing substrate (Premier Tech) and arranged in trays at a density of 266 plants/m<sup>2</sup>. The substrate was soaked in a solution of ‘Spurt’ liquid organic fertilizer (2.0N–0.0P–0.83K; EZ-GRO Inc.) at a rate that supplied 123 mg N·L<sup>-1</sup>.

The experiment was a full factorial completely randomized design with four factors (rooting hormone, leaf number, cutting position, and leaf tip removal), two levels per factor and 10 replications per factor combination. For leaf number, cuttings had either one fully-expanded leaf removed (two leaves remaining) or were left with three leaves. For cutting position, cuttings from terminal shoots were taken from either an apical position (node 10 and higher) or a basal position (below node 10). For the leaf tip removal treatment, a portion of leaf tips ( $\approx$ 1/3 of the leaf area) was removed from the fully expanded leaves or the leaves were left uncut. For the rooting hormone factor, the base ( $\approx$ 5 cm) of each stem was dipped in either 0.2% indole-3-butyric acid gel (synthetic rooting hormone; EZ-GRO Inc.) or in a 0.2% willow extract rooting gel (organic rooting hormone; EZ-GRO Inc.).

## **Propagation environment**

Trays were randomly arranged in a walk-in growth chamber (Conviron ATC60; Controlled Environments Ltd., Winnipeg, MB, Canada) and cuttings were misted with reverse osmosis (RO) water once, when they were placed in the chamber. From days 0 to 4 after cuttings were placed in the substrate (DAP), RH was maintained at 95% (s.d.  $\pm 1.3$ ), reduced to 80% (s.d.  $\pm 1.3\%$ ) for 5-8 DAP, and to 60% (s.d.  $\pm 1.5\%$ ) for 9-12 DAP. Temperature was maintained at 24 °C (s.d.  $\pm 0.04$ °C) (day/night) for the entire period. Fluorescent lighting (Philips Lighting, Markham, ON, Canada) was used to maintain an 18-hour photoperiod. Photosynthetically active radiation (PAR) at canopy level was maintained at 50  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (s.d.  $\pm 0.6$   $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 0-4 DAP 1-5, 80  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (s.d.  $\pm 0.7$   $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 6-8 DAP, and 115  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (s.d.  $\pm 0.5$   $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 10-12 DAP.

## **Rooting assessment and harvest**

The bottom of the trays was observed daily from 7 DAP onwards for protruding roots, and cuttings were harvested at 12 DAP when approximately more than 50% of the cuttings showed visible roots at the bottom of the tray. Rooting success rate was measured on a binomial scale in which any visible adventitious root formation was considered rooted. Rooting success was calculated as the percentage of cuttings with roots in each treatment. Successfully rooted cuttings were assigned to either of two classifications based on degree of adventitious rooting: A root quality index (RQI) score of '1' or '2' was assigned by a third party without knowledge of the applied treatments based on a visual reference (Figure 2.1). Before RQI measurements, the substrate was washed from rooted cuttings with RO water.

## **Statistical analysis**

Data were analyzed using JMP Statistical Discovery Version 13.0 (SAS Institute Inc., Cary, NC) at a Type 1 error rate of  $\leq 0.05$ . Rooting success rate and RQI data were analysed assuming a binomial error distribution using a generalized linear model (GLM) and logit link function. Stepwise regression with minimum AICC was used to remove non-significant interactive effects. Chi-square contrasts were used to compare treatment means and interactive effects between treatments.

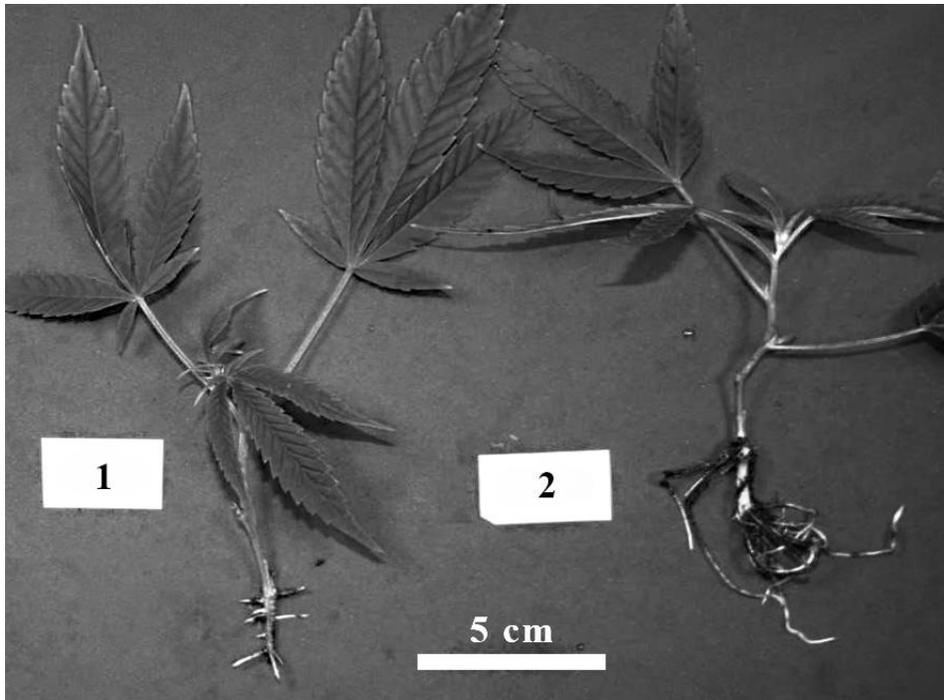


Figure 2.1. Visual scale for Rooting Quality Index (RQI) assessment of cannabis cuttings.

## Results

Cuttings under all treatment combinations had some degree of successful rooting. Rooting hormone had the greatest effect on both rooting success rate and root quality (Figure 2.2, 2.3). The synthetic hormone delivered a 2.1 times higher rooting success rate (84% vs. 40%;  $\chi^2 = 39.0$ ,  $P < 0.0001$ ) and 1.6 times higher root quality (1.6 vs. 1.0;  $\chi^2 = 41.1$ ,  $P < 0.0001$ ) than the organic hormone. Removing leaf tips had the second greatest effect on rooting success rate. When leaf tips were removed, rooting success rate was lowered from 71% to 53% ( $\chi^2 = 9.8$ ,  $P = 0.0018$ ), though there was no effect on root quality. Leaf number had no effect on rooting success rate, but rooted cuttings with three leaves had 15% higher root quality than those with two (1.5 vs. 1.3;  $\chi^2 = 4.3$ ,  $P = 0.038$ ). Cutting position did not influence rooting success rate or root quality. There was however, an interactive effect between cutting position and leaf tip removal on rooting success rate (Figure 2.4). When leaf tips were removed, cuttings of basal origin had lower rooting success rate than apical cuttings (43% vs. 63%;  $\chi^2 = 5.7$ ,  $P = 0.0169$ ).

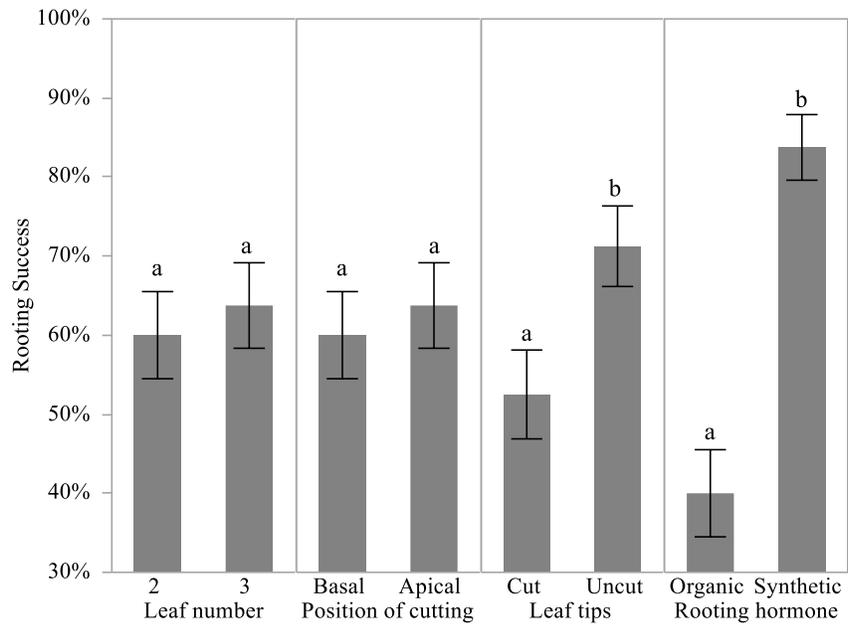


Figure 2.2. Rooting success rate of cannabis cuttings (means  $\pm$  SEM;  $n = 80$ ). Cut leaves had about 1/3 of leaf tips were removed. Cuttings were from terminal shoots with apical cuttings taken from  $\geq$  node 10 and basal cutting from  $<$  node 10. The synthetic rooting hormone was a 0.2% indole-3-butyric (IBA) acid gel and the organic was a 0.2% willow extract gel. Bars within each factor (e.g. leaf number) bearing different letters are significantly different at  $P < 0.05$  using chi squared contrasts.

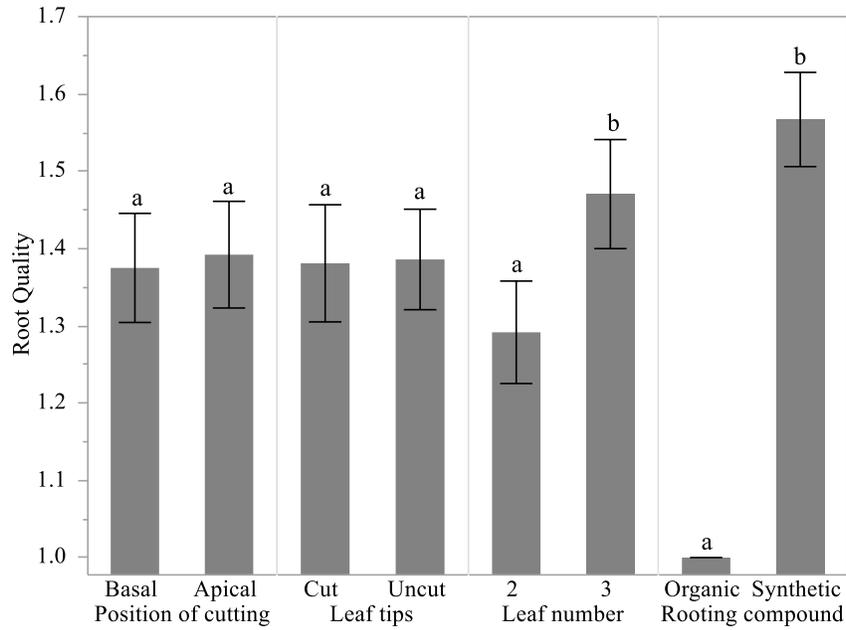


Figure 2.3. Root Quality Index of cannabis cuttings (means  $\pm$  SEM;  $n = 80$ ). Cut leaves had about 1/3 of leaf tips were removed. Cuttings were from terminal shoots with apical cuttings taken from  $\geq$  node 10 and basal cutting from  $<$  node 10. The synthetic rooting hormone was a 0.2% indole-3-butyric (IBA) acid gel and the organic was a 0.2% willow extract gel. Bars within each factor (e.g. leaf number) bearing different letters are significantly different at  $P < 0.05$  using chi squared contrasts.

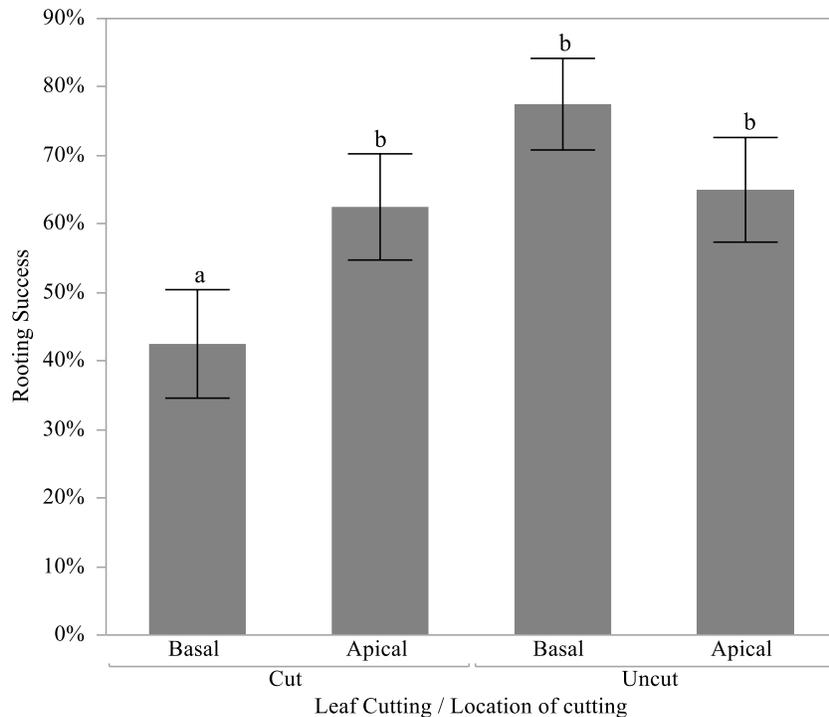


Figure 2.4. Rooting success rate of cannabis cuttings (means  $\pm$  SEM; n = 40). Cut leaves had about 1/3 of leaf tips were removed. Cuttings were from terminal shoots with apical cuttings taken from  $\geq$  node 10 and basal cutting from  $<$  node 10. Treatments with different letters are significantly different at  $P < 0.05$  using Chi Squared contrasts.

## Discussion

### Rooting hormone

The use of IBA led to markedly higher rooting success rate and root quality than the organic hormone. Similar success with IBA has been documented in other species propagated by stem cuttings (Al-Saqri and Alderson 1996; Saffari and Saffari 2012). In studies on the effects of centrifuged willow shoot extracts on willow and mung bean stem cuttings, willow extract application increased adventitious rooting in mung bean but had no effect in willow cuttings (Kawase 1964, 1970). The authors attributed the improved rooting in mung bean to a synergistic effect between indole-3-acetic acid (IAA) in the cuttings and two unknown root promoting fractions identified in willow extract. This synergistic effect was explored on adventitious rooting, also in bean cuttings, in Gesto et al. (1977) and attributed to the presence of the

compound catechol in willow extract. Kawase (1970) suggested that only cuttings with sufficient IAA, such as mung bean, would see improved rooting from the synergism. A recent evaluation on the effects of willow extract on olive stem cuttings (Al-Amad and Qrunfleh 2016) showed that, similar to willow cuttings, willow extract had no effect on adventitious rooting in olive cuttings. The relatively poor rooting success rate of cannabis cuttings treated with willow extract in the present study could be attributed to a lack of IAA in the cuttings. Further study is required to measure IAA in cannabis cuttings and to further explore the synergistic effect between IAA and catechol on adventitious rooting. Also, more organic rooting hormones need to be explored and evaluated for cannabis propagation to provide alternatives for growers that choose organic production.

### **Leaf number/ leaf tip removal**

Rooting success was similar between cuttings with two and three leaves, suggesting that two leaves may provide sufficient carbohydrates, auxin, and rooting co-factors (Haissig 1974) for successful rooting in cannabis. In the propagation of stem cuttings, increased photosynthetic surface area and resultant carbohydrate supply generally increased rooting success rate until another factor such as evapotranspiration stress became limiting (Davis and Potter 1989). Cuttings with three leaves showed no signs of wilting or other indications of evapotranspiration stress. It is likely that both two- and three-leaf treatments exhibited little evapotranspiration-induced water stress in the stable, high humidity provided in this trial. It is estimated that under conditions of lower or less stable humidity, fewer leaves would deliver improved rooting success rate as these cutting would have a lower evaporative demand from humidity.

Notably, three leaves increased root quality over two. The observed quality improvement was likely caused by the additional carbohydrates, rooting co-factors, endogenous auxin (Haissig 1974) or a combination of these factors provided by the additional foliage. Further study is required to evaluate the effect of each of these factors and their interactions on cannabis stem cuttings to discern their relative importance. Based on this finding, it is recommended that cannabis cuttings be taken with three or potentially more leaves (to improve rooting quality) so long as humidity during propagation can be adequately maintained.

It was expected that cutting leaf tips would have a similar effect to reducing leaf number since the source and thus potentially the amount of photosynthetic material was manipulated; however, cutting leaf tips reduced rooting success and had no effect on rooting quality.

Both cutting leaf tips and leaf number altered surface area for evapotranspiration and photosynthesis; however, there was a notable difference in the effects of these treatments. Leaf cutting influenced rooting success rate while leaf number influenced root quality. Further study is necessary to discern the reason for these differing effects. Based on these findings, it is recommended that leaf tips not be cut in cannabis cuttings; and, if less leaf material is desired to conserve space in the propagation environment or to prevent evapotranspiration stress, then fewer whole leaves be used instead.

### **Cutting position**

There was no indication that basal cuttings had improved rooting success rate or quality over apical cuttings. Similar results were found in stem cuttings of fever tea (Soundy et al. 2008) and may be attributed to the lack of distinct stages of maturation in these herbaceous plants in contrast to most hardwood species (Schreiber and Kawase 1975; Morgan and McWilliams 1976; Husen and Pal 2006). These findings suggested that in cannabis, cutting position does not play an important role in rooting.

### ***Interactive effects***

There was an interactive effect between leaf tip removal and cutting position. Cuttings from basal positions with two leaves had lower rooting success rate than any other combination of these two factors. In general, basal cuttings had smaller leaves (general observation without measurement) than apical cuttings which, through cutting of leaf tips, were left with less overall leaf surface area. The reduction in photosynthesis resulting from the smaller leaf area might have then resulted in the lower rooting success rate.

### **Conclusions**

Type of rooting hormone strongly influenced the success and quality of adventitious rooting in cannabis cuttings, with the 0.2% IBA gel delivering higher rooting success rate than the 0.2%

willow extract. Removing 30% of leaf tips from cuttings reduced rooting success rate and three leaves had higher root quality compared to two leaves without influencing rooting success rate. Position of cutting on the stock plant did not influence either rooting success rate or root quality. To achieve maximum rooting success and root quality, cuttings from either apical or basal positions should have at least three fully expanded, uncut leaves and be dipped in an IBA rooting hormone. If a reduction in leaf area is desired, either because high humidity cannot be maintained or more airflow is desired in the propagation environment, then lowering leaf number to two fully expanded leaves is preferential to cutting leaf tips.

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## CHAPTER THREE:

### Optimal rate of organic fertilizer during the vegetative stage for cannabis grown in two coir-based substrates<sup>2</sup>

#### Abstract

Cannabis producers, especially those with organic operations, lack reliable information on the fertilization requirements for their crops. To determine the optimal organic fertilizer rate for vegetative-stage cannabis (*Cannabis sativa* L.), five rates that supplied 117, 234, 351, 468, and 585 mg N·L<sup>-1</sup> of a liquid organic fertilizer (4.0N–1.3P–1.7K) were applied to container-grown plants with one of two coir-based organic substrates. The trial was conducted in a walk-in growth chamber and the two substrates used were ABCann UNIMIX 1-HP with lower water holding capacity (WHC) and ABCann UNIMIX 1 with higher WHC. No differences in growth or floral dry weight (yield) were found between the two substrates. Pooled data from both substrates showed that the highest yield was achieved at a rate that supplied 389 mg N·L<sup>-1</sup> (interpolated from yield-fertilizer responses) which was 1.8 times higher than that of the lowest fertilizer rate. The concentration of Δ<sup>9</sup>-tetrahydrocannabinol (THC) in dry floral material was maximized at a rate that supplied 418 mg N·L<sup>-1</sup> and no fertilizer rate effects were observed on Δ<sup>9</sup>-tetrahydrocannabinolic acid (THCA) or cannabinol (CBN). The highest yield, cannabinoid content and plant growth was achieved around an organic fertilizer rate that supplied 389 mg N·L<sup>-1</sup> during the vegetative growth stage when using the two coir-based organic substrates.

**Key words:** *Cannabis sativa*, cannabis growth, floral dry weight, marijuana, THC, CBN

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## Introduction

Cannabis (*Cannabis sativa* L.) legislation in North America continues to move rapidly towards liberalization and in some instances legalization, shifting cultivation from a largely illicit practice to one that is not only legal, but in high demand. In the United States, with only a handful of states having legalized recreational cannabis as of 2017, the market for legal cannabis was estimated at \$2.7 billion USD in 2014 and it is expected to reach \$11 billion by 2019 (ArcView Market Research and New Frontier, 2014). The current Canadian government has pledged to follow suit and pass legislation to legalize cannabis for recreational purposes beginning in Fall of 2018. Until then, current legislation allows a limited number of private, licensed facilities to produce and distribute cannabis for medicinal purposes as well as conduct scientific research (Government of Canada, 2016).

Cannabis is an annual, predominantly dioecious species, producing separate male and female plants. Archeological evidence of cultivation dates to 10,000 BCE in China where cannabis was used primarily for fiber. Later, the medicinal use of cannabis became widespread, with evidence of cultivation and use in ancient Egypt around 2800 BCE and in China around 2000 BCE (Russo, 2007). The medicinal value of cannabis is attributed primarily to a group of secondary metabolites unique to cannabis called cannabinoids which are concentrated mostly in the essential oils of unfertilized female flowers (Potter, 2014).

Over 100 unique cannabinoids have been identified (Ahmed et al., 2008, 2015; ElSohly and Slade, 2005); though,  $\Delta^9$ -tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most abundant and considered the primary psychoactive and medicinal components (Elzinga et al., 2015; Mechoulam et al., 1970). In live plants, cannabinoids exist primarily as carboxylic acids such as  $\Delta^9$ -tetrahydrocannabidiolic acid (THCA) and cannabidiolic acid (CBDA) (Muntendam et al., 2012). These acids undergo decarboxylation during storage (Ross and ElSohly, 1997; Taschwer and Schmid, 2015) and upon heating (Kimura and Okamoto, 1970) to become neutral cannabinoids such as THC and CBD. Varieties of cannabis with low THC and high CBD are termed hemp or fiber-type cannabis while those with high THC and low CBD are termed marijuana or drug-type cannabis, hereafter referred to as cannabis (van Bakel et al., 2011; Vollner et al., 1986). Selective breeding has produced hundreds of cultivars of cannabis with

varying chemical compositions and growth characteristics (Vollner et al., 1986). Selection has mostly been for high floral THC concentration; but, the medicinal effects of CBD have recently been identified (Russo, 2011) leading some breeders to select for high CBD. Most indoor production of cannabis occurs in two growth stages, vegetative and flowering, which are controlled by photoperiod (Farag and Kayser, 2015). Modern commercial cultivation of cannabis takes place almost exclusively indoors under artificial lighting using either solution culture systems or soilless growing substrates (Leggett, 2006; Potter, 2014). Additionally, many cannabis growers favor organic production practices since consumers and regulating bodies often demand pesticide-free cannabis (Government of Canada, 2016).

Online horticultural resources are available for cannabis production; however, limited information is available in peer-reviewed scientific literature. Furthermore, there is scant published scientific research on any aspect of organic cannabis production. Due to a lack of systematic horticultural research, current cannabis producers rely on cultivation methods derived largely from anecdotal information. Information on fiber-type cannabis cultivation techniques allows for some parallels to be drawn; however, fiber-type cannabis is field-grown and has been selectively bred for fiber production rather than for essential oil content (Amaducci et al., 2015). A chemotaxonomic study found low gene flow between drug- and fiber-type cannabis (Hillig and Mahlberg, 2004) and was supported by a recent genomic study comparing fiber and drug-type cannabis (van Bakel et al., 2011). This makes it difficult to relate cultivation techniques between the two crops (Amaducci et al., 2015).

Fertilization is one of the most important factors for indoor organic cannabis production. For fiber-type cannabis, the suggested fertilization rate is around 50 - 200 kg N·ha<sup>-1</sup> (Aubin et al., 2015; Ehrensing, 1998; Vera et al., 2004), which is similar to other high-yielding field crops such as wheat (*Triticum spp.*; Baxter, 2008). It is difficult however, to estimate fertilizer requirements of drug-type cannabis based on fiber-type cannabis, or other crops due to the differences in species and growing conditions (Wright and Niemiera, 1987). Furthermore, it is well-known that different growth stages of the same species have varying nutrient demand; when the demand is met, plant performance is improved (Raviv, 2007; Wang, 2000). Most studies on fertilizer application in other crops have been conducted using conventional fertilizers and there are few on the use of organic fertilizers for container crops. Fertigation rates of 190 - 400 mg

$\text{N}\cdot\text{L}^{-1}$  have been reported for container production of organic greenhouse-grown tomatoes (*Solanum lycopersicum* L.; Surrage et al., 2010; Zhai et al., 2009). To our knowledge, neither organic nor conventional fertilizer application rates have been published for indoor cannabis production in scientific literature.

Appropriate choice of a growing substrate is essential for soilless crop production, since it directly affects root zone water, air and nutrient availability and balance (Zheng, 2016). While there are no experimental data on growing substrates for cannabis, the information we collected from the industry indicates that many North American cannabis producers are using either coir- or peatmoss-based substrates, or inert substrates such as rockwool. Different substrates have different physical and chemical properties; therefore, it is essential to fertigate plants accordingly to ensure an adequate root zone environment (Zheng, 2016).

The objective of this study was to determine the optimal organic fertilizer rates for growing vegetative-stage cannabis plants in two coir-based organic growing substrates in a controlled environment growth chamber.

## **Materials and Methods**

### **Plant culture and treatments**

Seventeen-day-old rooted cuttings ( $\approx 10$  cm high with  $\approx 6$  leaves) of *Cannabis sativa* cv. ‘OG Kush  $\times$  Grizzly’ were transplanted into round peat-based pots (9.5 cm diameter  $\times$  10.2 cm high) with one plant per pot. Pots were filled with one of two growing substrates, ABcann UNIMIX 1-HP (U1-HP) or ABcann UNIMIX 1 (U1) (Physical and chemical properties presented in Table 3.1 and Table 3.2 respectively; ABcann Medicinals Inc., Napanee, ON, Canada). The two organic substrates were coir-based and with two distinct water-holding capacities (WHC): U1-HP with lower WHC and more drainage than U1.

Table 3.1. Physical properties of growing substrates ABcann UNIMIX 1-HP (U1-HP) and ABcann UNIMIX 1 (U1).

Growing Substrate	Total Porosity <sup>z</sup>	CC <sup>z</sup>	Air Space <sup>z</sup>	Bulk Density <sup>z</sup>
		%		g/cm <sup>3</sup>
U1-HP	93 ± 0.4	61 ± 1.2	31 ± 1.3	0.09 ± 0.001
U1	91 ± 0.3	72 ± 0.2	19 ± 0.3	0.10 ± 0.001

<sup>z</sup>Data are means ± SEM (n = 3). CC = container capacity.

Table 3.2. Electrical conductivity (EC), pH and nutrient content measured using the saturated paste method for growing substrates ABcann UNIMIX 1-HP (U1-HP) and ABcann UNIMIX 1 (U1).

Growing Substrate	EC <sup>z</sup>	pH <sup>z</sup>	Nitrate N	P	K	Ca	Mg	SO <sub>4</sub> <sup>2-</sup>	Na	Cl <sup>-</sup>	Zn	Mn	Cu	Fe	B	Mo
	mS·cm <sup>-1</sup>								mg L <sup>-1</sup>							
U1-HP	1.8 ± 0.07	6.30 ± 0.01	5	9.2	338.1	<1	2.7	31.2	104.5	413	<0.01	<0.01	<0.01	0.12	0.09	<0.01
U1	2.3 ± 0.12	6.28 ± 0.01	8	10.4	431.2	2.3	5.3	41.3	136.3	724	<0.01	0.01	<0.01	0.84	0.13	<0.01

<sup>z</sup>Data are means ± SEM (n = 3). EC = electrical conductivity, N = Nitrogen, P = Phosphorus, K = Potassium, Ca = Calcium, Mg = Magnesium, SO<sub>4</sub><sup>2-</sup> = Sulfate, Na = Sodium, Cl<sup>-</sup> = Chloride, Zn = Zinc, Mn = Manganese, Cu = Copper, Fe = Iron, B = Boron and Mo = Molybdenum

Pots were randomly arranged in a growth chamber at a density of 97 plants·m<sup>-2</sup>. The growth chamber was set at 22 °C, 85% RH, 500 ppm CO<sub>2</sub> (day and night) and a photosynthetically active radiation (PAR) of 250 ± 50 μmol·m<sup>-2</sup> s<sup>-1</sup> at canopy level with an 18-hour photoperiod under fluorescent lighting. Beginning 3 days after transplant, plants were hand-fertigated with corresponding nutrient solution to saturation with a 20% leaching fraction when mean substrate moisture was ≈30%, measured using a WET-2 soil moisture sensor (Delta-T Devices Ltd., Cambridge, UK). This was considered the first day of treatment application. Plants were re-randomized after each irrigation event.

The experiment was a completely randomized design with two factors: five fertilizer rates and two substrate types, with 10 replicates for each factor combination. Each potted plant was an experimental unit. Plants were fertilized at one of the five rates of Nutri Plus Organic Grow liquid organic fertilizer (4.0N–1.3P–1.7K; Nutri Plus Grow; EZ-GRO Inc., Kingston, ON, Canada), supplying 117, 234, 351, 468 or 585 mg N·L<sup>-1</sup>, diluted with reverse osmosis (RO) water. Other nutrient element concentrations of Nutri Plus Grow were (in mg·L<sup>-1</sup>): 0.0 Ca, 0.0 Mg, 14.5 Zn, 12.0 B, 2.6 Mo, 2.1 Cu, and 8.5 Fe.

At the end of the vegetative growth period (21 days after transplanting), six plants with representative height and canopy size from each treatment were selected and transferred into a growth chamber for the flowering stage. Plants were potted into 6 L blow-molded black pots (22 cm diameter × 22 cm height) containing a custom blended organic growing substrate (60% sphagnum peatmoss and 40% bulk coconut coir; Premier Tech, Rivière-du-Loup, QC, Canada). Agricultural dolomitic lime (Premier Tech) was incorporated at a rate of 3.0 kg·m<sup>-3</sup> of substrate. Plants were spaced on tables to a density of 6.5 plants·m<sup>-2</sup>. The PAR was maintained at 500 ± 50 μmol·m<sup>-2</sup> s<sup>-1</sup> with a 12-hour photoperiod. Irrigation was administered with one emitter per plant. During the first 11 days in the flowering stage, plants were irrigated whenever the substrate moisture content reached 30% with Nutri Plus Grow at a recommended rate of 140 mg N·L<sup>-1</sup> and from then on with a flowering specific fertilizer, Nutri Plus Organic Bloom (2.00N–0.87P–3.32K; EZ-GRO Inc.). Other nutrient element concentrations in Nutri Plus Organic Bloom were (in mg L<sup>-1</sup>): 0.0 Ca, 0.0 Mg, 10.0 Zn, 12.8 B, 0.1 Mo, 2.3 Cu, and 6.8 Fe. Nutri Plus Organic Bloom was administered at the following manufacturer-recommended rates: 77 mg N·L<sup>-1</sup> from day 12 to 19 in the flowering stage, 103 mg N·L<sup>-1</sup> from day 20 to 27, and 129 mg N·L<sup>-1</sup> from day

28 to 39. Both vegetative and flowering fertilizers were amended with 2 mL·L<sup>-1</sup> of an organic Calcium-Magnesium supplement (3.0N–0.0P–0.0K–3.0Ca–1.6Mg; EZ-GRO Inc.). Between days 39 and 47 in the flowering stage, no fertilizer was applied, and the substrates were flushed, as per current industry practice, with RO water: 10 L per pot at 7 days before harvest and 6 L at 5 days before harvest.

### **Growth and yield measurements**

During the vegetative stage, leaf number, canopy area and plant height were measured every  $\approx 7$  days on 5 randomly selected plants from each treatment. Repeated measurements were made on these same plants throughout the vegetative stage. During the flowering stage, branch number, canopy area and plant height were measured on all plants every  $\approx 10$  days. Growth index for each plant was calculated as height (cm) x length (cm) x width (cm) x 300<sup>-1</sup> (Ruter, 1992). Plants were harvested after 47 days in the flowering stage on 14 December 2015 when floral resin on most plants had  $\approx 50\%$  amber coloration. Stems were cut at soil level; floral material was cut from stems and leaves were trimmed thereafter. Floral fresh weight was measured before the floral material was placed in paper bags for drying at 21 °C and 40% RH for 5 days until moisture content reached  $11 \pm 1\%$ . Dry material was then cured at 18 °C and 60% RH for 14 days before determining the floral dry weight (yield).

### **Substrate EC and pH measurement**

Substrate pH and EC were determined weekly using the pour-through method (Wright, 1986) during the vegetative stage and at 4 and 5 weeks of the flowering stage. Pour-through solutions were measured for pH and EC using a HI991300 portable pH/EC/TDS/Temperature Meter (Hanna Instruments, Woonsocket, R.I., USA).

### **Floral cannabinoid analysis**

Dried, cured floral material was stored in dark and cool conditions according to United Nations Office on Drugs and Crime (2009) before being analyzed by an independent laboratory (RPC Science and Engineering, Fredericton, NB, Canada). Cannabinoid analysis was performed on the floral material of plants grown in U1 substrate. Analysis of the neutral cannabinoids THC, CBD and cannabiol (CBN) as well as acid forms, THCA and CBDA were conducted by high-

performance liquid chromatography as described in section 5.4.8 of United Nations Office on Drugs and Crime (2009).

## **Statistical analysis**

Data were analyzed using JMP Statistical Discovery Version 13.0 (SAS Institute Inc., Cary, NC) at a Type 1 error rate of  $\leq 0.05$ . Full-factorial ANOVA with repeated measures was used to determine the effects of substrate, fertilizer and their interaction on substrate EC and pH as well as growth index, leaf number and branch number over time. Differences among means were tested with Tukey's multiple means comparison test. Two-way ANOVA was used to determine the effects of substrate, fertilizer and their interaction on yield and the effects of fertilizer on cannabinoid concentrations.

Pearson correlation coefficients were calculated to determine if there is a relationship between growth attributes and final yield. Orthogonal partition and regression analysis (Bowley, 1999) were used to relate substrate EC, pH, plant growth, yield and cannabinoid concentrations with fertilizer rate and/or yield. If the partitioning variance analysis indicated a significant treatment effect, then the treatment effects were partitioned into one or more regression effects followed by an estimation of regression parameters for the best-fit regression. In all analyses, if there was no significant treatment effect, then data were presented as the average of all the treatments (pooled). If cannabinoid concentrations were below the detection limit ( $<0.05\%$ ), the values were excluded from the analysis. The residuals of the above analyses were tested for normality and equality of variance using The Shapiro-Wilk test and Bartlett's test, respectively.

## **Results**

### **Growth**

There were no observed signs of nutrient toxicity or deficiency at any fertilizer rate during the vegetative or flowering stage. Identifying nutrient disorders based on visible foliar symptoms became difficult at 6 weeks of the flowering stage when all plants began showing signs of foliar senescence. Older leaves started to become chlorotic in week 6 and eventually necrotic before harvest. Substrate and substrate $\times$ fertilizer rate had no effect on leaf number, branch number or

growth index. During the vegetative stage, both leaf number and growth index responded similarly to fertilizer rate (Figure 3.1). Growth attributes did not respond to fertilizer rate at 5 days after the first treatment application (DAT) though, growth index responded to fertilizer rate linearly at 13 DAT and quadratically at 19 DAT; and leaf number responded to fertilizer rate quadratically at 13 and 19 DAT. At 19 DAT, maximum leaf number was 42, achieved at a rate that supplied 420 mg N·L<sup>-1</sup> and maximum growth index was 51 at a rate that supplied 477 mg N·L<sup>-1</sup>. Treatment effects carried forward into the flowering stage in which branch number responded quadratically to vegetative stage fertilizer rate (maximum of 17.6 at a rate that supplied 403 mg N·L<sup>-1</sup>) and growth index increased linearly with increasing vegetative stage fertilizer rate.

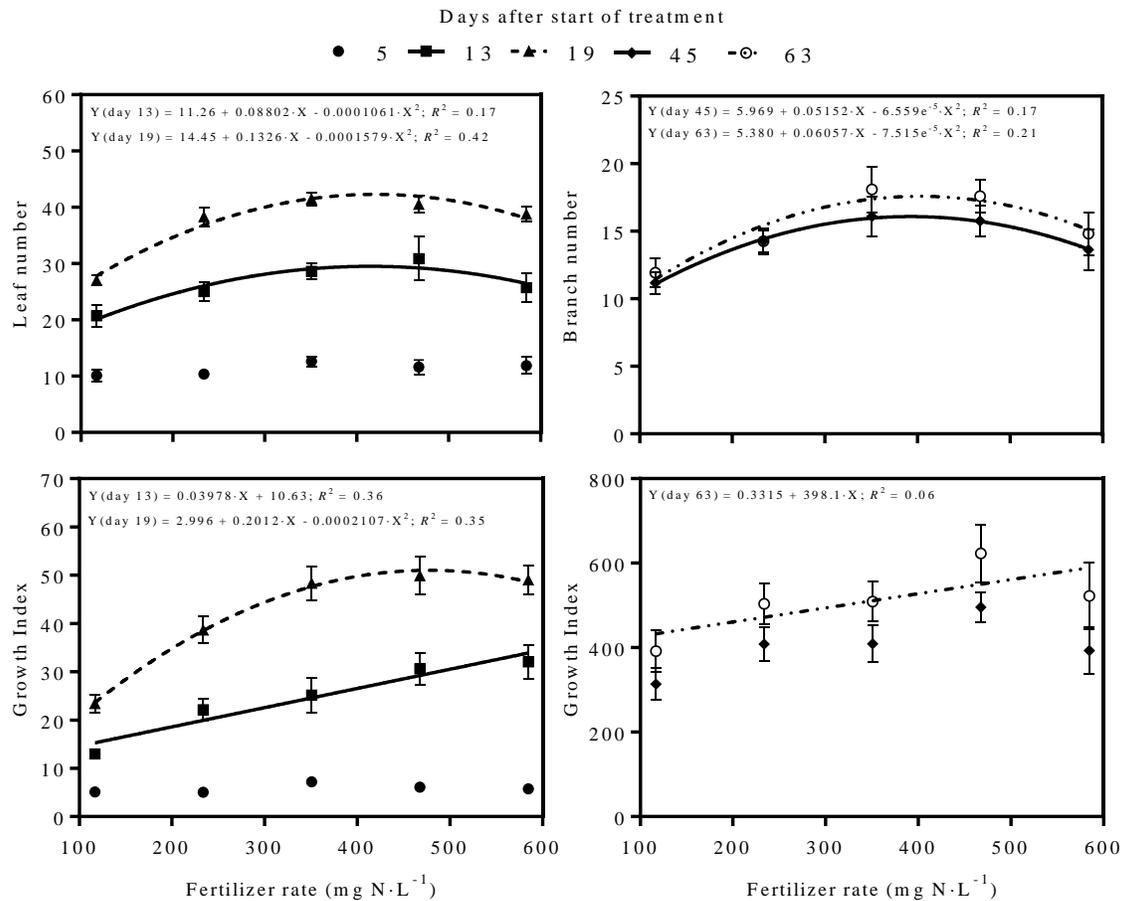


Figure 3.1. Response of cannabis growth attributes to organic fertilizer (4.0N–1.3P–1.7K) rate (indicated by nitrogen (N) concentration) applied during the vegetative stage. Values are means  $\pm$  SEM and lines are the best fit regression relationships at  $P < 0.05$ . For days 5 and 13,  $n = 10$ ; for day 19,  $n = 20$  (vegetative stage; left); for days 45 and 63 (flowering stage; right) at rates that supplied 117, 234 and 468 mg N·L<sup>-1</sup>,  $n = 12$ ; at rates that supplied 351 and 585 mg N·L<sup>-1</sup>,  $n = 11$ .

## Yield

There was no yield difference between substrates, and no substrate $\times$ fertilizer rate effect on yield. Based on the pooled data from both substrates, yield responded to fertilizer rate quadratically with the highest yield at a rate that supplied 389 mg N·L<sup>-1</sup> (Figure 3.2). Yield at this fertilizer rate was interpolated to be 41.6 g/plant which is 1.8 times higher than that at the lowest which supplied 117 mg N·L<sup>-1</sup>. Yield was positively correlated with growth index ( $r = 0.45$ ,  $P < 0.001$ ),

leaf number ( $r = 0.39$ ,  $P = 0.0027$ ), and branch number ( $r = 0.53$ ,  $P < 0.0001$ ) measured at the end of the vegetative stage (19 DAT;  $n = 58$ ).

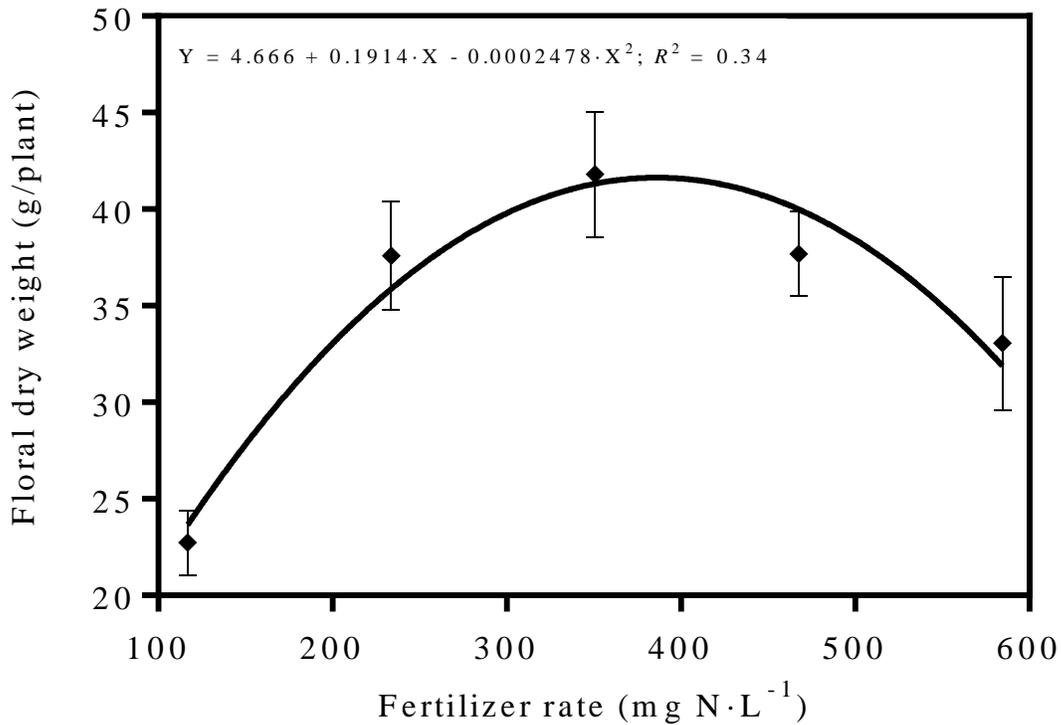


Figure 3.2. Response of cannabis yield to organic fertilizer (4.0N–1.3P–1.7K) rate (indicated by nitrogen (N) concentration) applied during the vegetative stage. Values are means  $\pm$  SEM. The curve is best fit regression relationship with  $P < 0.05$  ( $n = 12$  at rates that supplied 117, 234 and 468 mg N · L<sup>-1</sup>;  $n = 11$  for rates that supplied 351 and 585 mg N · L<sup>-1</sup>).

### Cannabinoids

Of the analyzed cannabinoids, only THC, THCA, and CBN were above the detection limit (0.05%). Floral THC concentration responded quadratically to increasing fertilizer rate, reaching a maximum of 0.31% at a rate supplying 418 mg N · L<sup>-1</sup> (Figure 3.3). There was no fertilizer rate effect on the floral THCA concentration (mean  $\pm$  SEM of 10.6  $\pm$  0.31%) or CBN concentration (mean  $\pm$  SEM of 0.08  $\pm$  0.018%). Cannabinoid concentrations also varied with yield. THC and

CBN were positively correlated with yield while THCA was not correlated with yield (Figure 3.4).

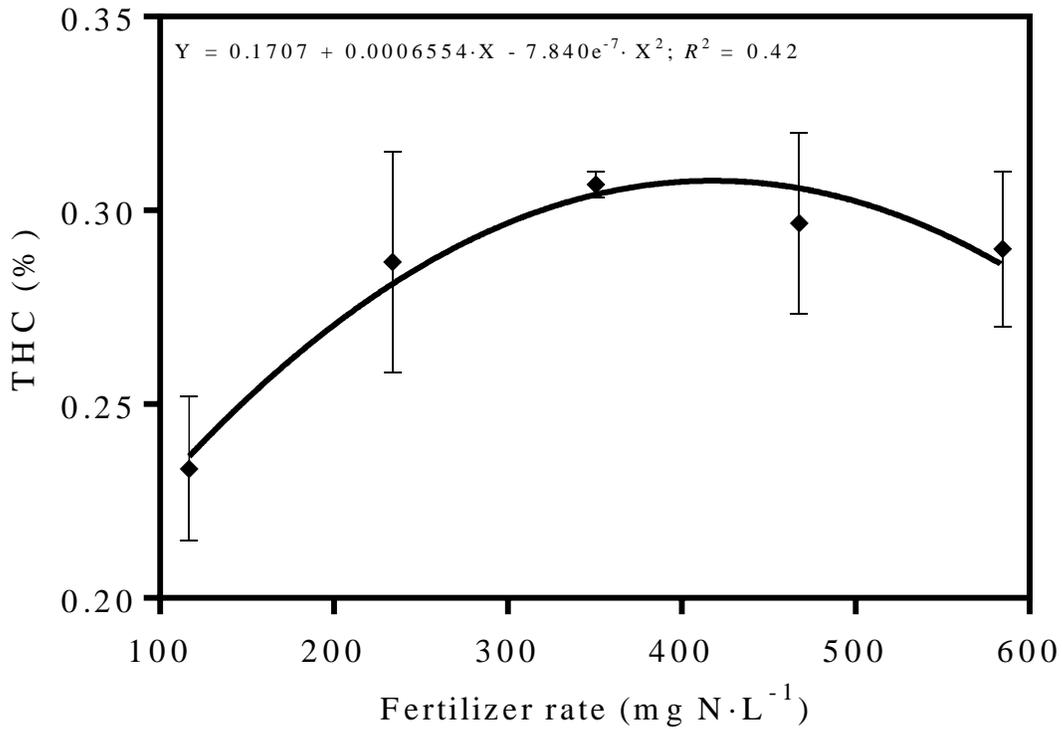


Figure 3.3. Relationship between  $\Delta^9$ -tetrahydrocannabinol (THC) concentration in dry floral material of cannabis and organic fertilizer (4.0N–1.3P–1.7K) rate (indicated by nitrogen (N) concentration) applied during the vegetative stage. Values are means  $\pm$  SEM. The curve is the best fit regression relationship with  $P < 0.05$  ( $n = 3$ ).

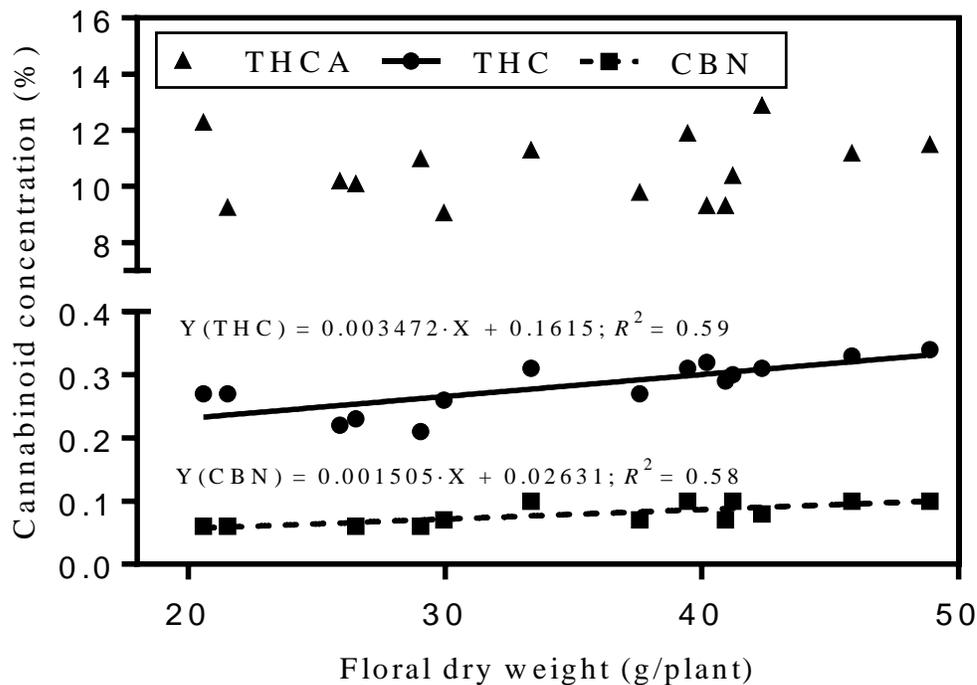


Figure 3.4. Relationships between cannabinoid concentrations in dry floral material of cannabis and dry floral weight. THCA =  $\Delta^9$ -tetrahydrocannabinolic acid, THC =  $\Delta^9$ -tetrahydrocannabinol and CBN = cannabiniol. Values are means  $\pm$  SEM (n = 15 for THCA and THC; n = 13 for CBN). Lines are the best fit regression relationships with  $P < 0.05$ .

### Substrate EC and pH

Substrate pH decreased over time for all fertilizer rates during the vegetative stage (Figure 3.5), decreasing linearly or responding quadratically to increasing fertilizer rate. The lowest mean pH was 6.19 at the 351 mg N·L<sup>-1</sup> rate, measured at 17 DAT. Substrate EC, measured at 5, 13, and 17 DAT, increased linearly over time and with increasing fertilizer rate. Mean EC ranged from 0.9 to 3.9 mS·cm<sup>-1</sup> from the lowest to the highest fertilizer rate at 17 DAT. In the flowering stage, pH (measured at 47 and 59 DAT) increased linearly with increasing vegetative stage fertilizer rate with means ranging from 6.74 to 7.16 (Figure 3.6). No difference was observed in EC among vegetative stage fertilizer rates during the flowering stage with substrate EC at 1.3  $\pm$  0.03 and 1.6  $\pm$  0.02 mS·cm<sup>-1</sup> (mean  $\pm$  SEM) at 47 and 59 DAT, respectively. No differences in

substrate EC or pH were observed between the two tested substrates in both the vegetative and flowering stages.

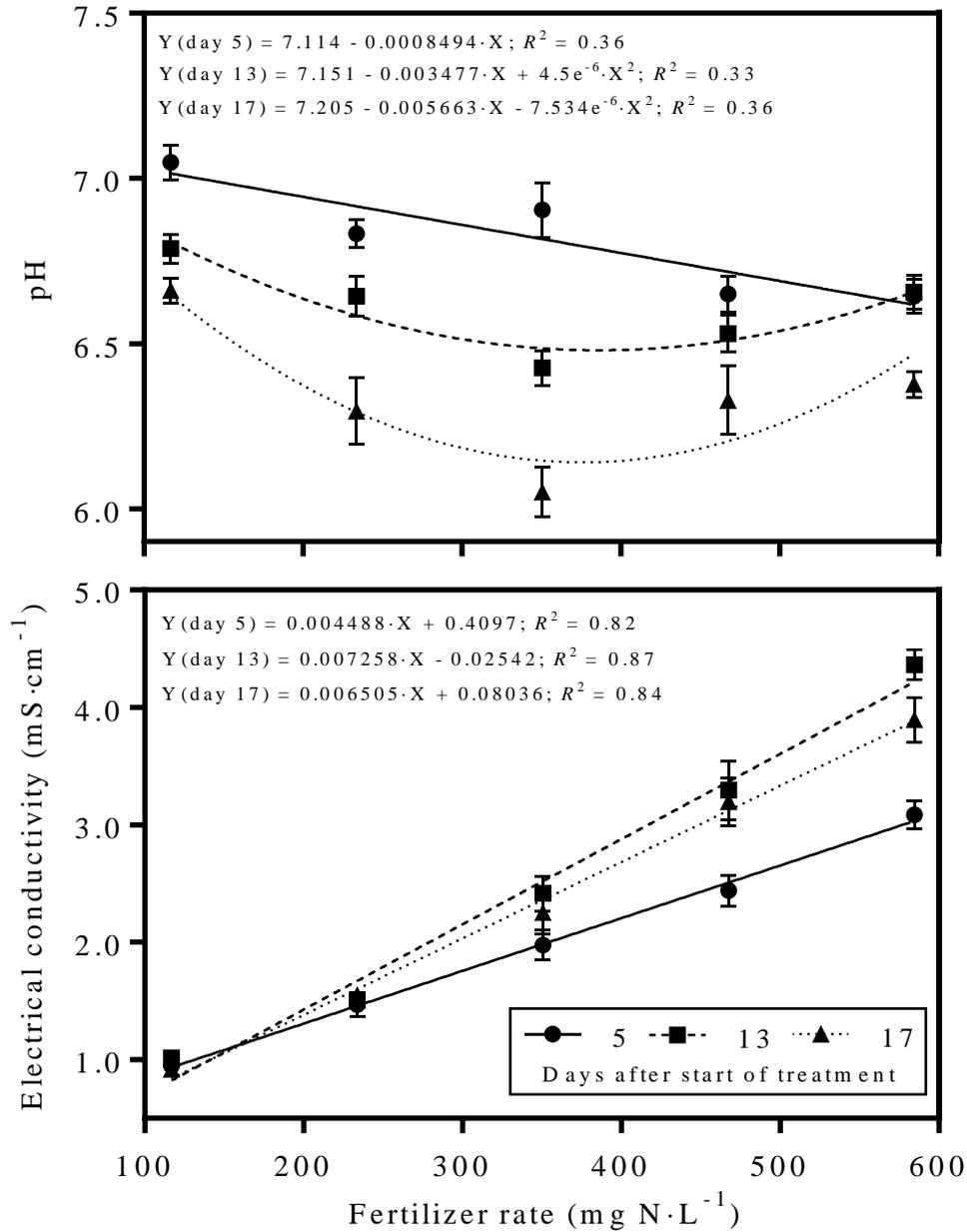


Figure 3.5. Response of substrate pH and electrical conductivity to organic fertilizer (4.0N–1.3P–1.7K) rate (indicated by nitrogen (N) concentration) applied during the vegetative stage. Data are means  $\pm$  SEM ( $n=5$  for pH at the 585 mg N · L<sup>-1</sup> rate on day 17 and  $n=10$  for all other means) and lines are the best fit regression relationships with  $P < 0.05$ .

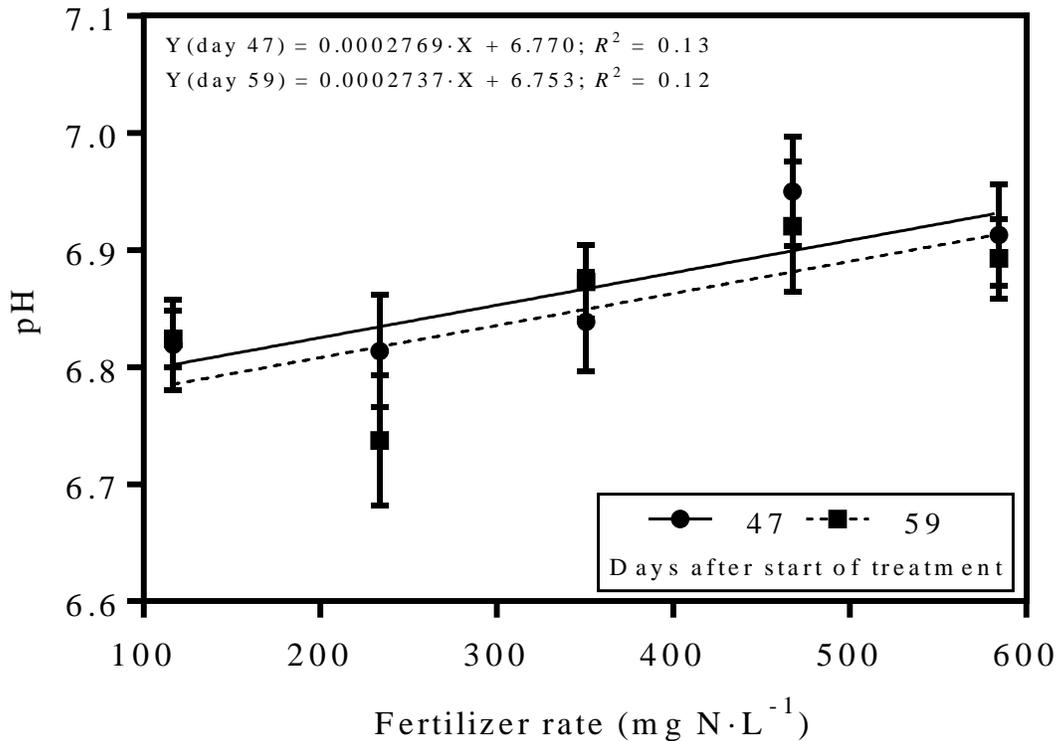


Figure 3.6. Response of substrate pH during the flowering stage to organic fertilizer (4.0N–1.3P–1.7K) rate (indicated by nitrogen (N) concentration) applied during the vegetative stage. Data are means  $\pm$  SEM ( $n = 8$ ) and lines are the best fit regression relationships with  $P < 0.05$ .

## Discussion

No visual signs of nutrient disorders were observed in this trial which suggests that the fertilizers used had nutrient elements and ratios within an acceptable range. Both growth attributes and yield of the cannabis plants exhibited a typical response to varying fertilizer application rates. Yield increased with increasing fertilizer until reaching a maximum at a rate supplying 389 mg N · L<sup>-1</sup>. Optimal organic fertilizer application rates in this experiment were higher than synthetic fertilizer recommendations for most conventional crops (Raviv, 2007). Organic fertilizers contain slower releasing and less soluble forms of Nitrogen and Phosphorus compared to most synthetic fertilizers and may release only 25% to 60% of their Nitrogen content (Prasad et al., 2004). Therefore, it is important to establish organic-specific fertilizer rates rather than using

conventional standards as guidelines. Results from our present study showed a fertilizer rate supplying 389 mg N·L<sup>-1</sup> provided the highest yield while increasing or having no effect on the concentration of the cannabinoids measured.

Substrate EC increased over time during the vegetative stage and the increase was more apparent at higher fertilizer rates. Sub-optimal yields were seen at fertilizer rates that supplied 468 and 585 mg N·L<sup>-1</sup> under which substrate EC was  $3.0 \pm 0.13$  and  $3.8 \pm 0.13$  mS·cm<sup>-1</sup>, respectively. These yield reductions may have been caused by high substrate salinity. High salinity can damage crops through increased osmotic potential, depressing the external water potential in the root zone. In greenhouse-grown flowering crops, salinity thresholds vary dramatically among species, ranging in EC from of 1.0 to > 4.2 mS·cm<sup>-1</sup> (Sonneveld et al., 1999). In the current study cannabis tolerated substrate EC up to 3.0 mS·cm<sup>-1</sup> without reduction in yield.

In all fertilizer rates, pH decreased gradually during the vegetative stage; and the highest yielding rates, which supplied 234, 351, and 468 mg N·L<sup>-1</sup>, exhibited the lowest pH values. In most organic fertilizers, nitrogen exists primarily as NH<sub>4</sub><sup>+</sup> (i.e., high NH<sub>4</sub><sup>+</sup>-N/NO<sub>3</sub><sup>-</sup>-N ratio; Gül et al., 2007) and can be taken up by plants directly or as other forms after being converted by microorganisms in the substrate via ammonification and nitrification (Shinohara et al., 2011). Reductions in pH under organic fertilization can be caused by NH<sub>4</sub><sup>+</sup> nitrification and the excretion of protons by the roots after NH<sub>4</sub><sup>+</sup> uptake (Johnson et al., 2011; Silber et al., 2004). It is possible that larger plants, those fertilized at rates identified as, or close to, optimal in this study, had higher rates of NH<sub>4</sub><sup>+</sup> uptake which decreased root zone pH. There is no experimental data in the literature on ideal growing substrate pH range for cannabis in soilless production system; however, information we collected from the industry and gray resources (Cervantes, 2006) suggest a range of 5.8 - 6.8 to avoid causing nutrient disorders. In the current study, there were no visual signs of pH-induced disorder in plants within the pH ranges measured (means of 6.2 to 7.1 in the vegetative stage and 6.7 and 7.2 in the flowering stage) suggesting that these ranges are suitable for container production of organic cannabis. More research is needed to determine the optimal growing substrate pH ranges for cannabis.

Around the optimal fertilizer rate, both growing substrates tested in the current study demonstrated acceptable qualities for the growth of cannabis in the vegetative stage. There were

no growth or yield differences observed between plants grown in the lower water holding capacity (drier) substrate (U1-HP) and the higher water holding capacity (wetter) substrate (U1) with fertigation administered when substrate moisture content dropped to 30%. This indicated that both substrates were appropriate for container production of organic cannabis.

The positive correlations between growth attributes in the vegetative stage and final yield may indicate that growing larger plants during the vegetative stage will increase yield. Since larger plants, those fertilized at rates around the optimal fertilizer rate, had increased THC concentration in floral material (maximized at the rate supplying  $418 \text{ mg N}\cdot\text{L}^{-1}$ ) and the concentrations of other cannabinoids were not affected, it may be concluded that to optimize yield and total THC content, cultivation techniques to increase vegetative growth, specifically branching, should be used. Besides fertigation, other cultural practices such as topping (Tanaka and Fujita, 1974) may also be used to increase branching.

The highest yielding plants, those fertilized around the optimal rate, had higher concentrations of THC and CBN. In fact, as yield increased, so did the concentration of these neutral cannabinoids. During the flowering stage, THCA transcription in floral material slows between weeks 1 and 3 whereas total cannabinoid concentration continues to increase until weeks 3 and 4, as THCA breaks down into neutral cannabinoids such as THC and CBN (Muntendam et al., 2012). This leads to an accumulation of neutral cannabinoids as plants mature through the flowering stage. It is estimated that higher concentrations of neutral cannabinoids, as seen in plants fertilized around the optimal rate, would be observed in plants which mature early. Optimal fertigation during the vegetative stage may, therefore reduce maturation time in cannabis. Early maturation is desirable as it could decrease time to harvest and result in more frequent crop turnover. To evaluate whether fertigation can, in fact, reduce maturation time, further study is required with cannabinoid analyses throughout the flowering stage.

In the current study, treatments were applied only in the vegetative stage whereas cannabinoid production occurs primarily during flowering because of an increase in glandular trichome development in the flowering stage (Muntendam et al., 2012; Vogelmann et al., 1988). Treatment effects carried forward to some final floral cannabinoid concentrations; however, effects may have been more apparent with variable fertilizer treatments during the flowering

stage. Further research is needed to evaluate the effects of fertilizer rate on flowering-stage cannabis.

Yields in the current study were slightly lower than industry standards and reports from recent horticultural studies on cannabis (Potter and Duncombe, 2012; Vanhove et al., 2011, 2012). The 47-day flowering period in the current study was relatively short, compared to the 7-9-week range in these cited studies. A shorter flowering period is known to reduce yields (Potter, 2014). Other factors including cannabis cultivar and the use of organic fertilizer may have also played a role.

## **Conclusions**

Our results demonstrated that to produce high-yielding, cannabinoid rich plants, the optimal fertilizer rate was that supplying about 389 mg N·L<sup>-1</sup>, for Nutri Plus Organic Grow liquid organic fertilizer (4.0N–1.3P–1.7K) in the vegetative growth stage of cannabis using coir-based organic substrates. These recommendations should be acceptable for similar organic fertilizer and substrates; however, different cannabis varieties may have different fertilization requirements. To provide cultivar-specific fertilization requirements, further study may be needed. Both organic substrates ABCann UNIMIX 1-HP and ABCann UNIMIX 1 maintained suitable pH (between 6.2 and 7.1 in the vegetative stage and between 6.7 and 7.2 in the flowering stage) and were effective for vegetative-stage cannabis growth; however, U1-HP may require more frequent fertigation than U1. Growing substrate EC of up to 3.0 mS·cm<sup>-1</sup> was tolerated without yield reductions. Further, larger plants (e.g., higher growth index, branching and leaf number) generally had higher yield and floral THC concentrations which may indicate that plants should be grown as large as possible during the vegetative stage.

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## CHAPTER FOUR:

### Optimal rate of organic fertilizer during the flowering stage for cannabis grown in two coir-based substrates<sup>3</sup>

#### Abstract

In the expanding North American medical cannabis industry, growers lack reliable and systematically investigated information on the horticultural management of their crops, especially with regards to nutrient management and growing substrates. To evaluate organic substrates and their optimal nutrient management, five rates that supplied 57, 113, 170, 226, and 283 mg N/L of a liquid organic fertilizer (2.00N–0.87P–3.32K) were applied to container-grown plants [*Cannabis sativa* L. ‘WP:Med (Wappa)’] in two coir-based organic substrates. The trial was conducted in a walk-in growth chamber and the two substrates used were ABcann UNIMIX 2-HP (U2-HP) with lower container capacity (CC) and ABcann UNIMIX 2 (U2) with higher CC. U2-HP produced 11% higher floral dry weight (yield), 13% higher growth index, 20% higher  $\Delta^9$ -tetrahydrocannabinol (THC) concentration, 57% higher THC yield (per plant), 22% higher  $\Delta^9$ -tetrahydrocannabinolic acid (THCA) yield, and 20% higher cannabigerolic acid (CBGA) yield than U2. Increasing fertilizer rate led to increased growth and yield but also to a dilution of THC, THCA and CBGA. In U2-HP, to maximize both yield and cannabinoid yield, the optimal organic fertilizer rates were those which supplied 212 to 261 mg N/L. For U2, the highest applied rate, that supplied 283 mg N/L, maximized yield; though, lower rates delivered higher cannabinoid concentrations in dry floral material. The results on these substrates and recommended fertilizer rates can serve as a guide when using other organic fertilizers and substrates; though, results may differ with cannabis cultivar.

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**Key words:** *Cannabis sativa*, cannabis growth, floral dry weight, marijuana, THC, THCA, CBGA

## Introduction

The North American market for government-regulated cannabis (*Cannabis sativa* L.) is expanding at an increasing pace. In 2016, spending on medical cannabis was reported at 4.9 billion USD and is projected to exceed 7 billion USD by 2020 in North America (ArcView Market Research, 2017).

Cannabis is an annual herbaceous species which has been widely cultivated and used as a medicinal plant since around 2800 BCE (Russo, 2007). The medicinal value of this species is attributed to a group of secondary metabolites, called cannabinoids which are concentrated in the essential oils of unfertilized female flowers (Potter, 2014). Over 100 unique cannabinoids have been identified (Ahmed et al., 2008, 2015; ElSohly and Slade, 2005; Radwan et al., 2015); though,  $\Delta^9$ -tetrahydrocannabinol (THC) and cannabidiol (CBD) have been most widely studied for their psychoactive and medicinal properties (Elzinga et al., 2015; Mechoulam et al., 1970; Vemuri and Makriyannis, 2015). In live plants, cannabinoids exist predominantly as carboxylic acids such as  $\Delta^9$ -tetrahydrocannabidiolic acid (THCA) and cannabidiolic acid (CBDA) (Muntendam et al., 2012). These acids decarboxylate during storage (Ross and ElSohly, 1997; Taschwer and Schmid, 2015) and upon heating (Kimura and Okamoto, 1970) to become neutral cannabinoids such as THC and CBD. Some varieties of cannabis have been selectively bred and cultivated mainly for fiber or seed production; these are characterized by low THC and high CBD concentrations and are generally termed hemp or fiber-type cannabis. Varieties with high THC and low CBD are termed marijuana or drug-type cannabis (van Bakel et al., 2011; Vollner et al., 1986), hereafter referred to as cannabis.

Our communications with Canadian medical cannabis producers, relevant horticulture literature relating to cannabis (Knight et al., 2010; Potter and Duncombe, 2012; Vanhove et al., 2011, 2012) and reviews on global cannabis production (Frag and Kayser, 2015; Leggett, 2006; Potter, 2014) suggest that modern day production occurs primarily in controlled environments using artificial lighting and either soilless growing substrates (e.g. Caplan et al., 2017) or solution culture. Further, most indoor production of cannabis occurs in two growth stages,

vegetative and flowering, which are controlled by photoperiod (Potter, 2014). Cannabis production has been and continues to be illegal in much of the world which has limited scientific research on this species, particularly with regards to its production. Growers have access to horticultural guides and online resources, but few are based on scientific research. Published information on hemp production allows for some parallels to be drawn; however, hemp is a field-grown crop and has been selectively bred for fiber or seed production rather than for flower and essential oil production (Amaducci et al., 2015). Additionally, recent studies have found low gene flow between cannabis and hemp (Hillig and Mahlberg, 2004; van Bakel et al., 2011) making it difficult to relate cultivation techniques between the two crops (Amaducci et al., 2015). The lack of horticultural information on cannabis limits producers and patients seeking to grow or consume consistent, high quality medicine.

Fertilization is one of the most important factors for indoor cannabis production. Over-fertilization can lead to salt accumulation in the root zone, whereas under-fertilization can cause nutrient deficiency and lower yields (Bar-Yosef, 1999). The suggested fertilization rate for hemp ranges from 50 to 200 kg N·ha<sup>-1</sup> (Aubin et al., 2015; Ehrensing, 1998; Vera et al., 2004) which is similar to other high-yielding field crops such as wheat (*Triticum spp.*; Baxter, 2008). It is difficult however, to base fertilizer rates for cannabis on suggestions for hemp or other crops due to the differences in species and growing conditions (Wright and Niemiera, 1987). Further, it is common for nutrient requirements to vary based on growth stage in flowering plants. The vegetative growth stage in flowering plants is characterized by an exponential growth rate (biomass increase) and often a higher nutrient demand (Raviv and Lieth, 2007). The flowering stage is characterized by a linear growth rate. In this stage, carbohydrates are translocated to reproductive organs such as flowers and seeds and nutrient demand decreases (Raviv and Lieth, 2007). Varying fertilizer requirements by growth stage have been reported in greenhouse-grown crops such as sweet peppers (*Capsicum annum* L.), for which the highest total plant and fruit yield was achieved by supplying 30% N from NH<sub>4</sub><sup>+</sup>-N in the vegetative stage and using only NO<sub>3</sub><sup>-</sup>-N in the flowering stage (Xu et al., 2001). A recent evaluation on the effects of organic fertilization during the vegetative-stage for cannabis suggests that over-fertilization during the vegetative stage may decrease both THC concentration in floral material and floral dry weight (yield; Caplan et al., 2017) upon harvest. An optimal fertilizer rate of 389 mg N/L was proposed

using a liquid organic fertilizer (4.0N–1.3P–1.7K) in two coir-based organic substrates. To our knowledge, there is no research on flowering-stage fertilizer rates for cannabis.

Appropriate choice of a growing substrate is also important for indoor cannabis production. Substrates vary in physical and chemical properties; therefore, to ensure a suitable root zone environment it is important for fertigation to be tailored to the growing substrate (Zheng, 2016). Substrates with low container capacity (drier substrates) require more frequent irrigation to keep moisture levels constant, whereas substrates with higher container capacity (wetter substrates) require less frequent irrigation and may conserve irrigation water (Raviv and Lieth, 2007). While there is scant research on growing substrates for cannabis, the information we collected from the industry indicates that many North American cannabis producers are using either coir- or peat-based substrates, or inert substrates such as stone wool. Caplan et al., (2017) evaluated two coir-based substrates for vegetative-stage cannabis production. At the end of the vegetative stage, plants were transferred into a growth chamber for the flowering stage under similar conditions to determine if treatment effects carried forward to harvest. The substrates differed in container capacity by  $\approx 11\%$  but no differences in growth, yield or cannabinoid content were reported between the two. Vegetative-stage cannabis may grow well in substrates with container capacities within certain range; however, there are no similar evaluations for flowering stage cannabis production.

The objectives of this study were to: (1) evaluate two coir-based organic growing substrates for flowering-stage cannabis production in a controlled environment growth chamber, and (2) determine the optimal organic fertilizer rates for these substrates.

## **Materials and Methods**

### **Plant culture**

Fifteen-day-old rooted cuttings ( $\approx 10$  cm high with  $\approx 6$  leaves) of *Cannabis sativa* L. ‘WP:Med (Wappa)’ were transplanted into round peat-based pots (9.5 cm diameter  $\times$  10.2 cm high; Jiffy Products N.B. Ltd., NB, Canada) filled with ABcann UNIMIX 1 - HP growing substrate (ABcann Medicinals Inc., Napanee, ON, Canada) with one plant per pot. Pots were placed in a walk-in growth chamber (15 m<sup>2</sup>) at a density of 97 plants·m<sup>-2</sup>.

The chamber air temperature was maintained at 24/23 °C (s.d.  $\pm$  0.04/1.0°C) and relative humidity (RH) was 76/76% (s.d.  $\pm$  3.8/3.9%) during the light/dark period throughout the vegetative stage. Carbon dioxide (CO<sub>2</sub>) concentration was maintained at 545 ppm (s.d.  $\pm$  45 ppm) from 1-4 days after transplant (DAT), 570 ppm (s.d.  $\pm$  18 ppm) from 5-10 DAT and 613 ppm (s.d.  $\pm$  18 ppm) from days 11-19 DAT during the light period and 529 ppm (s.d.  $\pm$  18 ppm) during the entire dark period. Using dimmable fluorescent lighting (Philips Lighting, Markham, ON, Canada) with an 18-hour photoperiod, the photosynthetically active radiation (PAR) at the top of the canopy was maintained at 100  $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$  (s.d.  $\pm$  1  $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$ ) from 1-4 DAT, 199  $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$  (s.d.  $\pm$  6  $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$ ) from 5-7 DAT, 300  $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$  (s.d.  $\pm$  2  $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$ ) from 8-10 DAT and 337  $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$  (s.d.  $\pm$  49  $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$ ) from 11-19 DAT.

Beginning three DAT, plants were hand-fertigated, as per Caplan et al., (2017), using Nutri Plus Organic Grow liquid organic fertilizer (4.0N–1.3P–1.7K; EZ-GRO Inc., Kingston, ON, Canada) at a rate that supplied 389 mg N/L amended with 1 mL·L<sup>-1</sup> of Calcium-Magnesium supplement (0.0N–0.0P–0.0K–3.0Ca–1.6Mg; EZ-GRO Inc.), and with a 20% leaching fraction. Other nutrient element concentrations of Nutri Plus Organic Grow were (in mg·L<sup>-1</sup>): 14.5 Zn, 12.0 B, 2.6 Mo, 2.1 Cu, and 8.5 Fe. Irrigation was administered when mean substrate moisture was  $\approx$ 30%, measured using a WET-2 soil moisture sensor (Delta-T Devices Ltd., Cambridge, UK).

## **Treatments**

At 19 DAT, 60 plants with similar height and canopy size were selected and transferred into a larger walk-in growth chamber (130 m<sup>2</sup>) for the flowering stage. This was considered the first day of the flowering stage (DFS). Four additional plants were harvested at this stage to measure initial growth attributes. Plants were transplanted into 6 L blow-molded black pots (220 mm diameter  $\times$  220 mm height) filled with one of two growing substrates, ABcann UNIMIX 2 - HP (U2-HP) or ABcann UNIMIX 2 (U2) (physical and chemical properties presented in Table 4.1 and 4.2, respectively; ABcann Medicinals Inc.). The substrates were coir-based organic substrates with two distinct container capacities (CC): U2-HP with lower CC and better drainage than U2. Coir weed control disks were used on top of the growing substrate to prevent algae growth.

Table 4.1. Physical properties of growing substrates ABcann UNIMIX 2-HP (U2-HP) and ABcann UNIMIX 2 (U2).

Growing substrate	Total porosity <sup>z</sup>	CC <sup>z</sup>	Air space <sup>z</sup>	Bulk density <sup>z</sup>
		%		g/cm <sup>3</sup>
U2-HP	83 ± 0.5	49 ± 0.4	34 ± 0.4	0.10 ± 0.001
U2	91 ± 0.9	55 ± 2.2	35 ± 1.3	0.09 ± 0.001

<sup>z</sup>Data are means ± SEM (n = 3). CC = container capacity.

Table 4.2. Electrical conductivity, pH, and nutrient content measured using the saturated media extract procedure (Warncke, 1986) for growing substrates ABcann UNIMIX 2-HP (U2-HP) and ABcann UNIMIX 2 (U2).

Growing Substrate	EC <sup>z</sup>	pH <sup>z</sup>	Nitrate N	P	K	Ca	Mg	SO <sub>4</sub> <sup>2-</sup>	Na	Cl <sup>-</sup>	Zn	Mn	Cu	Fe	B	Mo
	mS·cm <sup>-1</sup>		mg L <sup>-1</sup>													
U2-HP	2.2 ± 0.02	6.2 ± 0.03	5	11.8	423	<1	4.0	34.0	118.9	534	<0.01	0.02	<0.01	0.2	0.09	0.01
U2	1.9 ± 0.04	6.4 ± 0.02	5	9.8	353	<1	3.2	37.0	109.4	488	<0.01	0.02	<0.01	0.7	0.12	<0.01

<sup>z</sup>Data are means ± SEM (n = 3). EC = electrical conductivity, N = Nitrogen, P = Phosphorus, K = Potassium, Ca = Calcium, Mg = Magnesium, SO<sub>4</sub><sup>2-</sup> = Sulfate, Na = Sodium, Cl<sup>-</sup> = Chloride, Zn = Zinc, Mn = Manganese, Cu = Copper, Fe = Iron, B = Boron and Mo = Molybdenum

The experiment was a completely randomized design with two factors: five fertilizer rates and two substrate types, with 6 replicates for each factor combination. Each potted plant was an experimental unit. Plants were fertilized at one of five rates that supplied 57, 113, 170, 226 and 283 mg N/L using Nutri Plus Organic Bloom (2.00N–0.87P–3.32K; EZ-GRO Inc.), diluted with RO water. Other nutrient element concentrations in Nutri Plus Organic Bloom were (in mg L<sup>-1</sup>): 100 Mg, 10.0 Zn, 12.8 B, 0.1 Mo, 2.3 Cu, and 6.8 Fe. All fertigation solutions were amended with 1 mL·L<sup>-1</sup> of Calcium-Magnesium supplement (3.0Ca–1.6Mg; EZ-GRO Inc.) and with Organa ADD micronutrient supplement, at a rate that supplied 22.9 mg N/L (2.0N–0.0P–0.0K; EZ-GRO Inc.). Other nutrient element concentrations in Organa ADD were (in mg L<sup>-1</sup>): 100.0 Ca, 29851 Zn, 4892 Mn, 1239 B, 12.7 Mo, 2419 Cu, and 2917 Fe. Fertilizer rates were selected based on recommendations for vegetative-stage organic fertilizer rates from Caplan et al., (2017) and previous studies on organic fertigation of greenhouse-grown tomatoes (*Solanum lycopersicum* L.; Surrage et al., 2010; Zhai et al., 2009).

Plants were spaced on growing tables at a density of 5.3 plants·m<sup>-2</sup>. PAR was maintained at 581  $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$  (s.d.  $\pm 93 \mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$ ) throughout the flowering stage under MASTER GreenPower Plus 1000W high pressure sodium lamps (Philips Lighting) with a 12-hour photoperiod. Growth chamber air temperature was maintained at 22/21 °C (s.d.  $\pm 0.8/0.5^\circ\text{C}$ ) from 1-6 DFS, 20/20 °C (s.d.  $\pm 0.5/0.8^\circ\text{C}$ ) from 7-9 DFS, and 18/17 °C (s.d.  $\pm 0.7/1.0^\circ\text{C}$ ) from 10-53 DFS during light/dark periods. RH was maintained at 70/76% (s.d.  $\pm 2.9/2.3\%$ ) from 1-6 DFS, 64/66% (s.d.  $\pm 2.5/2.7\%$ ) from 7-43 DFS, and 56/62% (s.d.  $\pm 2.6/1.5\%$ ) from 44-53 DFS. Chamber CO<sub>2</sub> was maintained at 594/659 ppm (s.d.  $\pm 56/42$  ppm) from 7-9 DFS, 673/705 ppm (s.d.  $\pm 83/44$  ppm) from 7-9 DFS, and 781/838 ppm (s.d.  $\pm 83/78$  ppm) from 44-53 DFS during light/dark periods.

During the first 11 DFS, plants were hand-fertigated at a rate that supplied 389 mg N/L of Nutri Plus Organic Grow and from then on, with the corresponding nutrient solution of Nutri Plus Organic Bloom (including calcium-magnesium and micronutrient supplements) whenever the mean substrate moisture content reached  $\approx 30\%$ . Between days 45 and 53 in the flowering stage, no fertilizer was applied, and the substrates were flushed, as per current industry practice, with RO water when mean substrate moisture content reached  $\approx 30\%$ . Fertigation solution pH was

adjusted to maintain substrate pH between 5.5 and 6.3, measured using the pour-through method (Wright, 1986) during both vegetative and flowering stages.

### **Substrate EC and pH measurement**

Substrate pH and EC during the flowering stage were determined weekly using pour-through method. Pour-through solutions were measured using a HI991300 portable pH/EC/TDS/Temperature Meter (Hanna Instruments, Woonsocket, R.I., USA).

### **Growth and yield measurements**

During the flowering stage, branch number, canopy area and plant height were measured every  $\approx 7$  days on 5 randomly selected plants from each treatment. Repeated measurements were made on the same plants throughout the vegetative stage. During the flowering stage, branch number, canopy area and plant height were measured on all plants at 15, 27, and 53 DFS. Canopy area for each plant was calculated using two perpendicular length measurements at the widest part of the canopy using plant tags as reference points for repeated measurements. Growth index (GI) for each plant was calculated as height (cm) x length (cm) x width (cm) x  $300^{-1}$  (Ruter, 1992). At 34 DFS, leaf greenness was measured as Chlorophyll Content Index (CCI), using a CCM-200 Chlorophyll Content Meter (Opti-Sciences Inc., Hudson, NH, USA) from the center of the most recent fully expanded leaf. Plants were harvested at 53 DFS when floral resin on most plants had  $\approx 50\%$  amber coloration. Stems were cut at substrate level; above-ground fresh weight was measured; large leaves were removed from stems and plants were hung to dry at 18 °C (s.d.  $\pm 0.1$  °C) and 49% RH (s.d.  $\pm 4.4\%$ ) for 6 days then cured at 18 °C (s.d.  $\pm 0.5$  °C) and 58% RH (s.d.  $\pm 3.5\%$ ) for 11 days. Floral material was then cut from stems and leaves were trimmed using a Twister T4 mechanical trimming machine (Keirton Inc., Surrey, BC, Canada) before floral dry weight (yield) measurement.

### **Floral cannabinoid analysis**

Dried, cured floral material was stored in dark and cool conditions according to United Nations Office on Drugs and Crime (2009) before being analyzed by an independent laboratory (RPC Science and Engineering, Fredericton, NB, Canada). Analysis of the neutral cannabinoids  $\Delta^9$ -Tetrahydrocannabinol (THC), Cannabidiol (CBD), Cannabinol (CBN), Cannabichromene (CBC)

and Cannabigerol (CBG) as well as acid forms,  $\Delta^9$ -Tetrahydrocannabinolic Acid (THCA), Cannabidiolic Acid (CBDA), and Cannabigerolic Acid (CBGA), were conducted by high-performance liquid chromatography as described in section 5.4.8 of United Nations Office on Drugs and Crime (2009).

### **Statistical analysis**

Data were analyzed using JMP Statistical Discovery Version 13.0 (SAS Institute Inc., Cary, NC) at a Type 1 error rate of  $\leq 0.05$ . Full-factorial ANOVA with repeated measures was used to determine the effects of substrate, fertilizer and their interaction on substrate EC, pH, plant height, GI, leaf number, and branch number over time. Differences among means were tested using Tukey's multiple means comparison test. Pearson correlation coefficients were calculated to compare cannabinoid concentrations to yield; and CCI to GI index and yield. Orthogonal partition and regression analysis (Bowley, 1999) were used to relate substrate EC, pH, plant growth, yield and cannabinoid yield/concentrations with fertilizer rate. If the partitioning variance analysis indicated a significant treatment effect, then the treatment effects were partitioned into one or more regression effects followed by an estimation of regression parameters for the best-fit regression. If there was no significant treatment effect, then data were presented as the average of all the treatments (pooled). If cannabinoid concentrations were below the detection limit ( $<0.05\%$ ), the values were excluded from the analysis. The residuals of the above analyses were tested for normality and equality of variance using The Shapiro-Wilk test and Bartlett's test, respectively.

## **Results**

### **Growth**

During both the 19-day vegetative stage and the 53-day flowering stage, plants grew normally and without any symptoms of nutrient disorder. Pistillate flowers were visible around 12 DFS in all treatments. During the flowering stage (from 0 DFS to 53 DFS), the average aboveground fresh weight increased from 7.1 to 296 g/plant, branch number increased from 4.5 to 11 branches/plant and growth index increased from 29 to 462 combined across treatments.

Four plants were removed during the trial because of root rot, three in U2 (two at the rate that supplied 57 mg N/L and one at the rate that supplied 113 mg N/L) and one in U2-HP (at the rate that supplied 57 mg N/L). In these cases, both younger and older leaves began to show interveinal purpling, leading to chlorosis and necrosis. There was also visible leaf purpling in treatments of U2, at the rates that supplied 223 and 286 mg N/L; most of these plants had entirely purple leaves from 32 DFS until harvest.

Generally, plants with higher fertilizer rates had greener leaves. At 34 DFS, there was a positive linear relationship between fertilizer rate and CCI measurements taken from the center of the newest fully expanded leaf (Figure 4.1; pooled from both substrates), and there was no difference in CCI between substrate treatments. There was also a positive correlation between CCI and floral dry weight ( $r = 0.64$ ,  $P < 0.0001$ ) and GI at 53 DFS ( $r = 0.39$ ,  $P < 0.0184$ ). Taking CCI measurements became difficult after 34 DFS when trichomes were abundant on the proximal region of leaves which interfered with the readings. There were signs of foliar senescence beginning around week 6 of flowering when older leaves became chlorotic and ultimately necrotic before harvest. These signs of senescence are typical for cannabis.

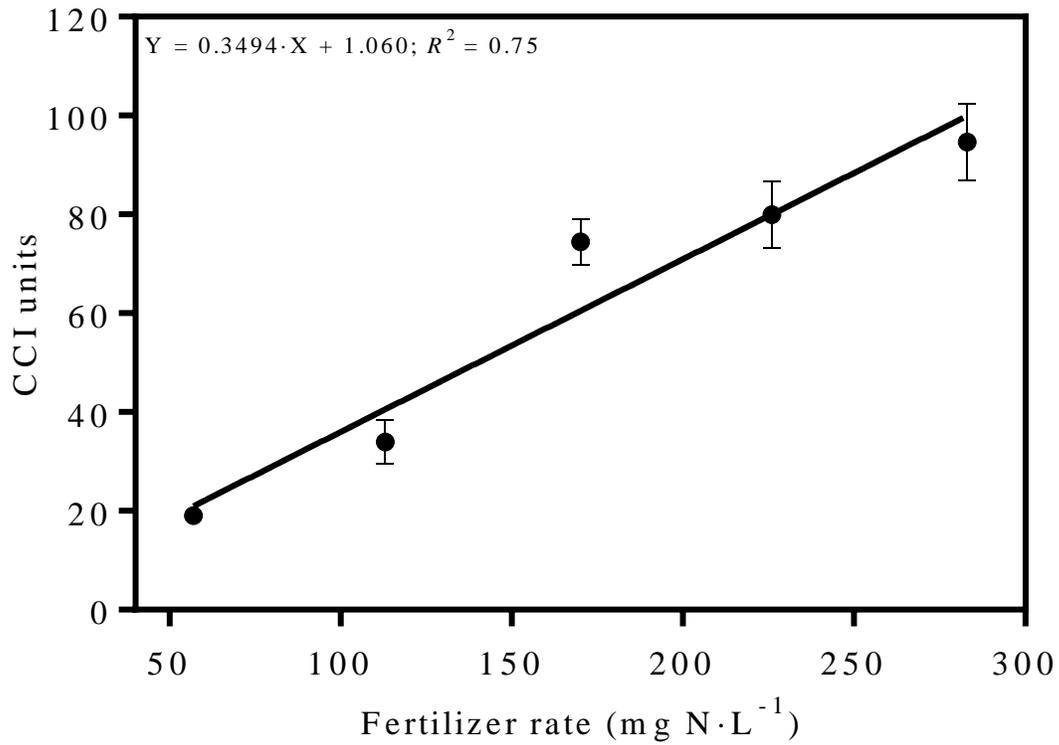
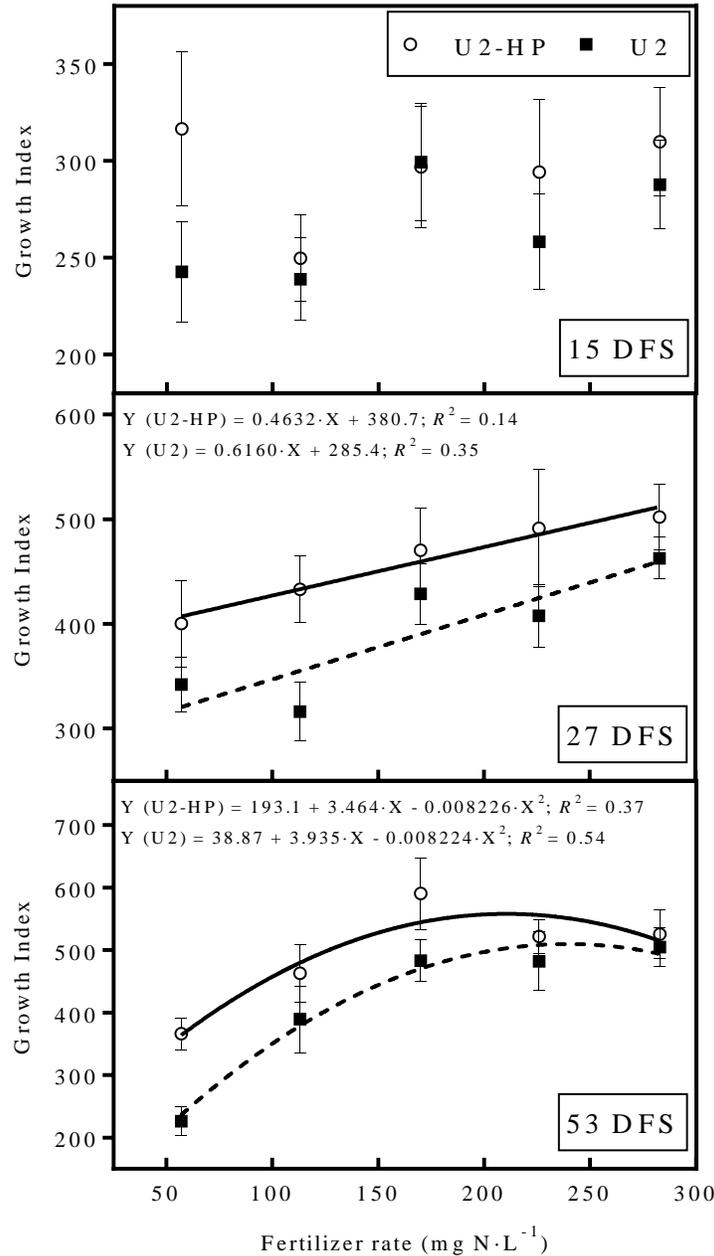


Figure 4.1. Response of leaf ‘greenness’ (CCI readings), measured on the newest fully expanded leaf, to organic fertilizer rate [indicated by nitrogen (N) concentration] at day 34 of the flowering stage. Data were pooled from both substrates. Values are means  $\pm$  SEM ( $n = 8$ ) and line is the best fit regression relationships at  $P < 0.05$ .



S

Figure 4.2. Response of cannabis growth index to organic fertilizer rate [indicated by nitrogen (N) concentration] in two growing substrates (U2-HP and U2) measured on different days during the flowering stage (DFS). Values are means  $\pm$  SEM (n = 5 for U2-HP at the rate that supplied 170 mg N/L on day 53; for U2 at the rate that supplied 57 mg N/L on day 15 and at the rate that supplied 113 mg N/L on day 53; n = 4 for U2 at the rate that supplied 57 mg N/L on day 27 and 53 and n = 6 for all other means) and lines are the best fit regression relationships at P < 0.05.

Growth index did not show any treatment effect on 15 DFS, increased linearly with increasing fertilizer rate at 27 DFS and exhibited a quadratic response at 53 DFS (Figure 4.2) for both substrates. At the final measurement (53 DFS), the interpolated maximum GI (558) for U2-HP was achieved at a rate supplying 211 mg N/L, and for U2 the interpolated maximum (510) was achieved at a rate supplying 239 mg N/L. Averaged across all fertilizer rates, plants grown in U2-HP had a 13% higher final GI than in U2 ( $F = 9.6$ ,  $P < 0.01$ ). No interactive effect was detected between substrate and fertilizer rate on GI.

Branch number did not differ among fertilizer rates or between substrate treatments, and there was no interactive effect of these treatments on branch number. Pooled across all treatments, branch number was  $11 \pm 0.1$  ( $\pm$  SEM) at 53 DFS.

### **Yield**

In both substrates, yield increased with increasing fertilizer rate; however, in U2-HP yield reached a maximum, and in U2 yield increased linearly (Figure 4.3). The interpolated maximum yield in U2-HP was 50 g/plant at a rate supplying 261 mg N/L. In U2, the highest yield was  $47 \pm 3.0$  g/plant (mean  $\pm$  SEM) at the rate that supplied 283 mg N/L. At the highest fertilizer rate, yield was 2.1 times greater than the lowest administered rate in U2-HP, and 2.2 times higher yield in U2. Pooled across fertilizer rates, yield was 11% higher in U2-HP than in U2 ( $P = 0.0013$ ;  $n = 29$  for U2-HP and  $n = 27$  for U2) with means ( $\pm$  SEM) of  $40 \pm 2.2$  g and  $36 \pm 2.0$  g, respectively. No interactive effect was detected between substrate and fertilizer rate on yield.

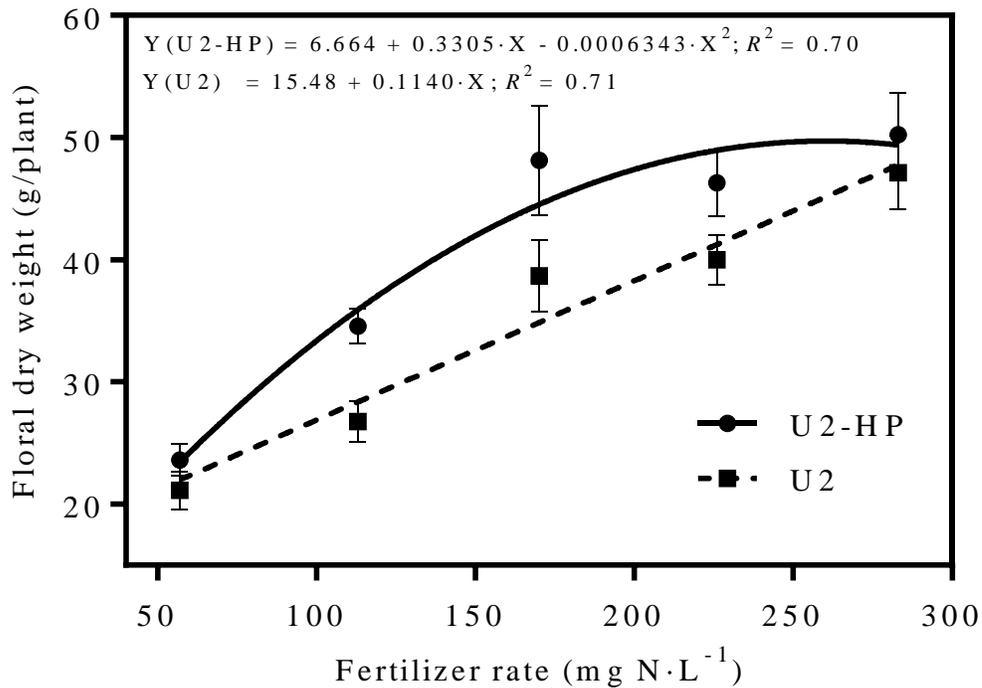


Figure 4.3. Response of cannabis yield to organic fertilizer rate [indicated by nitrogen (N) concentration] applied during the flowering stage in two growing substrates (U2-HP and U2). Values are means  $\pm$  SEM and the curves are best fit regression relationship with  $P < 0.05$ . For U2-HP,  $n = 5$  at the rate that supplied 170 mg N/L and  $n = 6$  at each other fertilizer rate; for U2,  $n = 4$  at the rate that supplied 57 mg N/L,  $n = 5$  at the rate that supplied 113 mg N/L and  $n = 6$  at each other rate.

## Cannabinoids

### *Fertilizer rate*

Of the analyzed cannabinoids, only THC, THCA, CBG and CBGA were above the detection limit (0.05%). Also, CBG concentration data were not normally distributed and could not be fit into the model; therefore, only treatment means are presented for this data.

As fertilizer rate increased, there were varied responses in floral concentrations and cannabinoid yield per plant (Figure 4.4). In U2, THC concentration had a quadratic response to increasing

fertilizer rate with a minimum of 0.36% at a rate supplying 193 mg N/L. Averaged across fertilizer rates, THC concentration in U2-HP was  $0.44\% \pm 0.018$  (mean  $\pm$  SEM) and in this substrate, there was no effect of fertilizer rate on THC concentration. In both substrates, THCA concentration decreased linearly with increasing fertilizer rate. THCA concentration from lowest to highest fertilizer rate was  $21.6 \pm 0.64\%$  to  $16.7 \pm 0.51\%$  for U2-HP and  $21.0 \pm 0.63\%$  to  $18.1 \pm 0.85\%$  for U2 (mean  $\pm$  SEM). In U2, CBGA concentration decreased linearly as fertilizer rate increased, ranging from  $0.64 \pm 0.02\%$  to  $0.54 \pm 0.03\%$  from lowest to highest fertilizer rate. In U2-HP, floral CBGA concentration averaged across fertilizer rates was  $0.57 \pm 0.01\%$  (mean  $\pm$  SEM) and in this substrate, there was no effect of fertilizer rate on CBGA concentration. Mean CBG concentration pooled across all treatments was  $0.06 \pm 0.002\%$  ( $\pm$  SEM).

Fertilizer effects were more evident when analyzing cannabinoid yield (g/plant) as a function of fertilizer rate (Figure 4.4, right). In U2-HP yield of floral THC responded quadratically to increasing fertilizer rate, reaching a maximum of 0.27 g/plant at a rate supplying 223 mg N/L and in U2, reaching minimum of 0.11 g/plant at a rate supplying 103 mg N/L. Yield of floral THCA increased linearly in U2 and responded quadratically in U2-HP with a maximum of 9.4 g/plant at a rate supplying 212 mg N/L. For both substrates, yield of floral CBG increased linearly with increasing fertilizer rate. Finally, yield of floral CBGA responded quadratically to fertilizer rate in U2-HP with a maximum of 0.29 g/plant at a rate supplying 228 mg N/L and increased linearly in U2.

To determine if increasing yield influenced cannabinoid concentrations, Pearson correlation coefficients were calculated to relate cannabinoid concentrations with floral dry weight. Pooled across treatments, floral THCA concentration decreased with increasing dry floral weight ( $r = -0.44$ ,  $P = 0.0047$ ) but there was no correlation between THC, CBG or CBGA concentration and dry floral weight.

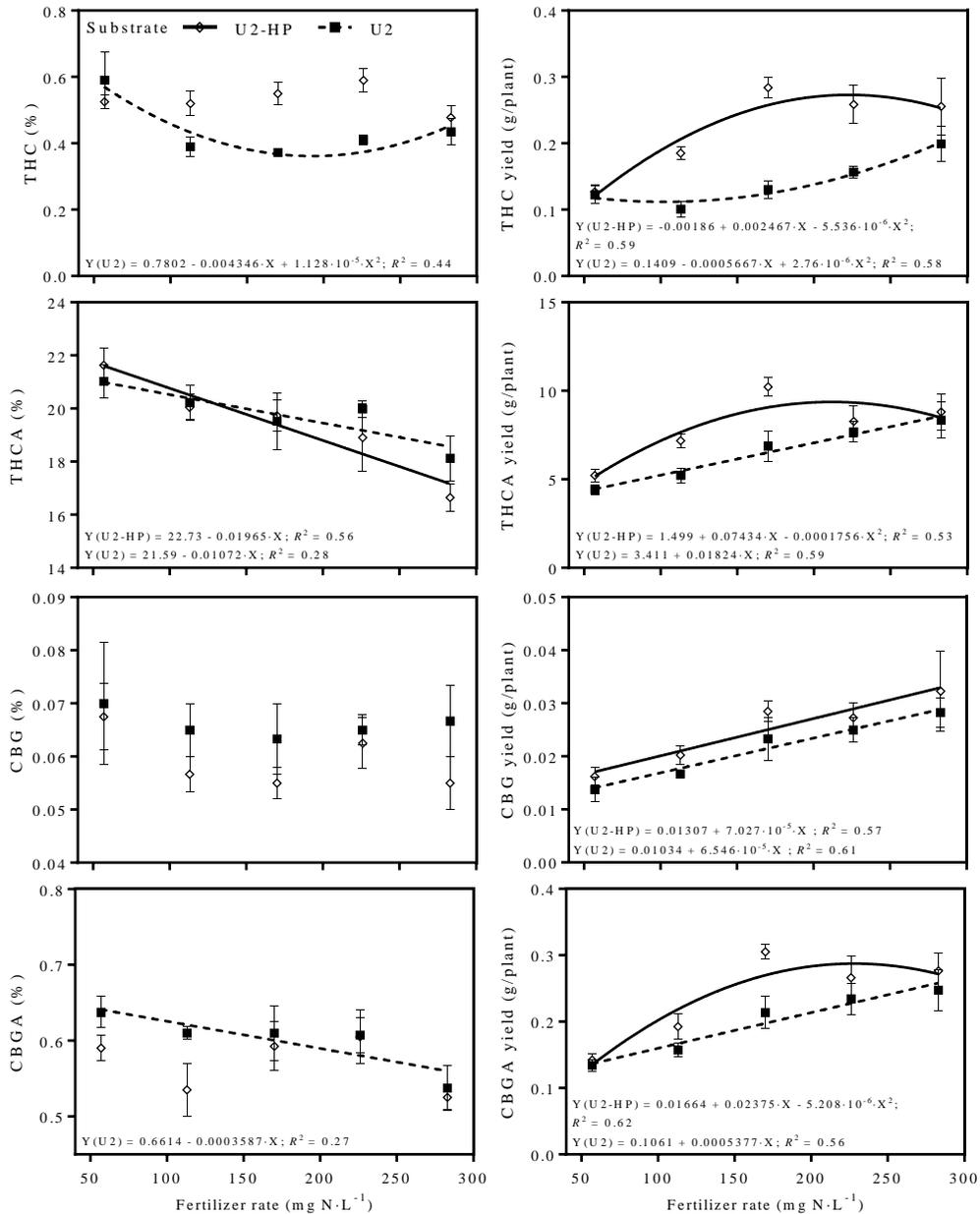


Figure 4.4. Relationship between cannabinoid concentration in dry floral material (left) and cannabinoid yield per plant (right) and organic fertilizer rate applied during the flowering stage in two substrates (U2-HP and U2). Fertilizer rate is indicated by nitrogen (N) concentration. Values are means  $\pm$  SEM. The curve is the best fit regression relationship with  $P < 0.05$ . In U2-HP, for CBG concentration and yield at the rate that supplied 113 mg N/L,  $n = 3$  and at the rate that supplied 283 mg N/L,  $n = 2$ . In U2, for CBG concentration and yield at the rates that supplied 57, 170, and 283 mg N/L,  $n = 3$  and at the rate that supplied 113 mg N/L,  $n = 2$ . For all other values,  $n = 4$ . THC =  $\Delta^9$ -tetrahydrocannabinol, THCA =  $\Delta^9$ -tetrahydrocannabidiolic acid, CBG = cannabigerol and CBGA = cannabigerolic acid.

## Substrate type

In presenting the effects of substrate on cannabinoids, means were pooled across fertilizer rates; however, statistical analysis was based on the complete model. There were no differences in the floral concentration of THCA or CBGA between the two substrates; though, U2-HP had a THC concentration 20% higher than U2 [ $0.53 \pm 0.016\%$  and  $0.44 \pm 0.025\%$  (means  $\pm$  SEM), respectively;  $F = 16.9$ ,  $P = 0.0008$ ;  $n = 20$ ]. There was also an interactive effect of substrate and fertilizer rate on THC concentration ( $P = 0.01852$ ;  $n = 4$ ). At the lowest and highest fertilizer rates, THC concentrations did not differ between substrates; but, at all other rates, THC concentration was higher in U2-HP.

As with fertilizer rate, differences between substrates were more evident when comparing cannabinoid yield per plant. THC yield, THCA yield, and CBGA yield were all higher in U2-HP than in U2: THC yield by 57% ( $F = 9.3$ ,  $P = <.0001$ ;  $n = 20$ ), THCA yield by 22% ( $F = 10.7$ ,  $P = <.0001$ ;  $n = 20$ ), and CBGA yield by 20% ( $F = 8.3$ ,  $P = <.0001$ ;  $n = 20$ ). THC yield in U2-HP was  $0.22 \pm 0.017$  g/plant (means  $\pm$  SEM) and in U2 was  $0.14 \pm 0.010$  g/plant; THCA yield in U2-HP was  $7.9 \pm 0.47$  g/plant and in U2 was  $6.5 \pm 0.44$  g/plant; and CBGA yield in U2-HP was  $0.24 \pm 0.016$  g/plant and in U2 was  $0.20 \pm 0.013$  g/plant. Substrate had no effect on CBG yield [ $0.019 \pm 0.002$  g/plant (mean  $\pm$  SEM) across all treatments].

## Substrate EC, pH, and irrigation

In both substrates, EC increased linearly or responded quadratically over time at each fertilizer rate except at the lowest rate that supplied 57 mg N/L, in which EC decreased linearly (Figure 4.5). At the final measurement before harvest (at week 6 of the flowering stage), EC from the lowest to the highest fertilizer rate was  $1.6 \pm 0.01$  to  $6.0 \pm 0.31$   $\text{mS}\cdot\text{cm}^{-1}$  for U2-HP and  $1.9 \pm 0.08$  to  $6.3 \pm 0.35$   $\text{mS}\cdot\text{cm}^{-1}$  for U2 (means  $\pm$  SEM). Averaged across fertilizer rates and time, EC was 6.3% higher in U2-HP than in U2 ( $3.1 \pm 97.2$   $\text{mS}\cdot\text{cm}^{-1}$  and  $3.3 \pm 119.0$   $\text{mS}\cdot\text{cm}^{-1}$  (means  $\pm$  SEM), respectively;  $F = 4.8$ ,  $P = 0.03$ ).

Substrate pH decreased over time in all fertilizer rates and in both substrates (Table 4.3). Starting at 4 weeks in the flowering stage (WFS) until the end of the trial, pH was lower in U2-HP than in

U2, differing by 0.4 to 0.9 during this time. There were no interactive effects of substrate and fertilizer rate on substrate EC or pH.

To maintain a minimum substrate moisture content of 30%, plants grown in U2-HP were fertigated 17 times during the flowering stage compared to 13 times for U2. Fertigation volume was  $\approx 1$  L per plant each time; therefore, during the flowering stage, plants grown in U2-HP were given 31% more water ( $\approx 4$  L more) and fertilizer than those grown in U2. For example, at the rate supplying 170 mg N/L, plants grown in U2-HP received 2.89 g N/L of Nutri Plus Organic Bloom during the flowering stage compared to 2.21 g N/L for U2.

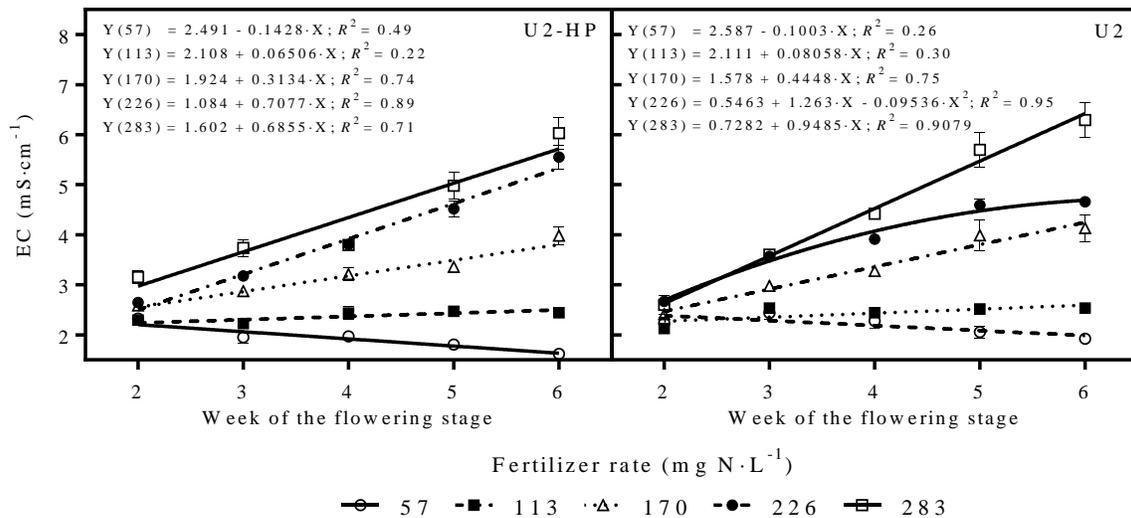


Figure 4.5. Response of growing substrate EC (electrical conductivity) to organic fertilizer rate applied during the flowering stage for cannabis in two substrates (U2-HP and U2). Fertilizer rate is indicated by nitrogen (N) concentration. Data are means  $\pm$  SEM (For U2-HP, n = 4 at weeks 2, 4 and 6 and n = 8 at weeks 3 and 5; for U2, n = 4 at each week) and lines are the best fit regression relationships with P < 0.05.

Table 4.3. Response of substrate pH to organic fertilizer rate applied during the flowering stage for cannabis.

WFS <sup>y</sup>	Growing substrate												Substrate <sup>x</sup>
	U2-HP						U2						
	Fertilizer rate [indicated by nitrogen (mg L <sup>-1</sup> N) concentration]												
	57	113	170	226	283	All <sup>z</sup>	57	113	170	226	283	All <sup>z</sup>	
	Growing Substrate pH												
2	7.4 a <sup>w</sup>	7.2 a	7.3 a	7.2 a	7.2 a	7.3 ± 0.04	7.4 a	7.3 a	7.1 a	7.6 a	6.9 a	7.2 ± 0.09	NS
3	7.0 a	6.7 ab	6.4 b	6.7 ab	6.5 ab	6.7 ± 0.06	7.0 a	6.4 a	6.6 a	6.4 a	6.3 a	6.6 ± 0.09	NS
4	6.0 a	6.0 a	5.7 a	6.0 a	5.9 a	5.9 ± 0.10	6.6 a	6.2 a	6.3 a	6.3 a	6.0 a	6.3 ± 0.09	**
5	5.9 a	5.6 a	5.6 a	5.9 a	5.8 a	5.8 ± 0.08	6.9 a	6.4 a	6.1 a	6.4 a	6.4 a	6.5 ± 0.09	***
6	6.0 a	5.5 ab	5.5 ab	5.8 ab	5.1 b	5.6 ± 0.10	6.9 a	6.4 a	6.5 a	6.5 a	6.4 a	6.5 ± 0.07	***
All <sup>v</sup>	6.5 ± 0.13	6.2 ± 0.14	6.1 ± 0.15	6.3 ± 0.13	6.1 ± 0.14	6.2 ± 0.06	7.0 ± 0.08	6.5 ± 0.12	6.5 ± 0.10	6.6 ± 0.12	6.4 ± 0.09	6.6 ± 0.05	

<sup>z</sup>Data are means of substrates pooled across fertilizer rates

<sup>y</sup>WFS = Weeks in the flowering stage

<sup>x</sup>NS, \*, \*\*, \*\*\* Nonsignificant, or significant at  $P < 0.05$ , 0.01, and 0.0001, respectively, within week intervals among substrate treatments.

<sup>w</sup>Data followed by the same letter within the same column do not differ at  $P < 0.05$ .

<sup>v</sup>Data are means of 6 weeks; for U2-HP, n = 4 at 2, 4 and 6 WFS and n = 8 at 3 and 5 WFS; for U2, n = 4 at each WFS.

## Discussion

Both growing substrates U2-HP and U2 performed well for cannabis production during the flowering stage; however, plants grown in U2-HP had higher GI, yield, THC concentration, THC yield, THCA yield and CBGA yield than those grown in U2.

Yield increased with increasing fertilizer rate, reaching a plateau in U2-HP and until the highest applied rate for U2; however, as fertilizer rate increased, the concentration of most measured cannabinoids (THC, THCA, and CBGA) decreased. This suggests that for cannabis, high fertilizer rate during the flowering stage may have a dilution effect on THC, THCA and CBGA. While the dilution effect was apparent with increasing fertilizer rate, it did not have a substantial impact on the total per-plant yield of most cannabinoids. This was evidenced by a lack of correlation between yield and THC, CBG or CBGA concentrations. There was however, a negative correlation between floral THCA concentration and yield, suggesting a slight dilution of THCA as yield increased. This illustrates that growing higher yielding cannabis plants is appropriate to maximize the yield of THC and CBGA with only minor losses of THCA. To maximize floral yield without sacrificing THC, THCA, and CBGA concentration due to the dilution effect, it is recommended that excessive organic fertilizer application during the flowering stage be avoided.

In U2-HP, peak yield, THC yield, THCA yield, and CBGA yield were at rates supplying 261 mg N/L, 223 mg N/L, 212 mg N/L and, 228 mg N/L, respectively. Therefore, for U2-HP, the optimal rate of this organic fertilizer is between 212 and 261 mg N/L, depending on grower preference for high yield or individual cannabinoid yield. For U2, yield did not reach a plateau over the applied fertilizer rates; therefore, growers may choose to maximize yield using the highest fertilizer rate that supplied 283 mg N/L or chose a lower rate to maximize cannabinoid concentration. Growing substrate U2-HP is preferential to achieve maximum yield and cannabinoid content whereas U2 has the potential to reduce water and fertilizer use if the nutrient solution is not reused.

Some of the differences between the two substrates may have been accounted for by differences in irrigation frequency. Since irrigation frequency was based on substrate moisture content, the

drier substrate (U2-HP) was irrigated, and consequently fertilized, more frequently than U2. The differences in performance between the two substrates were greater than could be accounted for by the additional fertilizer applied in U2-HP over the duration of flowering stage. For example, yield was higher in U2-HP at the rate that supplied 170 mg N/L than yield in U2 at the rate that supplied 226 mg N/L while the total fertilizer applied was 2.89 g N/L and 2.94 g N/L respectively. High irrigation frequency is known to increase plant growth in some species (de Kreij and Straver, 1988; Katsoulas et al., 2006; Morvant et al., 1998; Silber et al., 2003, 2005). In greenhouse-grown roses (*Rosa hybrid* L., 'First Red'), doubling irrigation frequency while maintaining constant total irrigation volume increased the dry weight of cut flower shoots by approximately 30% over a lower frequency irrigation (Katsoulas et al., 2006). Similar effects have been reported in greenhouse-grown *Codiaeum variegatum* L.; using a flood and drain irrigation system, de Kreij and Straver (1988) found that high irrigation frequency combined with high-porosity substrates increased plant growth and reduced substrate nutrient leaching. Increased irrigation frequency can also increase aboveground biomass in lettuce (*Lactuca sativa* L., 'Iceberg'; Silber et al., 2003) and peppers (*Capsicum annum* L., 'Selika'; Silber et al., 2005). The increased growth from frequent irrigation has been attributed to enhanced nutrient uptake, specifically improved P mobilization and uptake (Silber et al., 2003, 2005).

Another factor that may have contributed to the differences in growth and cannabinoid concentrations/yield between the two substrates is root zone oxygen availability. With a higher container capacity (Table 4.1), U2 holds more water, consequentially can displace more air and reduce oxygen diffusing rate in the root zone than U2-HP. A well-oxygenated root zone is vital for good plant health, improving nutrient uptake, root growth, and preventing root-borne disease (Jackson and Colmer, 2005; Zheng et al., 2007). Further, there was some evidence of root disease in the present study that may have been caused by low root zone oxygen. While there was no statistical difference, three plants were removed during the trial due to root rot in U2 compared to one in U2-HP. To our knowledge, there is no research into the effects of irrigation or root zone oxygen on cannabinoid concentrations, but it is speculated that these factors may have contributed to the higher cannabinoid concentrations seen in U2-HP. Results from the current study suggests that cannabis may benefit from high irrigation frequency and/or high root zone oxygen; however, further study is required to control for these variables and to optimize irrigation frequency and root zone oxygen concentration for cannabis.

Plant growth responded as expected to varying fertilizer application rates; plants increased in size as fertilizer rate increased until a maximum at a rate supplying 211 mg N/L for U2-HP and at a rate supplying 239 mg N/L for U2 at final harvest. The fertilizer rates which delivered optimal growth and yield in this trial were generally higher than typical recommended synthetic fertilizer rates used in container crop production, which rarely exceed 200 mg N/L (Raviv, 2007); this was likely due to the slow releasing and less soluble nature of N and P in organic fertilizer when compared to most synthetic fertilizers (Prasad et al., 2004). Improved performance from high organic fertilizer rates (at rates supplying around 389 mg N/L) was also reported for vegetative stage cannabis (Caplan et al., 2017), suggesting that cannabis has high organic fertilizer requirements in both growth stages.

Caplan et al., (2017) also demonstrated that with varying fertilizer rate and substrate type applied to vegetative-stage cannabis, that cannabinoid yield and concentration did not differ substantially in the cultivar ‘OG Kush × Grizzly’ when the plants were grown under similar flowering-stage conditions. During the vegetative stage, substrate had no effect on floral cannabinoid concentration, and of all the detected cannabinoids only THC concentration responded to fertilizer rate, increasing to a maximum at a rate supplying 418 mg N/L. Fertilizer effects were more substantial in the present study, likely because cannabinoid concentration increases mainly during the flowering stage when glandular trichome development is at its peak (Aizpurua-Olaizola et al., 2016; Muntendam et al., 2012). Additionally, Caplan et al., (2017) found that an increase in yield due to optimal vegetative-stage fertigation was associated with an increase in the concentration of THC and cannabinol (CBN) in dry floral material. In the present study, there was negligible CBN detected in the floral material of the cultivar ‘WP:Med (Wappa)’, and results differ from Caplan et al., (2017) with regards to THC concentration. The cannabinoid dilution effect, attributed to increased fertilizer rate, may therefore only apply to the flowering stage. This difference illustrates a varying effect between vegetative stage and flowering stage fertilizer rate for cannabis.

The relationship between fertilizer rate and yield differed between the two substrates. In U2-HP, yield decreased at rates that supplied above 261 mg N/L, yet yield increased linearly with increasing fertilizer rate in U2. The concentration of fertilizer in the irrigation water remained constant; therefore, more fertilizer was used for U2-HP than U2 over the 53-day period. It was

presumed that since more fertilizer was administered for U2-HP, that substrate EC would accumulate to a higher level in U2-HP compared to U2. In fact, the opposite was observed; substrate EC was slightly higher in U2 than in U2-HP (Figure 4.5). Plants grown in U2-HP also grew larger, potentially because of increased irrigation frequency and/or substrate aeration as previously described. Increased plant growth may have facilitated greater nutrient uptake, accounting for lower EC accumulation in U2-HP. Silber et al. (2003) found that in lettuce, increased irrigation frequency improved the availability of immobile elements such as P and K allowing for increased uptake. In U2-HP, at rates that supplied above 261 mg N/L, plants may have accumulated nutrients to above-optimum levels, which could account for the yield reduction at the higher rates. In contrast, the lesser amount of total administered fertilizer in U2 might have not reached an optimum level.

Low substrate pH may have also contributed to the yield reductions seen at the highest fertilizer rate in U2-HP. The optimal pH range for cannabis that is suggested by grey resources (Cervantes, 2006) and found to be acceptable in our previous trial during the vegetative stage (Caplan et al., 2017) is 5.8 to 7.2. The pH in both substrates remained mostly within this range; however, at the highest fertilizer rate at 6 WFS, pH in U2-HP was 5.1. Within the measured range of substrate pH (means of 5.1 to 7.4 in the flowering stage) there were no visual signs of pH-induced disorders. This observation overlaps the acceptable range cited in Caplan et al., (2017) of 6.7 to 7.2 for the flowering stage. If the lowest substrate pH measured (5.1) is excluded, a range of substrate pH of 5.5 to 7.4 appears suitable in the flowering stage for container production of organic cannabis. More research is needed to confirm the optimal range for multiple varieties.

Leaf 'greenness' increased linearly with increasing fertilizer rate and was positively correlated to the preceding GI measurement as well as yield. The chlorophyll content index is often a reliable indicator of leaf N-status; however, the relationship must be determined for a given species using leaf tissue analysis for accurate results (Xiong et al., 2015), and to our knowledge has not been characterized for cannabis. Current results suggest that CCI may be an indicator of plant N-status for cannabis since yield and growth generally increase with optimal N fertilization.

Finally, further research is required to evaluate the effects of fertilizer rate and substrate on other cannabinoids of interest, such as CBD, which was not detected in the current cultivar ‘WP:Med (Wappa)’. This would require the use of additional cannabis varieties.

## **Conclusions**

Our results suggest that to produce high-yielding, cannabinoid rich, organic cannabis plants, lower container capacity coir-based substrates, like U2-HP, are preferable to those with higher container capacity, like U2. The drier substrate produced higher floral yield, GI, THC concentration, THC yield, THCA yield and, CBGA yield than U2, possibly because of higher fertigation frequency and/or adequate root zone oxygen leading to a more favorable root zone environment. Both substrates generally maintained suitable substrate pH between 5.5 and 7.4 and were effective for cannabis production during the flowering stage. Increasing fertilizer rate was found to have a dilution effect on THC, THCA and CBGA; therefore, excessive organic fertilizer application during the flowering stage should be avoided despite increased biomass yield. To maximize both yield and cannabinoid yield, the optimal organic fertilizer rate for U2-HP was determined to be within a range supplying 212 to 261 mg N/L using the Nutri Plus Organic Bloom liquid organic fertilizer during the flowering growth stage. Higher rates within this range favor increased floral yield, while lower rates favor higher yield of some cannabinoids. For U2, the optimal rate was one supplying 283 mg N/L to maximize yield; though, lower rates may be desirable to increase cannabinoid concentrations. These recommendations could be generalized for similar organic fertilizer and substrates; however, results may vary with cannabis cultivar.

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## CHAPTER FIVE:

### **Increasing floral dry weight and cannabinoid content in medical cannabis using controlled drought stress<sup>4</sup>**

#### **Abstract**

Controlled application of drought can increase secondary metabolite concentrations in essential oil-producing crops. To evaluate the effects of drought on cannabis (*Cannabis sativa* L.) dry floral weight (yield) and cannabinoid content, drought stress was applied to container-grown cannabis plants through gradual growing substrate drying in a controlled environment. Fertigation was withheld during week seven in the flowering stage until mid-day plant water potential (WP) was -1.5 MPa (drought stress threshold) which correlated with a 50% increase in leaf angle (wilting). This occurred after eleven days without fertigation. A well-irrigated control was used for comparison. Leaf net photosynthetic rate ( $P_n$ ), plant WP, wilting (leaf angle) and volumetric moisture content (VMC) were monitored throughout the drying period until the day after the drought group was fertigated. At the drought stress threshold,  $P_n$  was 42% lower and plant WP was 50% lower in the drought group than the control. Drought increased the concentration of major cannabinoids tetrahydrocannabinol acid (THCA) and cannabidiolic acid (CBDA) by 12% and 13%, respectively, compared to the control. Further, yield per unit growing area of THCA was 43% higher than the control, CBDA yield was 47% higher,  $\Delta^9$ -tetrahydrocannabinol (THC) yield was 50% higher and cannabidiol (CBD) yield was 67% higher. Controlled drought stress may therefore be an effective horticultural management technique to maximize both floral weight and cannabinoid yield in cannabis, although results may differ with cannabis cultivar or chemotype.

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<sup>4</sup> Formatted for submission to *HortScience*. Authorship: Deron Caplan, Mike Dixon, and Youbin Zheng.

**Key words:** *Cannabis sativa*, marijuana, deficit irrigation, plant water potential, medicinal crops, volumetric soil water content

## Introduction

The historic prohibition of cannabis (*Cannabis sativa* L.) has stunted scientific research on its production, leaving growers to rely on guides and online resources based heavily on anecdotal information. In the past decade, the regulations surrounding cannabis production and use, especially for medicinal purposes have become increasingly liberalized in North America and in some parts of Europe (Chandra et al., 2017) allowing research into this field.

The essential oil of female cannabis flowers gives the crop its value as a medicinal and recreational product; these oils are concentrated mostly in glandular trichomes and contain a diverse array of secondary metabolites, including a class of meroterpenoid compounds known as phytocannabinoids (cannabinoids; Chandra et al., 2017; Potter, 2014). Some cannabinoids, including  $\Delta^9$ -tetrahydrocannabinol (THC) and cannabidiol (CBD) have been widely studied for their psychoactive and medicinal properties (Elzinga et al., 2015; Mechoulam et al., 1970; Vemuri and Makriyannis, 2015); though, the medicinal properties of other cannabinoids and cannabinoid interactions are still mostly unknown (McPartland and Russo, 2001; Russo, 2011).

The dry floral weight (yield) and secondary metabolite content in cannabis is largely controlled through breeding and phenotype selection (Muntendam et al., 2012); however, horticultural management techniques such as fertilization (Bócsa et al., 1997; Caplan et al., 2017a; 2017b), choice of growing substrate (Caplan et al., 2017a; 2017b), air temperature in the growing environment (Chandra et al., 2011; Latta and Eaton, 1975), horticultural lighting intensity/quality (Lydon et al., 1987; Potter and Duncombe, 2012) and photoperiod (Potter, 2009) also have a substantial impact. Further, controlled exposure to stress may be an effective method to increase the production of some secondary metabolites in cannabis. For example, treatment with UV-B radiation, which is not utilized in photosynthesis, may increase THC concentration in cannabis floral material under controlled environment conditions (Lydon et al., 1987).

Drought stress is a major stimulator of secondary metabolites in plants. This is exemplified in herbs and spices cultivated in semi-arid regions such as the Mediterranean. Intermittent drought

and high solar radiation in these areas has been attributed to aromatic herbs and spices with abundant essential oil (Kleinwächter and Selmar, 2015). In the literature, there are no reports on the effects of drought stress in cannabis; however, secondary metabolite accumulation due to drought stress has been documented in a number of other herbaceous species (Baher et al., 2002; Bettaieb et al., 2009; Kleinwächter and Selmar, 2015). In Summer savory (*Satureja hortensis*), plants that were highly drought stressed during the flowering stage had 31% higher essential oil concentration than a well-watered control (Baher et al., 2002). Likewise, drought stress increased essential oil concentration in lemon balm (*Melissa officinalis* L.) and lemon catmint (*Nepeta cataria* L. f. *citriodora*) compared to a well-watered control but did not for sage (*Salvia officinalis* L.). Though concentrations were higher, essential oil yield (by growing area) of lemon catmint and lemon balm was lower in the drought-stressed plants because of reduced growth and harvestable plant material.

In contrast, both Bettaieb et al. (2009) and Nowak et al. (2010) have documented not just increased essential oil concentration in sage, by up to four times, but also higher essential oil yield in drought stressed plants compared to a non-stressed control. Other than the notable exceptions in sage, increased essential oil yield per unit growing area is rarely cited (Kleinwächter and Selmar, 2015), possibly because drought stress has well-documented negative impacts on plant growth and can reduce harvestable plant material. Drought reduces rates of carbon assimilation as a result of both stomatal and metabolic limitations (Chaves, 1991; Flexas et al., 2002; Tezara et al., 1999). To maximize essential oil or secondary metabolite yield, the level and timing of the of drought stress should be such that yield losses are minimized (Nakawuka et al., 2014).

In applying drought stress over extended periods, researchers generally aim to maintain constant levels of root zone water potential (WP), either through use of a solute-infused substrate (Charles et al., 1990; Van Der Weele et al., 2000) or by regulating soil/growing substrate moisture content (Baher et al., 2002; Blanch et al., 2009; Manukyan, 2011; Nowak et al., 2010). This allows for long-term assessment of the drought stress response; however, these methods involve a sustained level of drought rather than mimicking natural substrate saturation and drying cycles. Allowing the growing substrate to dry before irrigation increases the level of root zone oxygen, which can improve nutrient uptake, root growth and prevent root-borne disease (Caplan et al., 2017a;

Jackson and Colmer, 2005; Zheng et al., 2007). Substrate-drying techniques that incorporate a wetting and drying cycle are preferred to observe both the immediate effects of the stressor as well as subsequent acclimation. This technique requires the use of a growing substrate that can effectively re-saturate after an extended dry period. Peat-based substrates without incorporated wetting agents, for example may not be effective (Fields et al., 2014).

Drought stress timing is also essential to minimize yield losses and maximize essential oil yield and the concentration of secondary metabolites; differences in growth stage and natural timing of phytochemical accumulation must be considered by species (Petropoulos et al., 2004). The cannabis life cycle includes two growth stages, vegetative and flowering, which are controlled by photoperiod. A short-day photoperiod (~12h) triggers flowering which may last around seven to twelve weeks depending on cultivar and growing conditions (Potter, 2014). Cannabinoids accumulate mostly during the flowering stage, but timing of peak cannabinoid concentration varies by chemotype and cultivar. Drug-type varieties of chemotype I have a high THCA:CBDA ratio (>1.0) while varieties of chemotype II have an intermediate ratio (generally 0.5–2.0) (Pacifico et al., 2008). For chemotype I, peak THCA concentration is approximately week nine of the flowering stage and for chemotype II, the peak is approximately week seven. Peak CBDA in chemotype I is in week eleven of the flowering stage and, in chemotype II, it varies minimally from week eight onwards (Aizpurua-Olaizola et al., 2016; Muntendam et al., 2012).

In the present study, drought stress was applied to a chemovar II cultivar during week seven of the flowering stage. It was hypothesised that controlled drought stress may be a valuable tool for growers to improve the yield and quality of their cannabis crops. The objective was to evaluate the effects of drought stress on floral yield and cannabinoid content and yield in cannabis.

## **Methods**

### **Plant culture**

Fourteen-day-old rooted cuttings ( $\approx$ 10 cm high with  $\approx$ 6 leaves) of *Cannabis sativa* L. ‘NC:Med (Nebula)’ were transplanted into round blow-molded black pots (102 mm diameter  $\times$  89 mm height) containing a custom blended organic growing substrate [40-45% (vol/vol) sphagnum peatmoss, 20-25% chunk coconut coir, 20-25% horticultural grade perlite and 5-10% worm

casings; Premier Tech Horticulture, Rivière-du-Loup, QC, Canada] with one plant per pot. Pots were placed in a walk-in growth chamber (15 m<sup>2</sup>) at a density of 97 plants·m<sup>-2</sup>. Growth chamber environmental parameters are presented in Table 5.1.

Plants were hand-fertigated, as per Caplan et al., (2017), using Nutri Plus Organic Grow liquid organic fertilizer (4.0N–1.3P–1.7K; EZ-GRO Inc., Kingston, ON, Canada) at a rate that supplied 389 mg N/L amended with 2 mL·L<sup>-1</sup> of Calcium-Magnesium supplement (0.0N–0.0P–0.0K–3.0Ca–1.6Mg; EZ-GRO Inc.), diluted with reverse osmosis (RO) water and with a 20% leaching fraction. Other nutrient element concentrations of Nutri Plus Organic Grow were (in mg·L<sup>-1</sup>): 14.5 Zn, 12.0 B, 2.6 Mo, 2.1 Cu, and 8.5 Fe. Fertigation was administered when mean substrate moisture was ≈30%, measured using a WET-2 soil moisture sensor (Delta-T Devices Ltd., Cambridge, UK).

At 15 days after transplant (DAT), 8 plants with similar height and canopy size were selected and transferred into a larger walk-in growth chamber (130 m<sup>2</sup>) for the flowering stage. This was considered the first day of the flowering stage (DFS). Plants were up-potted into 11 L blow-molded black pots (279 mm diameter × 241 mm height) containing Pro-Mix HP Mycorrhizae (Premier Tech Horticulture) and spaced on growing tables at a density of 6.4 plants·m<sup>-2</sup>. Trial plants were bordered on all sides by cannabis plants of the same age and size.

During the first 10 DFS, plants were hand-fertigated at a rate that supplied 389 mg N/L of Nutri Plus Organic Grow, as per Caplan et al., 2017b, whenever substrate moisture content reached ≈20%. From then on, plants were fertigated as per Caplan et al., 2017a, using Nutri Plus Organic Bloom (2.00N–0.87P–3.32K; EZ-GRO Inc.) at a rate that supplied 170 mg N/L, diluted with RO water. Other nutrient element concentrations in Nutri Plus Organic Bloom were (in mg L<sup>-1</sup>): 100 Mg, 10.0 Zn, 12.8 B, 0.1 Mo, 2.3 Cu, and 6.8 Fe. Flowering-stage fertigation solutions were also amended with 5 mL·L<sup>-1</sup> of Calcium-Magnesium supplement (0.0N–0.0P–0.0K–3.0Ca–1.6Mg; EZ-GRO Inc.) and with Organa ADD micronutrient supplement, at a rate that supplied 22.9 mg N/L (2.0N–0.0P–0.0K; EZ-GRO Inc.). Other nutrient element concentrations in Organa ADD were (in mg·L<sup>-1</sup>): 100.0 Ca, 29851 Zn, 4892 Mn, 1239 B, 12.7 Mo, 2419 Cu, and 2917 Fe. Fertigation solution pH was adjusted to maintain substrate pH between 5.5 and 6.3, measured using a soil pH probe (Hanna HI 99121; Hanna Instruments, Woonsocket, R.I., USA).

Table 5.1. Growth chamber environmental parameters during the trial.

		Days after transplant				
		0 - 2	3 - 4	5 - 9	10 - 15	
Vegetative stage (18-hour photoperiod)	PAR <sup>y</sup> ( $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$ )	100 $\pm$ 1.3 <sup>z</sup>	200 $\pm$ 1.9	300 $\pm$ 2.6	400 $\pm$ 4.1	
	Air Temperature ( $^{\circ}\text{C}$ )	..... 24 $\pm$ 0.1/ 23 $\pm$ 0.9 .....				
	Relative Humidity (%)	..... 73 $\pm$ 5.1/ 73 $\pm$ 4.4 .....				
	CO <sub>2</sub> Conc. (ppm)	..... 691 $\pm$ 99.1 / 601 $\pm$ 31.9 .....				
		Days in the flowering stage				
		0 - 5	6 - 9	10 - 48	49 - 54	
Flowering stage (12-hour photoperiod)	PAR <sup>x</sup> ( $\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$ )	..... 262 $\pm$ 40.7 .....		..... 427 $\pm$ 70.5 .....		
	Air Temperature ( $^{\circ}\text{C}$ )	..... 22 $\pm$ 0.2/ 22 $\pm$ 0.3 .....		..... 20 $\pm$ 0.4/ 18 $\pm$ 0.7 .....		
	Relative Humidity (%)	70 $\pm$ 0.4 /	65 $\pm$ 0.7 /	60 $\pm$ 0.9 /	55 $\pm$ 1.4 /	
		70 $\pm$ 0.5	65 $\pm$ 0.3	61 $\pm$ 1.2	60 $\pm$ 1.4	
	CO <sub>2</sub> Conc. (ppm)	..... 731 $\pm$ 190.8 / 666 $\pm$ 151.3 .....				

<sup>z</sup>Values are mean  $\pm$  standard deviation during light/dark periods.

<sup>y</sup>Photosynthetically active radiation (PAR) was maintained using fluorescent lighting (Philips Lighting, Markham, ON, Canada) and measured the at canopy-level.

<sup>x</sup>PAR was maintained using 315-watt Green Power Master Elite Agro ceramic metal halide lamps (Philips Lighting) and measured at the canopy-level.

## Treatments

At 39 DFS, plants were randomly assigned to drought or control treatment groups, with 4 plants in each group. Each potted plant was an experimental unit. The control was irrigated as previously described for the flowering stage, with a fertigation event triggered when substrate moisture content of an individual plant reached  $\approx$ 20%. Fertigation was withheld from drought treatment until plant water potential (WP) reached between -1.4 and -1.5 MPa.

## **Drought stress indicators**

### ***Plant water potential***

Stem psychrometers and data loggers (PSY1; ICT International Pty Ltd., Armidale, NSW, Australia) were installed on each plant and plant WP measurements were taken every 15-minutes. The procedures outlined by Tran et al., 2015 were followed to install and maintain the psychrometers. Plant WP was noted immediately before fertigating the drought group and daily, at mid-day up until one day after the fertigation. Psychrometer reinstallations were necessary if plant WP readings suddenly dropped to zero or were positive while lights were on. These circumstances usually indicated that the vapor seal between the psychrometer and the stem was broken, condensation had accumulated inside the chamber or the thermocouple was damaged (Stoochnoff et al., 2018).

### ***Substrate moisture content and substrate water potential***

Capacitance-type substrate moisture sensors (ECH<sub>2</sub>O-TE; Decagon Devices Inc., Pullman, WA) were inserted vertically into the substrate surface of each pot and connected to two five-port data loggers (EM50; Decagon Devices Inc.) The moisture sensors measured dielectric permittivity every 15 minutes which was converted to volumetric moisture content (VMC) using a substrate-specific calibration. To ensure that the substrate in the drought treatment adequately rehydrated after fertigation, VMC at mid-day the day after fertigation of the drought group was compared to that of the control, measured at an equal interval after they were last irrigated.

### ***Leaf net photosynthetic rate***

Leaf net photosynthetic rate ( $P_n$ ) was measured each day between 8 and 9 h into the light cycle beginning at 39 DFS as well as immediately before fertigating the drought group. Measurements were made using a portable photosynthesis measurement system (LI-6400XT; LI-COR Biosciences, Lincoln, NE) on the youngest fully expanded leaf. Light was supplied by 6400-02B Red-Blue light emitting diodes (LI-COR) with photosynthetically active radiation (PAR) set to around chamber canopy level ( $450 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). CO<sub>2</sub> concentration in the leaf cuvette was maintained at  $800 \mu\text{mol}\cdot\text{mol}^{-1}$  and block temperature was maintained at 20 °C.

### *Relative leaf angle*

Initial leaf angle was measured at mid-day at treatment initiation using a hand-held, pivoting angle-finder and a protractor. Subsequent leaf angle measurements were taken when wilting was first evident then, 3-4 times/day after that until fertigation of the drought group. New fully expanded leaves on a side-branch from the first internode were selected for measurement and petioles were marked with colored tape for future measurement (Figure 5.1). The angle between the center of the middle leaflet and the stem from which it originates was measured. The leaflet tips were not used as reference points since ‘tip curl’ is common in cannabis, sometimes related to a nutrient disorder. As leaves wilted, increasing leaf angle relative to the initial angle was noted.

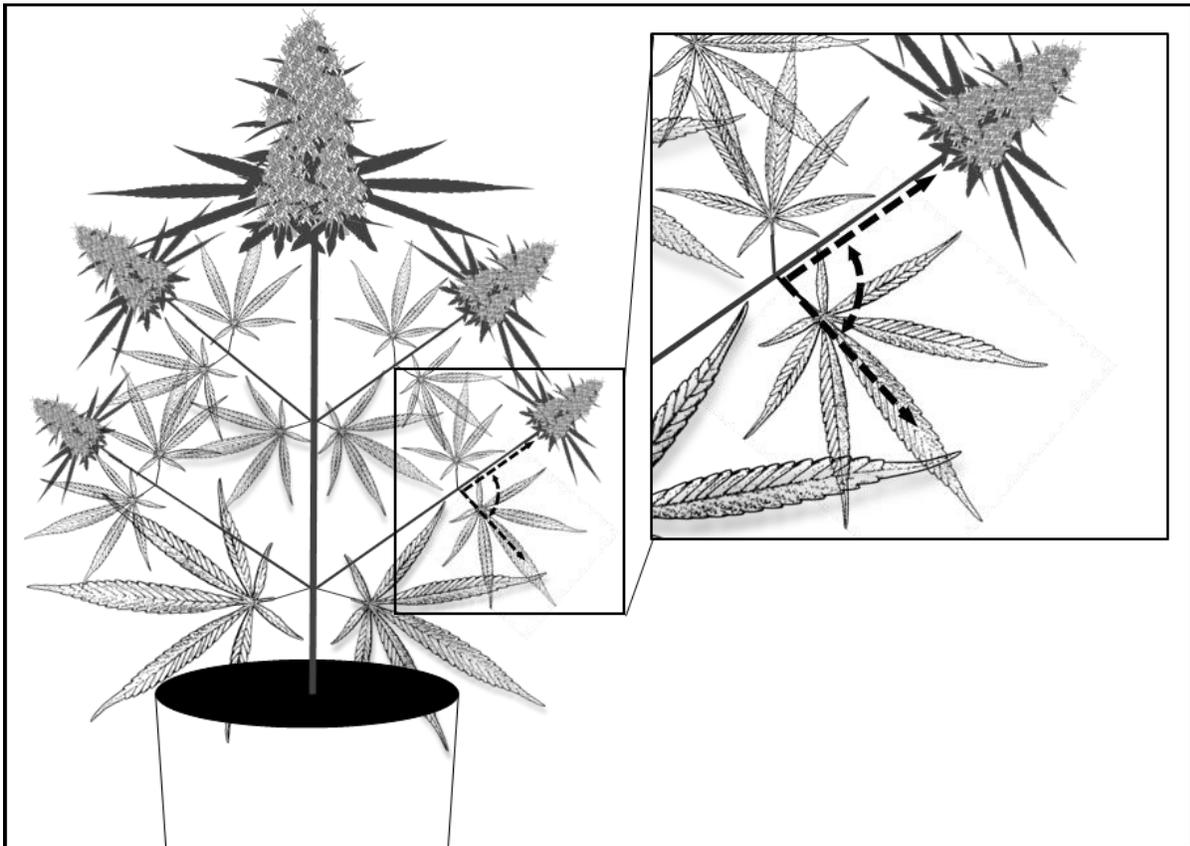


Figure 5.1. Location for leaf angle measurement to indicate degree of wilting in cannabis.

## **Yield and secondary metabolite measurements**

Plants were harvested at 54 DFS. Stems were cut at substrate level; large leaves were removed from stems and plants were hung to dry at 18 °C (s.d.  $\pm$  0.1 °C) and 45% RH (s.d.  $\pm$  1.9%) for 2 days then cured at 18 °C (s.d.  $\pm$  0.1 °C) and 57% RH (s.d.  $\pm$  4.3%) for 12 days. Floral material was then cut from stems and leaves were trimmed using a Twister T4 mechanical trimming machine (Keirton Inc., Surrey, BC, Canada) before floral dry weight (yield) measurement.

The dried, cured apical floral material of three plants from each group was stored under dark and cool conditions according to United Nations Office on Drugs and Crime (2009) before being analyzed by an independent laboratory (RPC Science and Engineering, Fredericton, NB, Canada). Analysis of the neutral cannabinoids  $\Delta$ 9-Tetrahydrocannabinol (THC), Cannabidiol (CBD), Cannabinol (CBN), Cannabichromene (CBC) and Cannabigerol (CBG) as well as acid forms,  $\Delta$ 9-Tetrahydrocannabinolic Acid (THCA), Cannabidiolic Acid (CBDA) and Cannabigerolic Acid (CBGA), were conducted by high-performance liquid chromatography as described in section 5.4.8 of United Nations Office on Drugs and Crime (2009). Moisture content of the dry floral material was determined using the methods described in the The United States Pharmacopeial Convention, section 921 method 3 (UPS 40, 2017) and cannabinoid concentration was corrected to zero percent moisture content. Cannabinoid yield was calculated as cannabinoid concentration multiplied by yield and expressed per unit area ( $\text{g}\cdot\text{m}^2$ ).

## **Statistical analysis**

Data were analyzed using JMP Statistical Discovery Version 13.0 (SAS Institute Inc., Cary, NC) at a Type 1 error rate of  $\leq 0.05$ . Differences among means were tested using Student's t-test. If cannabinoid concentrations were below the detection limit ( $<0.05\%$ ), the values were excluded from the analysis. The residuals of the above analyses were tested for normality and equality of variance using The Shapiro-Wilk and Bartlett's tests, respectively.

## Results

### Drought stress indicators

During the 54-day flowering period, there were no symptoms of nutrient disorder and no observable differences in plant appearance between control and drought groups until the drought treatment was without fertigation for around nine days. From nine days without fertigation to harvest, plants under drought treatment showed signs of veinal chlorosis on older leaves and, to a lesser extent, newly formed leaves on the entire plant. Wilting was observed after eleven days without fertigation when leaf angle in the drought treatment was  $52\% \pm 0.7$  higher than the initially measured angles.

Up until eleven days without fertigation in the drought treatment, plant WP did not differ from the control groups ( $P = 0.78$ ;  $n = 4$  for day 10). Immediately prior to fertigating the drought group, on the 11th day without fertigation, plant WP in the drought treatment was 50% lower than in the control (Table 5.2). The day after fertigating plants in the drought treatment, their mean mid-day plant WP recovered to be the same level as the control.

There were also notable differences in net photosynthetic rate ( $P_n$ ) and substrate volumetric moisture content (VMC) between drought and control treatments around the time of fertigating the drought group (Table 5.2). Immediately prior to that fertigation,  $P_n$  in the drought stressed plants was 42% lower than the control and VMC was 84% lower than the control. On the day after fertigation of the drought group at mid-day,  $P_n$  partially recovered in the drought stressed plants but was still 32% lower than the control. Further, VMC in the drought group on the day after fertigation did not differ from that of the control as measured the day after it was last irrigated during this period.

Table 5.2. Plant water potential, leaf net photosynthetic rate and substrate moisture of cannabis under drought conditions and after subsequent fertigation at seven weeks in the flowering stage.

	Treatment	Plant Water Potential (MPa)	Net Photosynthetic Rate ( $\mu\text{mol m}^{-2}\text{s}^{-1}$ )	Volumetric Substrate Moisture Content (%)
Immediately before fertigation (wilting point)	Control	$-1.0 \pm 0.05^z$	$13.2 \pm 1.14$	$33.3 \pm 2.89$
	Drought	$-1.5 \pm 0.12$	$7.7 \pm 0.80$	$5.3 \pm 1.23$
	Significance <sup>y</sup>	**	**	***
Mid-day after fertigation	Control	$-0.9 \pm 0.09$	$13.9 \pm 1.01$	$43.2 \pm 1.36^x$
	Drought	$-0.6 \pm 0.10$	$9.4 \pm 0.65$	$39.4 \pm 4.02$
	Significance	NS	**	NS

<sup>z</sup>Data are means  $\pm$  SEM; n = 3 for volumetric moisture content of the control and n = 4 for all other means.

<sup>y</sup>NS, \*, \*\*, \*\*\* Nonsignificant, or significant at  $P < 0.05$ , 0.01, and 0.0001, respectively.

<sup>x</sup>Measured the day after the control was last irrigated during this period.

### Yield and cannabinoids

Yield in the control was  $178 \pm 9.4 \text{ g}\cdot\text{m}^{-2}$  and was  $232 \pm 18.5 \text{ g}\cdot\text{m}^{-2}$  in the drought treatment but yield did not differ statistically between the two treatments ( $P = 0.06$ ; n = 3). The moisture content of the dried and cured floral material was  $8 \pm 0.1 \%$  in the control, 11% lower than that in the drought treatment, at  $9 \pm 0.1 \%$  ( $P = 0.01$ ; n = 3). Henceforward, the floral dry weight and cannabinoid contents are corrected to zero percent moisture.

Of the analyzed cannabinoids, all were detected in at least one sample, these included: THC, THCA, CBD, CBDA, CBG, CBGA, and CBN. In the drought treatment, only one sample had a detectable concentration of CBG and CBN, and in the control there were no samples with detectable CBN; therefore, comparisons could not be made for these cannabinoids, and the means for CBN were not presented.

The drought treatment elicited a 12% increase in THCA concentration and a 13% increase in CBDA concentration but had no effect on the concentrations of the other detected cannabinoids

(Table 5.3, top). Drought had substantial effects on cannabinoid yield, expressed as grams of cannabinoid from floral material per unit growing area ( $\text{g}\cdot\text{m}^{-2}$ ). In the drought treatment, THC yield was 50% higher, THCA yield was 43% higher, CBD yield was 67% higher, and CBDA yield was 47% higher than in the control (Table 5.3, bottom).

Table 5.3. Cannabinoid concentration and yield in the dry floral material of cannabis exposed to drought stress at week seven in the flowering stage.

Treatment	Yield	THC	THCA	CBD	CBDA	CBG	CBGA
		Concentration (%) <sup>z</sup>					
Control	-	0.3 ± 0.02	4.7 ± 0.03	0.2 ± 0.01	9.1 ± 0.05	0.06 ± 0.004	0.45 ± 0.012
Drought	-	0.3 ± 0.01	5.3 ± 0.09	0.2 ± 0.01	10.3 ± 0.09	0.08 <sup>w</sup>	0.49 ± 0.028
Significance <sup>x</sup>	-	NS	**	NS	**	ND <sup>y</sup>	NS
		Cannabinoid Yield (g·m <sup>-2</sup> ) <sup>z</sup>					
Control	164 ± 8.5	0.4 ± 0.03	7.7 ± 0.40	0.3 ± 0.02	15 ± 0.7	0.1 ± 0.01	0.7 ± 0.03
Drought	211 ± 16.5	0.6 ± 0.07	11 ± 0.9	0.5 ± 0.04	22 ± 1.7	0.1 <sup>w</sup>	1.0 ± 0.12
Significance	NS	*	*	*	*	ND	NS

<sup>z</sup>Data are means ± SEM and are corrected to zero percent moisture content; n = 3 unless otherwise indicated.

<sup>y</sup>ND = No data or insufficient data to compare means.

<sup>x</sup>NS, \*, \*\*, \*\*\* Nonsignificant, or significant at P < 0.05, 0.01, and 0.0001, respectively.

<sup>w</sup>n = 1.

## Discussion

The controlled drought treatment used in this study substantially increased the concentrations of both major cannabinoids, THCA and CBDA as well as yield of THCA, CBDA, THC, and CBD compared to the control. These results suggest that the level of drought stress applied in the present study was adequate to stimulate cannabinoid production without reducing yield for this cultivar.

Plant WP proved to be an effective indicator of drought stress; at wilting point, there was a significant difference in plant WP between drought and control groups. The stem psychrometer is a useful tool for non-destructive assessment of plant-environment interactions which may vary by species and between individual plants (Dixon and Tyree, 1984). Traits such as crown architecture, root structure and leaf morphology all affect water transport (Ali, 2010) and therefore drought responses. The combined effect of these and environmental parameters can be quantified through plant WP measurements (Dixon and Tyree, 1984; Stoochnoff et al., 2018). The use of stem psychrometers for irrigation scheduling is, however, not commercially viable. The sensors are costly and require significant technical training. Substrate VMC or leaf wilting are easier to measure and can be useful indicators of drought if correlated to plant WP data. Leaf angle measurements can be made in seconds using a protractor or angle finder; substrate VMC measurement generally requires several substrate moisture sensors but data can be collected remotely (Bogena et al., 2007).

In the present study, leaf wilting was an effective indicator of plant stress. At the irrigation threshold for the drought treatment, plants were visibly wilted, and the indicator leaf angle increased by about 50% from the turgid leaf angle. Using wilting as a drought-stress indicator may therefore be an effective method in cannabis production, especially since it is easily measured. In potato (*Solanum tuberosum* L.), for example, leaf wilting may be the most obvious visual indicator of drought stress (Banik et al., 2016). Notably, wilting response to drought may vary by species (Xu et al., 2010) and can depend on the degree to which an individual plant is acclimated to drought stress (Banik et al., 2016; Flexas et al., 2009); therefore, using a 50% increase in leaf angle wilting threshold as a drought stress indicator may be most effective if used

with varieties of cannabis (chemovar II) and under similar environmental conditions (Table 1) to the present study.

To our knowledge, this was the first evaluation of the effects of controlled drought stress on cannabis; though, as previously described, drought can increase essential oil yield in some herbaceous crops. In drought-stressed sage, essential oil and monoterpene yield can increase up to 281% (Bettaieb et al., 2009) and 20% (Nowak et al., 2010), respectively, over a well-watered control. Likewise, in curly-leafed parsley (*Petroselinum crispum* ssp. *crispum* L. cv. curly-leafed) grown under drought stressed conditions, plants were smaller but had higher oil concentrations than the well-watered control. The density of the plants could therefore be increased to accommodate the decreased size and essential oil yield per unit area would be higher (Petropoulos et al., 2008).

Increases in secondary metabolite concentration due to drought stress usually coincides with reduced growth; however, this was not the case in the present study in which there was no difference in yield between drought and control groups. Secondary metabolites are formed from photosynthetic carbon (Peñuelas and Llusà, 2002) and drought can reduce  $P_n$ , as exemplified in the present study. In fact, it is common for  $P_n$  of plants exposed to drought stress to only recover to 40-60% of their pre-drought levels on the day after irrigation and  $P_n$  may never fully recover (Delfine et al., 2005; Kirschbaum, 1987; Sofu et al., 2005). Nonetheless, in the present study, the yield of some cannabinoids increased irrespective of reduced carbon assimilation. Protective mechanisms help plants tolerate drought until some cumulative physiological threshold is exceeded, and only at this point, growth is impeded (Ali, 2010; Xu et al., 2010). A period of drought insufficient to impede growth, such as in the present study, may be the key to increasing secondary metabolite yield.

An understanding of the biochemical origins of cannabinoids and how they relate to other secondary metabolites may be useful to speculate how drought stress increased cannabinoid yield in the present study. For example, cannabinoids are closely related to some terpenoids which protect plants under stress. Cannabinoids and terpenoids share a similar biochemical pathway. Isopentenyl pyrophosphate (IPP) is the basic building block of all terpenoids and is produced either in the cytosol and mitochondria through the Mevalonate pathway (Banthorpe et al., 1972)

or in the plastids through the Mevalonate-independent (DXP) pathway (Eisenreich et al., 1998). The DXP pathway is the source of all mono-, di-, and tetraterpenes which include many essential oil components (Gershenzon et al., 2000). In cannabinoid synthesis, geranyl pyrophosphate (GPP) from the DXP pathway is combined with olivetolic acid (OA), a product of the polyketide pathway (Flores-Sanchez and Verpoorte, 2008; Hanuš et al., 2016), to produce cannabigerolic acid (CBGA) (Fellermeier et al., 2001). CBGA is then converted to more commonly known cannabinoids such as THCA and CBDA through various synthases (Taura et al., 1996).

Carotenoids and xanthophylls, which are also produced through the DXP pathway, are involved in mitigating photo-oxidative damage caused by environmental stress (Demmig-Adams, 1990). Stressors like drought elicit the formation of reactive oxygen species (ROS) in the chloroplasts, especially under high solar radiation (Penuelas et al., 2004). Production of antioxidant compounds in the chloroplasts such as carotenoids and the xanthophylls is upregulated in stressed plants to prevent cell damage (Eskling et al., 1997; Munné-Bosch and Alegre, 2000). This may also be the case for other terpenes with antioxidant properties (Delfine et al., 2005; Llusià and Peñuelas, 1998; Munné-Bosch and Alegre, 2000). Environmental stressors which normally up-regulate terpenoid synthesis and accumulation may do the same for some cannabinoids because of their related biochemical origins.

Since both water and fertilizer were applied together through fertigation, there were likely differences in the amount of fertilizer applied to the treatment groups. The concentration of fertilizer in the irrigation water remained constant and since the drought stress group was irrigated less than the control, it was also fertilized less. Nutrients are largely taken up through the roots along with water by mass transport so, it can be difficult to sustain adequate mineral nutrition uptake in dry substrates (Silber et al., 2003). This is a limitation applied to any method for long-term drought application. Nonetheless, research has shown that flowering-stage cannabis performs similarly under a range of organic fertigation rates (Caplan et al., 2017a) and in the present trial, the drought stressed group had similar yield to the control which would not be expected if nutrition was lacking (Caplan et al., 2017a).

Repetition of drought stress and subsequent acclimation can influence the way in which plants respond to the stressor (Banik et al., 2016). The present study evaluated the effects of drought at

a single point during the flowering stage, but timing of drought stress and drought stress frequency could also affect secondary metabolism in cannabis. Some higher plants have the ability to acclimate to drought stress and stress resistance may increase after exposure to a low level of stress (Banik et al., 2016; Flexas et al., 2009). The acclimation responses from repeated drought stress could, therefore, further stimulate secondary metabolites in cannabis; though, a longer drought event may be required to elicit the response in acclimated plants. More research is needed to evaluate the effects of drought stress timing and acclimation on cannabis yield and secondary metabolism. Lastly, since rates of cannabinoid accumulation vary by chemovar (Aizpurua-Olaizola et al., 2016; Muntendam et al., 2012), the effect of drought on other chemovars should be explored.

## **Conclusions**

This study suggested that controlled drought stress can increase the concentration of major cannabinoids THCA, CBDA and the yield of THCA, CBDA, THC, and CBD in chemovar II cannabis without reducing dry floral weight (yield) and irrespective of decreased  $P_n$ . These results were achieved by gradually drying the substrate over eleven days until plant WP reached around -1.5 MPa during week seven in the flowering stage. Comparable results can be expected using leaf wilting as a drought stress indicator with fertigation triggered at a leaf angle 50% higher than in its turgid state. This method for administering drought-stress and the results of this study should be applicable for similar varieties of chemovar II cannabis; however, other chemovars or varieties may respond differently.

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## CHAPTER SIX:

# Influence of drought stress timing and frequency on cannabis secondary metabolism and yield<sup>5</sup>

### Abstract

A single application of controlled drought stress in cannabis (*Cannabis sativa* L.) has been shown to increase the content of medically significant cannabinoids. To evaluate the effects of drought stress timing and frequency on cannabis dry floral weight (yield) and secondary metabolism, drought treatment was applied in weeks four, five, six, and seven during the flowering stage to container-grown cannabis plants in a controlled environment. In weeks four and five, two additional treatments were stressed twice, with one regular irrigation in between to simulate intermittent stress. Leaf wilting was used as the indicator of drought stress and drought treatments were compared to a well-watered control. Drought timing had substantial effects on the content of some terpenoids and there were no differences in yield or cannabinoid content among treatments. Intermittent stress did not increase secondary metabolites or yield more than single drought treatments. In early drought stress treatments, during weeks four and five, linalool yield (per unit growing area) was up to 46% higher than the control and cis-ocimene was detected exclusively in these treatments; however, in these earlier stress treatments, caryophyllene yield was lower than in the control. In later drought stress treatments, during week six and seven, yield of alpha-bisabolol was higher than the control by up to 40% and trans-ocimene was detected exclusively in these treatments and in the control. Controlled drought may therefore be an effective technique to increase the concentration and/or yield of specific terpenoids in cannabis; although, results may differ with different durations of stress or with cannabis cultivar.

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**Key words:** Cannabis sativa, marijuana, deficit irrigation, medicinal crops, irrigation scheduling

## **Introduction**

The market for legal medicinal and recreational cannabis (*Cannabis sativa* L.) in North America is quickly expanding (ArcView Market Research, 2017), but knowledge on the horticultural management of this crop is relatively limited and research efforts have been restricted in the past several decades (Potter, 2014). As for any medicinal crop, consumers demand a product that is both safe to consume and of consistent quality (Chandra et al., 2017). Improving horticultural management techniques through systematic research can provide growers the resources they need to grow safe and high-quality cannabis.

The essential oils of drug-type cannabis contain an array of secondary metabolites that are produced and concentrated mostly in glandular trichomes on female flowers (Dayanandan and Kaufman, 1976). Among them are a group of meroterpenoids known as phytocannabinoids (cannabinoids) which are found in uniquely high concentrations in this species (Dayanandan and Kaufman, 1976; Mechoulam, 2005). Cannabinoids such as  $\Delta^9$ -tetrahydrocannabinol (THC) and cannabidiol (CBD) have well studied psychoactive and medicinal properties (Elzinga et al., 2015; Mechoulam et al., 1970; Vemuri and Makriyannis, 2015); however, more than 100 unique cannabinoids have been identified in cannabis and little is known on their individual medicinal properties or how they interact with other cannabis secondary metabolites (McPartland and Russo, 2001; Russo, 2011). Terpenoids are another class of secondary metabolites found in cannabis essential oil. They are generally present at lower concentrations than THC and CBD, for example, but cannabinoid-terpenoid interactions may have medicinal significance (Russo, 2011).

The previous chapter of this thesis demonstrated that drought stress, applied to cannabis under controlled environment conditions, can increase cannabinoid yield per unit growing area without reducing yield. In the chemovar II cultivar, 'NC:Med (Nebula)', drought was applied gradually over eleven days until mid-day plant water potential (WP) was -1.5 MPa, during week seven of the flowering stage. Drought increased THCA yield per unit growing area by 43%, CBDA yield by 47%, THC yield by 50%, and CBD yield by 67% compared to the well-watered control.

Instances of increased essential oil or secondary metabolite concentration induced by drought stress are not uncommon (Kleinwächter and Selmar, 2015); yet, there are few examples of increased oil or secondary metabolite yield per plant or unit growing area in the literature. Notable exceptions have been documented in sage (Bettaieb et al., 2009; Nowak et al., 2010) and parsley (Petropoulos et al., 2008). Terpenoids usually accumulate in the leaves and floral parts of drought-stressed plants and play a protective role against the stressor (Blanch et al., 2009). In spearmint (*Mentha spicata* L.) and rosemary (*Rosmarinus officinalis* L.), essential oils, including many terpenoids, accumulate under drought stress and it was speculated that the terpenoids could mitigate photo-oxidative damage under stress conditions (Delfino et al., 2005; Peñuelas and Llusà, 2002).

Secondary metabolite accumulation can vary by growth stage within a plant's life cycle. For example, in parsley (*Petroselinum crispum* (Mill) Nym) harvest after the formation of six to eight leaves compared to harvest a month after can significantly alter essential oil composition (Petropoulos et al., 2004). The cannabis life cycle is divided into two distinct growth stages, vegetative and flowering. The flowering stage begins with a short-day photoperiod (~12h) and may last around seven to twelve weeks based on cultivar and growing conditions (Potter, 2014). Cannabinoids and terpenoids accumulate mostly during the flowering stage, but timing of peak concentrations varies by chemotype and cultivar (Aizpurua-Olaizola et al., 2016; Muntendam et al., 2012). Since secondary metabolite concentrations vary over time in cannabis, drought stress timing may affect cannabis secondary metabolism.

Acquired resistance can also influence a plant's physiological response to drought. Some higher plants can resist the negative impacts of drought stress after repeated exposure. This is known as acclimation (Banik et al., 2016; Flexas et al., 2009) and at times, growth can even return to above-control levels after recovery from mild drought stress (Acevedo et al., 1971; Xu et al., 2010). The protection conferred from acclimation and the extent of the subsequent biochemical changes in the plants may depend on when and how rapidly the drought stress is imposed. Under controlled environment conditions such as in greenhouse container production, drought often occurs quickly compared to under field conditions; this can limit the protective effects from acclimation (Banik et al., 2016). In studying acclimation, it is therefore important for drought stress to be imposed over periods of days rather than hours.

The previous chapter established precedent for the positive effects of controlled drought stress in cannabis production; however, drought was only applied once during the flowering stage. The effects of drought stress timing and frequency have not yet been explored for cannabis.

Furthermore, there is scant research on the effects of drought stress on terpenoid content in cannabis. The objective of the present study was to evaluate the effects of drought stress timing and frequency on cannabinoid and terpenoid content in cannabis.

## **Methods**

### **Plant culture**

Fifteen-day-old rooted cuttings ( $\approx 10$  cm high with  $\approx 6$  leaves) of *Cannabis sativa* L. ‘NC:Med (Nebula)’ were cultivated in a walk-in growth chamber ( $15\text{ m}^2$ ) for the vegetative stage, as described in the previous chapter. The environmental parameters of the vegetative-stage growth chamber are presented in Table 6.1.

At 15 days after transplant, 49 plants with similar height and canopy size were selected and transferred into a larger walk-in growth chamber ( $130\text{ m}^2$ ) for the flowering stage. This was considered the first day of the flowering stage (DFS). Plants were up-potted into 6 L blow-molded black pots (220 mm diameter  $\times$  220 mm height) containing Pro-Mix HP Mycorrhizae (Premier Tech Horticulture) and spaced on growing tables at a density of  $6.4\text{ plants}\cdot\text{m}^{-2}$ . The environmental parameters of the flowering growth chamber are presented in the lower portion of Table 6.1. Plants were fertigated during the flowering stage as described in the previous chapter, with substrate pH maintained between 5.5 and 6.3.

Table 6.1. Growth chamber environmental parameters during the trial.

		Days after Transplant			
		0 - 5	3 - 4	5 - 9	10 - 15
Vegetative stage (18-hour photoperiod)	PAR <sup>y</sup> ( $\mu\text{mol}\cdot\text{m}^{-2}\text{ s}^{-1}$ )	100 $\pm$ 1.2 <sup>z</sup>	200 $\pm$ 1.8	300 $\pm$ 2.5	439 $\pm$ 20.9
	Air Temperature ( $^{\circ}\text{C}$ )	..... 24 $\pm$ 0.1/ 23 $\pm$ 0.9 .....			
	Relative Humidity (%)	..... 73 $\pm$ 5.3/ 72 $\pm$ 5.6 .....			
	CO <sub>2</sub> Conc. (ppm)	..... 606 $\pm$ 85.1/ 577 $\pm$ 32.6 .....			
		Days in the Flowering Stage			
		0 - 5	6 - 9	10 - 45	46 - 56
Flowering stage (12-hour photoperiod)	PAR <sup>x</sup> ( $\mu\text{mol}\cdot\text{m}^{-2}\text{ s}^{-1}$ )	268 $\pm$ 29.2	..... 439 $\pm$ 76.2 .....		
	Air Temperature ( $^{\circ}\text{C}$ )	22 $\pm$ 0.1 / 22 $\pm$ 0.2	..... 20 $\pm$ 0.7/ 18 $\pm$ 0.9 .....		
	Relative Humidity (%)	70 $\pm$ 0.3 / 70 $\pm$ 0.4	65 $\pm$ 0.7 / 65 $\pm$ 0.2	60 $\pm$ 1.2 / 61 $\pm$ 1.2	56 $\pm$ 2.3 / 59 $\pm$ 1.5
	CO <sub>2</sub> Conc. (ppm)	..... 708 $\pm$ 165.3/ 656 $\pm$ 109.3 .....			

<sup>z</sup>Values are mean  $\pm$  standard deviation during light/dark periods

<sup>y</sup>Photosynthetically active radiation (PAR) was maintained using fluorescent lighting (Philips Lighting, Markham, ON, Canada) and measured the at canopy-level

<sup>x</sup>PAR was maintained using 315-watt Green Power Master Elite Agro ceramic metal halide lamps (Philips Lighting) and measured at canopy-level

## Treatments

The experiment was a completely randomized design with six drought stress treatments, a well-watered control and seven replicates within each treatment. Each potted plant was an experimental unit. The control was irrigated as described in the previous chapter for the flowering stage, with an irrigation event triggered when substrate moisture content of an individual pot reached  $\approx$ 20%. Drought treatments had irrigation withheld until leaf angle increased by  $\approx$ 50%, using a reference leaf for the initial angle, again described in the previous chapter. Treatments differed by drought stress timing during the flowering stage (Figure 6.1).

Stress was applied in either week four, five, six or seven of the flowering stage, hereby referred to as treatments: wk4(1), wk5(1), wk6(1) and wk7(1). Two intermittent stress treatments were also included; wk4(2) and wk5(2), in which drought was applied during week 4 and week 5, respectively, followed by a regular irrigation then a second stress application.

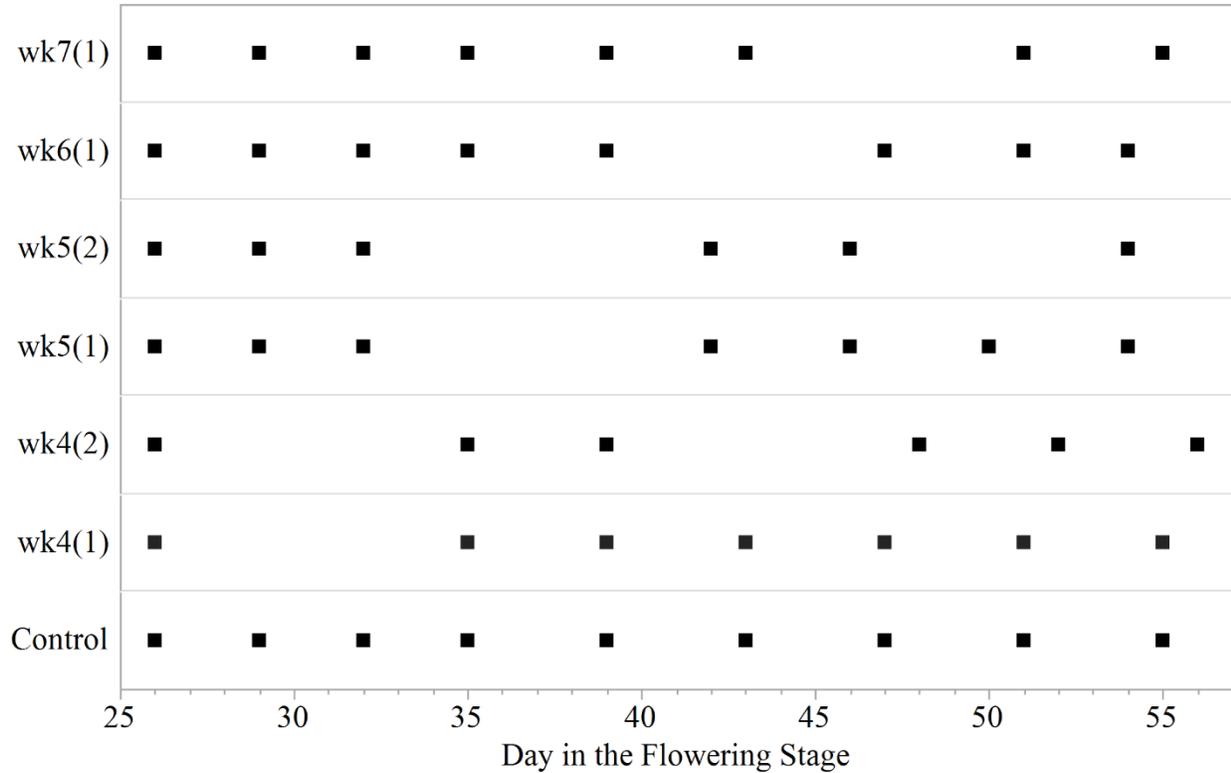


Figure 6.1. Irrigation timing for drought stress treatments. Square markers indicate an irrigation event and are means for each treatment.

### Substrate moisture content

Two to four capacitance-type substrate moisture sensors (ECH<sub>2</sub>O-TE; Decagon Devices Inc., Pullman, WA) were used per treatment to roughly indicate when wilting would occur, using the volumetric moisture content (VMC)-wilting relationships determined previously. When approaching the time of wilting and at the wilting threshold, a single hand-held WET-2 soil moisture sensor (Delta-T Devices Ltd.) was used to measure substrate VMC. The hand-held sensor was used to avoid the variability associated with multiple sensors and data loggers.

## Relative leaf angle

Leaf angle measurements were taken as an indicator of wilting as described in the previous chapter, using a hand-held, pivoting angle-finder and a protractor.

## Net photosynthetic rate and chlorophyll fluorescence

Leaf chlorophyll-a fluorescence parameters and net photosynthetic rate ( $P_n$ ) were measured at week 7 (51 - 52 DFS) for treatment wk7(1) immediately before fertigation (drought stress threshold), hourly for the four subsequent hours after fertigation and on the next day at mid-day. Three replicates from wk7(1) and three controls were measured for this analysis.

A portable photosynthesis measurement system (LI-6400XT; LI-COR Biosciences, Lincoln, NE) was used to measure  $P_n$  on the youngest fully expanded leaf. Light was supplied by 6400-02B Red-Blue light emitting diodes (LI-COR) with photosynthetically active radiation (PAR) set to around chamber canopy level ( $450 \mu\text{mol}\cdot\text{m}^{-2}\text{ s}^{-1}$ ).  $\text{CO}_2$  concentration in the leaf cuvette was maintained at  $800 \mu\text{mol}\cdot\text{mol}^{-1}$  and block temperature was maintained at  $20^\circ\text{C}$ . Chlorophyll-a fluorescence was measured using a portable, pulse amplitude modulation (PAM) fluorometer (FluorPen FP 100; Photon Systems Instruments; Drasov, Czech Republic). The youngest fully expanded leaf was dark-adapted for 45 min using a detachable leaf clip (Hansatech, Instruments, Kings Lynn, UK) which was then attached to the optical window of the fluorometer. Minimum fluorescence in the dark-adapted state ( $F_0$ ) was measured, then a saturating pulse of  $\sim 2100 \mu\text{mol photons m}^{-2}\text{ s}^{-1}$  was applied to measure maximal fluorescence intensity for the dark-adapted state ( $F_M$ ). A 10 s period of dark relaxation followed, then the sample was exposed to actinic irradiance ( $300 \mu\text{mol photons m}^{-2}\text{ s}^{-1}$ ) for 60 s to simulate growing conditions. During the period of actinic irradiance, a total of five saturating pulses were applied at 12 s intervals to probe non-photochemical quenching (NPQ). The value of maximum fluorescence during the last saturating pulse was considered the maximum fluorescence signal for the light adapted state,  $F_M'$ . During all fluorescence recordings,  $40 \mu\text{s}$  measuring pulses (475 nm) were applied at 1 s intervals at a photon flux density of  $900 \mu\text{mol m}^{-2}\text{ s}^{-1}$ . The fluorescence signal measured directly before and during each measuring pulse were subtracted which makes it a PAM-type of measurement (Humplík et al., 2015). The maximum quantum yield of PSII photochemistry ( $\Phi_{\text{PSII}}$ ) was estimated as:  $\Phi_{\text{PSII}} = F_v/F_M = (F_M - F_0)/F_M$  (Genty et al., 1989) and non-photochemical

chlorophyll fluorescence quenching (NPQ) was estimated as  $NPQ = (F_M - F_M') / F_M'$  (Oxborough and Baker, 1997).

### **Yield, growth, and secondary metabolite measurements**

Plants were harvested at 56 DFS. Just before harvest, growth index for each plant was calculated as height (cm) x length (cm) x width (cm) x  $300^{-1}$  (Ruter, 1992). Stems were cut at growing substrate level; large leaves were removed from stems and plants were hung to dry at 18 °C (s.d.  $\pm 0.1$  °C) and 45% RH (s.d.  $\pm 3.9\%$ ) for 2 days then cured at 18 °C (s.d.  $\pm 0.1$  °C) and 52% RH (s.d.  $\pm 2.5\%$ ) for 13 days. Floral material was then cut from stems and leaves were hand-trimmed before floral dry weight (yield) measurement. Dried, cured floral material from the apical flowers of each plant was stored under dark and cool conditions according to United Nations Office on Drugs and Crime (2009) before being analyzed by an independent laboratory (RPC Science and Engineering, Fredericton, NB, Canada).

Analysis of the neutral cannabinoids  $\Delta^9$ -Tetrahydrocannabinol (THC), Cannabidiol (CBD), Cannabinol (CBN), Cannabichromene (CBC), and Cannabigerol (CBG) as well as acid forms,  $\Delta^9$ -Tetrahydrocannabinolic Acid (THCA), Cannabidiolic Acid (CBDA), and Cannabigerolic Acid (CBGA), were conducted by high-performance liquid chromatography, as described in the previous chapter and terpenoids were analyzed using gas-chromatography. Total monoterpenes and sesquiterpenes were calculated by summing all analyzed terpenes of each type.

Moisture content of the dry floral material was determined using the methods described in the The United States Pharmacopeial Convention, section 921 method 3 (UPS 40, 2017) and cannabinoid/terpenoid concentration of floral material was corrected to zero percent moisture content. Cannabinoid and terpenoid yield were calculated as concentration multiplied by floral yield and expressed per unit area ( $g \cdot m^2$ ).

### **Statistical analysis**

Data were analyzed using JMP Statistical Discovery Version 13.0 (SAS Institute Inc., Cary, NC) at a Type 1 error rate of  $\leq 0.05$ . Differences between treatments were tested with a One-way ANOVA. Tukey's HSD test was used for multiple mean comparisons and Student's t-test was used for comparing means from the drought stress and recovery analysis at week seven [wk7(1)

compared to control]. The residuals of the analyses were tested for normality and equality of variance using The Shapiro-Wilk test and Bartlett's test, respectively. If cannabinoid or terpenoid concentrations were below the detection limit, <0.05% and <0.01%, respectively, the values were excluded from the analysis.

## **Results**

### **Growth and yield**

There were no symptoms of nutrient disorder in any of the drought treatments or in the control during the 56-day flowering period. Signs of drought stress were similar for all the drought treatments and included chlorosis in the leaf tissue immediately adjacent to veins in older leaves then newly formed leaves which became visually apparent about two or three days before wilting occurred.

There were no differences in yield between any of the treatments; growth index, measured upon harvest, also did not differ between treatments (Table 6.2).

Table 6.2. Yield (floral dry weight) and growth index of cannabis under six drought stress treatments during the flowering stage.

Treatment	n	Yield (g·m <sup>2</sup> )	Growth Index
Control	7	217 ± 9.9 <sup>z</sup> a	230 ± 16.4 a
wk4(1)	7	206 ± 13.0 a	237 ± 22.1 a
wk4(2)	7	197 ± 6.4 a	219 ± 16.2 a
wk5(1)	7	189 ± 9.9 a	213 ± 14.6 a
wk5(2)	7	191 ± 8.6 a	234 ± 18.6 a
wk6(1)	7	214 ± 8.7 a	218 ± 13.7 a
wk7(1)	6	223 ± 8.7 a	235 ± 19.3 a

<sup>z</sup>Means ± SEM followed by the same letter within the same column do not differ at  $P < 0.05$  using Tukey's HSD.

## Secondary metabolites

### *Terpenoids*

Twelve monoterpenes and four sesquiterpenes were detected in the floral material. Across treatments, the two most abundant terpenoid components were terpinolene (>4900 µg·g<sup>-1</sup>), and myrcene (>3000 µg·g<sup>-1</sup>). Wk4(2) and Wk5(1) had higher concentrations of terpinolene than wk7(1), but the concentration of this monoterpene did not differ in the other treatments. Myrcene concentration was higher in wk5(1) than in wk7(1) but again, there were no differences between the other treatments. Further, there were no differences in total terpenoid yield per unit growing area among treatments or when drought treatments were compared to the control except that the control had higher terpenoid yield than wk5(2), by 33%. There were, however, notable treatment effects on other terpenoids.

The monoterpenes, beta pinene, limonene, and linalool and the sesquiterpenes, caryophyllene, guaiaol and alpha-bisabolol were detected in relatively high concentrations (>250 µg·g<sup>-1</sup>). The

concentration and yield (Table 6.3 and 6.4, respectively) of some of these and other terpenoids varied between some drought treatments and the control.

For linalool and alpha-bisabolol, some drought treatments elicited higher terpenoid concentration and/or yield than control levels. Wk4(1), wk4(2), and wk5(1) all had higher concentrations of linalool than the control with the greatest increase in wk5(1), which was 68% higher than the control. Additionally, linalool yield was higher in wk4(1), wk4(2) and wk5(1) than the control, all by 49%. Notably, the control, wk7(1), and wk7(2) had the lowest linalool yield of all the treatments. For alpha-bisabolol, the highest concentration was in wk6(1) which was 40% higher than the control and higher than the other drought treatments by as much as 344% [when compared to wk5(2)]. Similarly, the yield of alpha-bisabolol in wk6(1) was higher than the control by 36% and higher than the other treatments by as much as 369% [when compared to wk5(2)]

The concentration of sesquiterpene caryophyllene was higher in wk6(1) than any other drought stress treatment, by as much as 47% when compared to wk4(1) and wk5(1) but did not differ from the control. Likewise, caryophyllene yield was higher in wk6(1) than all other treatments except wk7(1) and the control.

Cis and trans-ocimene were also found at relatively high concentrations ( $>600 \mu\text{g}\cdot\text{g}^{-1}$ ) but cis-ocimene was found only in earlier-stress treatments; wk4(1), wk4(2), wk5(1), and wk5(2), while trans-ocimene was found only in later-stress treatments: wk6(1), wk7(1) and the control.

Total monoterpene yield did not differ between treatments and monoterpene concentrations were similar with the exception that wk4(2) and wk5(1) had a 36% higher concentration than week wk7(1). Total sesquiterpene concentration was highest in wk6(1) and the control. Wk6(1) was 77% and 64% higher than earlier-stress treatments, wk4(1) and wk5(1), respectively. Likewise, total sesquiterpene yield was highest in the control and in later stress treatments, wk6(1) and wk7(1), than any earlier stress treatment.

Notably, other than having higher concentration and yield of alpha-bisabolol and lower concentration and yield of Terpeneol, wk6(1) did not differ from the control in the concentration or yield of any of the terpenoids.

Table 6.3. Terpenoid concentration in cannabis dry floral material under six drought stress treatments during the flowering stage.

n <sup>z</sup>	Drought Treatment						Control
	wk4(1)	wk4(2)	wk5(1)	wk5(2)	wk6(1)	wk7(1)	
	7	7	7	7	7	6	7
	(µg·g)						
<i>Monoterpenes</i>							
Alpha pinene	270 <sup>y</sup> bc	340 ab	370 a	320 abc	320 abc	240 c	300 abc
Beta pinene	580 ab	680 ab	740 a	630 ab	630 ab	520 b	570 ab
Myrcene	3500 ab	4000 ab	4200 a	3400 ab	3600 ab	3000 b	4000 ab
Limonene	960 ab	1000 ab	1200 a	920 ab	830 b	740 b	990 ab
Terpinolene	5600 ab	6500 a	6400 a	5600 ab	5700 ab	4900 b	6300 ab
Linalool	590 ab	610 ab	640 a	490 bc	380 cd	260 d	380 cd
Terpineol	340 ab	340 ab	320 ab	380 a	400 a	260 b	380 a
3-carene	180 ab	210 a	210 a	140 ab	190 ab	110 b	170 ab
Cis-ocimene	670 a	790 a	820 a	630 a	ND <sup>x</sup>	ND	ND
Trans-ocimene	ND	ND	ND	ND	850 a	720 a	890 a
Fenchol	210 a	180 a	180 a	180 a	140 a	130 a	190 a
Borneol	270 a	270 a	260 a	250 a	220 a	240 a	300 a
Total monoterpenes	13000 ab	15000 a	15000 a	13000 ab	13000 ab	11000 b	15000 ab
<i>Sesquiterpenes</i>							
Caryophyllene	510 c	530 bc	540 bc	510 c	750 a	580 bc	640 ab
Humulene	180 b	180 ab	210 ab	170 b	260 a	220 ab	220 ab
Guaiol	430 ab	440 ab	450 ab	410 b	530 a	450 ab	520 a
Alpha-bisabolol	310 c	240 c	270 c	180 c	800 a	560 b	570 b
Total Sesquiterpenes	1300 d	1400 cd	1400 cd	1200 d	2300 a	1800 bc	2000 ab
	(%)						
Total terpenoids	1.45 ab	1.64 ab	1.68 a	1.41 ab	1.55 ab	1.29 b	1.65 ab

<sup>z</sup>Number of replicates, unless otherwise noted

<sup>y</sup>Means ± SEM followed by the same letter within the same row do not differ at  $P < 0.05$  using Tukey's HSD.

<sup>x</sup>ND = Not detected or insufficient data to compare mean.

Table 6.4. Terpenoid yield per unit growing area in cannabis dry floral material under six drought stress treatments during the flowering stage.

n <sup>z</sup>	Drought Treatment						
	wk4(1)	wk4(2)	wk5(1)	wk5(2)	wk6(1)	wk7(1)	Control
	(mg·m <sup>2</sup> )						
<i>Monoterpenes</i>							
Alpha pinene	49 <sup>y</sup> a	59 a	63 a	54 a	61 a	48 a	59 a
Beta pinene	100 a	120 a	130 a	110 a	120 a	100 a	110 a
Myrcene	630 a	710 a	720 a	580 a	680 a	610 a	780 a
Limonene	170 a	180 a	200 a	160 a	160 a	150 a	190 a
Terpinolene	1000 a	1100 a	1100 a	970 a	1100 a	970 a	1200 a
Linalool	110 a	110 a	110 a	85 ab	72 bc	52 c	74 bc
Terpineol	61 abc	59 bc	55 c	64 abc	76 a	52 c	74 ab
3-carene	31 a	37 a	36 a	25 a	36 a	22 a (n=5)	33 a
Cis-ocimene	120 a	140 a	140 a	110 a	ND	ND	ND
Trans-ocimene	ND <sup>x</sup>	ND	ND	ND	160 a	140 a	170 a
Fenchol	38 a	31 a	30 a	32 a (n=6)	27 a	26 a	37 a
Borneol	49 a	48 a	43 a	43 a	43 a	48 a	59 a
Total monoterpenes	2400 a	2600 a	2600 a	2200 a	2500 a	2200 a	2800 a
<i>Sesquiterpenes</i>							
Caryophyllene	94 b	93 b	92 b	88 b	140 a	120 ab	130 a
Humulene	32 b	31 b	36 ab	30 b	49 a	45 ab	43 ab
Guaiol	80 ab	77 ab	76 ab	71 b	100 a	89 ab	100 a
Alpha-bisabolol	54 c (n=5)	42 c	46 c (n=5)	32 c (n=5)	150 a	110 b	110 b
Total sesquiterpenes	240 b	240 b	240 b	210 b	450 a	360 a	380 a
Total terpenoids	2600 ab	2900 ab	2800 ab	2400 b	3000 ab	2600 ab	3200 a

<sup>z</sup>Number of replicates unless otherwise noted

<sup>y</sup>Means ± SEM followed by the same letter within the same row do not differ at  $P < 0.05$  using Tukey's HSD.

<sup>x</sup>ND = No data or insufficient data to compare mean.

### *Cannabinoids*

None of the drought treatments had higher cannabinoid concentrations in dry floral material or yield per unit growing area than the control; and, there were no differences in the concentration or yield of most abundant cannabinoids, THCA and CBDA, among the treatments (Table 6.5). There were also no differences in cannabinoid concentration or yield among the drought treatments, though some had lower levels than the control.

Drought stress at week 7 [wk7(1)] had a lower concentration of THC than the control and wk5(1) had a lower concentration of CBG than the control. Compared to the control, yield of THC was lower in wk4(1), wk4(2), wk5(2), and wk7(1) and yield of CBD was lower in wk4(1), wk4(2), wk5(1), and wk5(2). Other than a slightly lower concentration of CBD, Wk6(1) did not differ from the control in the concentration or yield of any of the cannabinoids, as was observed with the terpenoids for this treatment.

Table 6.5. Cannabinoid concentration in dry floral material and yield per unit growing area in cannabis dry floral material under six drought stress treatments during the flowering stage.

	THC	THCA	CBD	CBDA	CBG	CBGA
<b>Treatment</b>	<b>Cannabinoid Concentration (%)</b>					
wk4(1)	0.58 <sup>z</sup> ab	6.0 a	0.36 ab	11.5 a	0.079 ab	0.59 a
wk4(2)	0.59 ab	6.4 a	0.37 ab	11.9 a	0.074 ab	0.58 a
wk5(1)	0.68 ab	6.0 a	0.40 ab	11.5 a	0.069 b	0.54 a
wk5(2)	0.62 ab	6.2 a	0.39 ab	11.6 a	0.072 ab	0.55 a
wk6(1)	0.58 ab	6.6 a	0.36 b	12.4 a	0.079 ab	0.57 a
wk7(1)	0.55 b	6.0 a	0.38 ab	11.5 a	0.075 ab	0.53 a
Control	0.81 a	6.2 a	0.48 a	11.8 a	0.095 a	0.56 a
	<b>Cannabinoid Yield (g·m<sup>2</sup>)</b>					
wk4(1)	1.1 b	11.0 a	0.66 b	21.1 a	0.15 ab	1.1 a
wk4(2)	1.0 b	11.1 a	0.65 b	20.8 a	0.13 b	1.0 a
wk5(1)	1.1 ab	10.1 a	0.67 b	19.4 a	0.11 b	0.9 a
wk5(2)	1.1 b	10.7 a	0.66 b	20.1 a	0.12 b	0.9 a
wk6(1)	1.1 a	12.5 a	0.68 b	23.7 a	0.15 ab	1.1 a
wk7(1)	1.1 b	12.0 a	0.74 ab	22.9 a	0.14 ab	1.1 a
Control	1.6 a	12.0 a	0.94 a	23.0 a	0.19 a	1.1 a

<sup>z</sup>Means followed by the same letter within the same column do not differ at  $P < 0.05$  using Tukey's HSD.  $n = 6$  for all cannabinoids for wk7(1), CGB for wk5(1), and CBG for 5(2);  $n = 7$  for all other means.

## Wilting

Averaged across treatments, the method used for assessing drought stress by measuring leaf angle proved successful; irrigation was triggered when leaf angle increased by 47% (SEM  $\pm$  0.7%;  $n = 56$ ), a 3% variation from the planned 50%.

Table 6.6 indicates the period required for each treatment to reach the defined drought stress threshold. There was a maximum variation of 2.5 days between treatments, with wilting taking longest for wk5(1) and occurring fastest for wk7(1).

Table 6.6. Period of drought required for cannabis plants to wilt during the flowering stage.

Treatment	n	Days Without Fertigation	
		First Stress	Second Stress
wk4(1)	7	9.0 ± 0.59 <sup>z</sup> abc	- <sup>y</sup>
wk4(2)	7	9.3 ± 0.25 abc	8.9 ± 0.25 a
wk5(1)	7	10.2 ± 0.28 a	-
wk5(2)	7	9.9 ± 0.35 ab	8.2 ± 0.35 a
wk6(1)	7	8.1 ± 0.29 bc	-
wk7(1)	6	7.7 ± 0.33 c	-

<sup>z</sup>Means ± SEM followed by the same letter within the same column do not differ at  $P < 0.05$  using Tukey's HSD.

<sup>y</sup>Dashes indicate that the treatment was drought-stressed only once

### Drought stress and recovery at week seven of the flowering stage

Leaf chlorophyll-a fluorescence parameters and  $P_n$  were assessed at week seven during the flowering stage to verify that the applied level of stress was similar to that of the previous chapter of this thesis and to observe the recovery of these parameters after fertigation. At the drought stress threshold in week seven of the flowering stage (51 DFS),  $P_n$  was  $3.7 \pm 0.07 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  in wk7(1) and  $19.4 \pm 0.46 \mu\text{mol CO}_2 \text{m}^{-2} \cdot \text{s}^{-2}$  in the control, 424% lower. The leaf chlorophyll-a fluorescence parameters,  $F_v/F_M$  and NPQ, measured at the same time, showed no differences (Table 6.7).

Three hours after fertigation,  $P_n$  in wk7(1) reached control levels; however, on the day after fertigation, at mid-day,  $P_n$  in wk7(1) was  $15.1 \pm 1.20 \mu\text{mol CO}_2 \text{m}^{-2} \cdot \text{s}^{-2}$ , still 32% lower than the control, at  $20.0 \pm 0.41 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  (Figure 6.2). Again,  $F_v/F_M$  and NPQ, did not differ when measured during the same period.

Table 6.7. Leaf chlorophyll-a fluorescence parameters of cannabis after drought stress and recovery after fertigation at 7 weeks into the flowering stage.

Treatment	F <sub>V</sub> /F <sub>M</sub>	NPQ
<u>Drought stress threshold</u>		
Control	0.80 ± 0.006 <sup>y</sup>	1.4 ± 0.11
Wk7(1)	0.80 ± 0.003	1.2 ± 0.20
Significance	NS	NS
<u>Next Mid-day after fertigation</u>		
Control	0.79 ± 0.015	1.3 ± 0.18
Wk7(1)	0.79 ± 0.006	1.3 ± 0.07
Significance	NS	NS

<sup>z</sup>NS, \*, \*\*, \*\*\* Nonsignificant, or significant at P < 0.05, 0.01, and 0.0001, respectively using student's t-tests

<sup>y</sup>Values are means ± SEM; n = 3

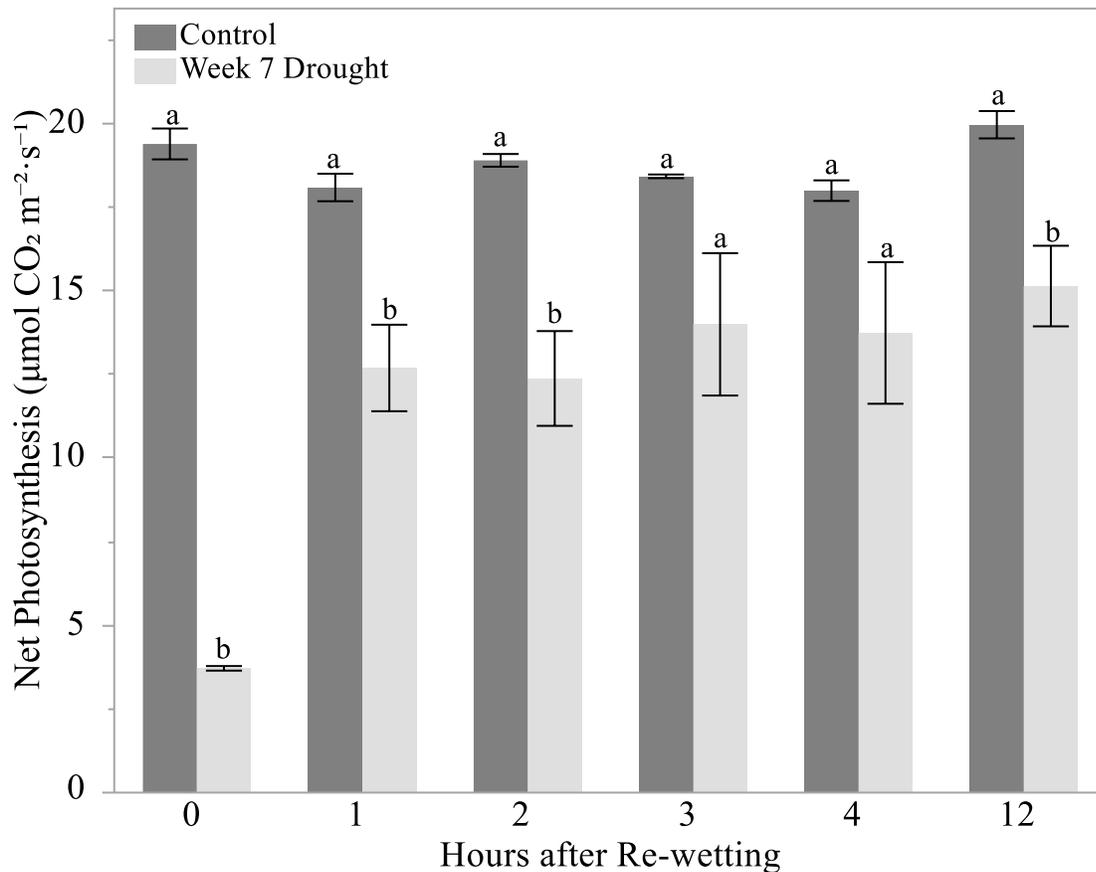


Figure 6.2. Net photosynthetic rate of cannabis after drought stress and subsequent fertigation at seven weeks into the flowering stage. Error bars indicate standard error. Adjacent bars bearing different letters are significantly different at  $P < 0.05$  using t-tests.

### Water/fertilizer use

The control was irrigated every  $3.2 \pm 0.08$  days ( $n = 7$ ) between 28 DFS to 55 DFS and fertigation volume was  $\approx 2$  L/plant at each irrigation event. For wk4(1), wk5(1), wk6(1) and wk7(1), between two and three irrigations were withheld and for wk4(2) and wk5(2), four to six were withheld (Table 6.6); therefore, drought application reduced fertigation water usage by between 4 and 12 L/plant or 28 and 86 L·m<sup>-2</sup> during the flowering stage.

## Discussion

There were no differences in growth, cannabinoid concentration or cannabinoid content per unit growing area between any of the treatments; however, some drought treatments increased the concentration and yield of specific terpenes compared to the control. For linalool, concentration and yield was higher for wk4(1), wk4(2), and wk5(1) than the control and, the concentration and yield of cis-ocimene was higher for wk4(1), wk4(2) wk5(1), and wk5(2) than the control. Further, both the concentration and yield of alpha-bisabolol were higher for wk6(1) than the control.

The effects of drought timing during the flowering stage were apparent when comparing earlier-stress treatments (during weeks four or five) to later-stress (during weeks six or seven). For example, cis-ocimene was only detected in earlier-stress treatments, wk4(1), wk4(2) wk5(1), and wk5(2) whereas trans-ocimene was only detected in later-stress treatments, wk6(1) wk7(1). Also, the concentration and yield of linalool was higher for wk4(1), wk4(2), and wk5(1) than for both wk6(1) and wk7(1).

In some cases, earlier drought stress increased terpenoid content. Total terpenoid concentration was higher for wk5(1) than for wk7(1). Also, total monoterpene concentration was higher in wk4(2) and wk5(1) than in wk7(1); however, total monoterpene yield did not differ between these treatments since dry floral weight was slightly (non-significantly) lower for the two earlier-stress treatments compared to wk7(1).

Drought applied later during the flowering stage increased the content of other terpenes, specifically some sesquiterpenes. Total sesquiterpene concentration was highest in wk6(1) and total sesquiterpene yield was highest in wk6(1) and wk7(1) compared to the other drought treatments. For alpha-bisabolol, both concentration and yield were higher in wk6(1) and wk7(1) compared to earlier drought treatments. Additionally, caryophyllene concentration and yield was higher in wk6(1) than any other drought treatment. Since caryophyllene concentration did not differ between wk6(1) and the control, it appeared that later-stress did not increase the yield of this terpenoid but rather that earlier-stress may have reduced its yield.

These results suggest that controlled drought stress applied during week four or five of the flowering stage may increase the yield of monoterpenes linalool and cis-ocimene and decrease yield of caryophyllene in cannabis. In contrast, controlled drought stress applied during week six or seven of the flowerings stage may increase the yield of alpha-bisabolol and trans-ocimene.

Sesquiterpenes and monoterpenes are both derived from precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) but the biochemical origin of the precursors is different between the two. For sesquiterpenes, IPP and DMAPP are produced in the cytosol and mitochondria through the Mevalonate pathway (Banthorpe et al., 1972) while for monoterpenes, they are produced in the plastids through the Mevalonate-independent (DXP) pathway (Eisenreich et al., 1998). Further, the immediate precursor for all monoterpenes is geranyl diphosphate (GPP) formed from the condensation of one DMAPP and one IPP molecule whereas the immediate precursor for all sesquiterpenes is farnesyl diphosphate (FPP), formed by the condensation of one DMAPP and two IPP molecules (Nagegowda, 2010). Various terpene synthases are responsible for the conversion of GPP and FPP into specific mono- and sesquiterpenes (Booth et al., 2017) and the expression of these synthases varies by plant tissue (Nagegowda, 2010) and growth stage (Aizpurua-Olaizola et al., 2016). Variable expression of terpene synthases as well as the biochemical origins of IPP and DMAPP for mono- and sesquiterpenes may account for some of the differences observed between the drought stress timings however, further research is required to elucidate the effects of drought and other stressors on terpenoid biosynthesis.

The stimulatory effects of drought stress on secondary metabolite production are generally considered to occur gradually as the plant acclimates to the stress (Banik et al., 2016; Grace and Logan, 1996). In chemotype II cannabis, floral concentration of sesquiterpenes varies minimally during the flowering stage whereas floral monoterpene concentration rises at an increasing rate after the second week of the flowering stage (Aizpurua-Olaizola et al., 2016). The earliest controlled drought stress treatments (during week four and five of the flowering stage) had the greatest stimulatory effects on monoterpenes. This may suggest that drought applied before the highest rates of accumulation of monoterpenes (and potentially other secondary metabolites), optimizes the stimulatory effects of the stress.

The cannabinoid results of the present trial are at odds with those of the previous chapter, in which drought stress increased THCA concentration by 12% and CBDA concentration by 13% over the well-watered control. Cannabinoid yield per unit growing area was also higher for THCA, CBDA, THC, and CBD in the drought-stressed group. Both studies employed the same cultivar of cannabis, the same drought stress threshold (during week seven), and similar environmental conditions; however, the pot size and volume of the growing substrate was different. In the previous chapter, the pots used during the flowering stage were 11 L in volume and in the present trial, they were 6 L. The larger volume of growing substrate in a larger pot can hold more water at container capacity than a smaller pot with the same substrate. Consequently, in a larger pot it can take longer for a plant to deplete root zone moisture to reach conditions of water deficit. This was exemplified by the fact that the drought-stressed plants in the previous trial took eleven days to reach the defined drought threshold whereas in the present trial, the plants that were drought-stressed in week seven took around eight days. A longer, likely more gradual, drought stress period as in the previous chapter may be required to increase cannabinoid yield in cannabis; however, further research is needed to determine the effects of substrate volume/pot size and duration of water deficit on cannabis secondary metabolism.

Leaf wilting can be an effective indicator of drought stress; however, the duration of the stress until a drought stress threshold is reached requires consideration. The time to wilting varied minimally between treatments from week four to week seven in the present trial; however, timing would likely vary by substrate volume based on comparison with the previous chapter. Also, environmental condition may influence time to wilting. For example, if the vapor pressure deficit (VPD) in the growing environment increases suddenly, plants may exhibit a rapid decline in plant water potential (Stoochnoff et al., 2018; Thompson et al., 2007; Tran et al., 2015) and possibly a wilting response (Banik et al., 2016) but this does not imply that the period of drought was sufficient to stimulate secondary metabolites. The use of a wilting threshold as a drought stress indicator should, therefore also coincide with a desired period for the wilting to occur if the objective is to increase secondary metabolite yield.

In the present study, VPD was increased during the ten days before harvest (Table 6.1). Based on our communications with Canadian medical cannabis producers, it is common for growers to increase the growing environment VPD during the final week or two during the flowering stage

with the intention of preventing microbial contamination of the floral material. Higher VPD may lead to higher rates of evapotranspiration and substrate drying (Thompson et al., 2007).

Accordingly, wilting occurred faster in wk7(1) than in week 5 drought stress treatments; though, the same was not observed between wk7(1) and other earlier-stress treatments (Table 6.6).

Difference between wilting rates between the earliest and latest drought stress treatments could be caused by differences in growth rates within the flowering stage in cannabis; however, further research is required to evaluate variability in growth rate during the flowering stage for cannabis.

At the week seven drought threshold,  $P_n$  was drastically reduced and did not fully recover to control levels on the day after fertigation. A reduction of  $P_n$  in cannabis plants under drought stress was also observed in the previous chapter. Drought causes both stomatal and metabolic limitations which reduce rates of carbon assimilation and therefore  $P_n$  (Tezara et al., 1999). Yet, leaf photosynthetic rate does not necessarily correlate with yield in cannabis. In both the present and the previous chapter, floral dry weight upon harvest did not differ from the control irrespective of reduced  $P_n$  and in the present trial, there were also no differences in growth index.

Fluorescence yield in dark-adapted plants ( $F_v/F_M$ ) and nonphotochemical quenching (NPQ) were also measured during week seven of the flowering stage. The  $F_v/F_M$  is an indicator of photochemical efficiency under water stress (Woo et al., 2008). Normal, non-stressed values of  $F_v/F_M$  for most species is around 0.8 and lower values may indicate stressful conditions and/or permanent damage to photosystem II (PSII; Johnson et al., 1993). The  $F_v/F_M$  values between drought and control treatments did not differ at the drought threshold or on the day after fertigation, both values were around 0.8. This indicates that the drought threshold in the present study was insufficient to reduce photochemical efficiency and/or cause permanent damage to PSII in cannabis. Comparable results have been documented in spearmint and rosemary in which increasing drought stress reduced  $P_n$  but  $F_v/F_M$  remained unchanged (Delfine et al., 2005). A substantial reduction in  $F_v/F_M$  may indicate terminal drought stress. For example, in *Arabidopsis thaliana*, a 33% reduction of the  $F_v/F_m$  from control plant levels indicates that the drought stress may have terminal effects (Woo et al., 2008). Similarly, NPQ values did not vary significantly between drought and control groups during week seven of the flowering stage in the present study. NPQ quantifies the energy dissipated as thermal radiation in the chlorophyll via the xanthophyll cycle, but variations in NPQ are sometimes only detectible under severe stress (Woo

et al., 2008). In *Arabidopsis*, NPQ varies minimally (between 0.8–1.6) under moderate levels of drought stress then decreases to near-zero levels once the stress is terminal (Woo et al., 2008). These results suggest that NPQ may not be an effective indicator of moderate drought stress in cannabis.

Treatments wk4(2) and wk5(2) were intended to simulate intermittent drought stress in which the first drought stress acclimates to subsequent stress. If these treatments prompted some degree of acclimation, wilting may occur slower during the second stress than in non-acclimated treatments. This has been observed in potato plants that have been drought-acclimated from previous exposure to water deficit (Banik et al., 2016); however, it was not the case in the present study, as time to wilting in intermittent stress treatments was similar in the first and second drought application. Also, there were no notable instances where the intermittent stress treatments outperformed treatments in which drought was applied once in terms of secondary metabolite concentration or yield per unit growing area. These results suggest that intermittent drought stress, as applied here, may not have an added benefit to cannabis production over controlled drought applied once. Other intensities or timings of intermittent drought stress may elicit another response so additional research is required.

Water and fertilizer were applied together through fertigation. Fertilizer rates remained constant; therefore, the amount of fertilizer applied to each treatment was slightly different based on the number of fertigation events. The drought treatments had similar yield to the control, as in the previous chapter, and flowering-stage cannabis performs similarly under a range of organic fertigation rates (Caplan et al., 2017), so it is unlikely that nutrition was lacking in the drought treatments.

## **Conclusions**

This study suggested that in cannabis, timing of controlled drought stress during the flowering stage influenced the concentration and yield per unit growing area of some terpenoids without reducing growth or yield. Earlier drought stress, during weeks four or five of the flowering stage increased the yield of linalool and cis-ocimene but decreased yield of caryophyllene. Later drought stress, during week six or seven of the flowerings stage, increased the yield of alpha-bisabolol and trans-ocimene. Intermittent drought stress did not increase secondary metabolites

or yield more than single drought applications suggesting that repeated stress may not be useful to increase cannabis secondary metabolites. Controlled drought can be effectively applied in cannabis through measurements of leaf wilting, but the duration of the stress must be considered as time to wilting can vary by root zone and environmental conditions such as pot size and changes in VPD in the growing environment. Further research is required to determine optimal duration of drought stress, subsequent acclimation, and the effects of drought on other chemovars or varieties of cannabis.

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## **CHAPTER SEVEN:**

### **Discussion, recommendations, and conclusions**

#### **Filling the gaps in horticulture research**

The importance of horticulture research for cannabis production is clear; growers of a high value, medicinal crop should not have to depend on unscientific or anecdotal information to guide them nor should the prescribers or users of medicinal cannabis. There is urgent need for evidence-based and scientifically-validated practices for the horticultural management of cannabis such as those presented in this thesis research. The scope of this research covers a portion of the study areas required and it will take significantly more effort to fill the gap left by more than half a century of cannabis prohibition.

In the experimental design of each manuscript of this thesis, there were several important horticulture decisions to be made that did not immediately relate to the applied treatments. These parameters included: lighting intensity/spectrum, growth chamber CO<sub>2</sub> concentrations, air temperature/humidity, duration of growth stages, and pot size; these parameters are interconnected, and they undoubtedly affect plant growth in some way. Since cannabis horticulture research is still in its infancy, any reliable horticultural information was valuable in deciding upon setpoints for these parameters. For the most part, these decisions were based on research involving other crops that are grown in controlled environments. For instance, the range of fertilizer rates selected as treatment levels in chapters three and four were based on previous studies on organic fertigation of greenhouse-grown tomatoes (Surrage et al., 2010; Zhai et al., 2009).

Researchers have been improving production practices for other crops, including medicinal plants, for decades, creating a large body of scientifically-validated information to draw upon. This information provides an ideal starting point for cannabis growers or researchers seeking to improve horticultural practices for this crop. Eventually, as the body of cannabis-specific research accumulates, researchers will be able to rely less and less on information from other crops.

## **Going forward using the integrated root zone management approach**

Our research suggested optimal fertilizer rates for cannabis and described how these optima varied by growth stage and choice of growing substrate. Further, results from our fertilizer and growing substrate trials, including the fertilizer rates, were instrumental in the subsequent design of the controlled drought stress trials.

Consideration of the interconnected nature of these horticultural management practices was valuable in the design of these trials and in presenting the significance of their results. The root zone is a complicated system and elements such as irrigation, growing substrate, and fertilizer are interconnected. To provide plants with a healthy root zone, it is essential to take an integrated approach to managing them. This is the basis of the integrated root zone management (IRM) approach (Zheng, 2016).

For example, the IRM approach was used when making irrigation decisions in chapters three and four of this thesis, pertaining to fertigation and growing substrates. Since irrigation frequency was not the subject of evaluation in these trials, it may be suggested that each substrate should be irrigated at the exact same frequency. The substrates in each trial had different physical properties, specifically different container capacities (CC; the water content remaining in the substrate after the substrate is well irrigated). If they were irrigated at the same frequency, those with higher CC (wetter substrates) may have been consistently waterlogged, reducing root zone oxygen and increasing the risk of root zone pathogens (Jackson and Colmer, 2005; Zheng et al., 2007). In this example, the interconnected nature of the growing substrate, irrigation, and root zone oxygen required consideration so that the substrates could be effectively compared. In situations such as these, the IRM approach will be useful for the design of future studies involving the horticultural management of container-crops, including cannabis.

The research presented in this thesis attempts to answer specific questions on cannabis production, pertaining to propagation, fertilizer management, growing substrates, and irrigation/controlled drought stress. Naturally, our findings have led to several unanswered questions. The following sections will describe some emerging questions and suggest avenues for further research.

## **Propagation**

The findings from the first chapter of this thesis, regarding propagation, were rather practical in nature. They suggested that for vegetative stem cuttings of cannabis, removing leaf tips is detrimental, and that cuttings with three fully-expanded leaves were better than those with two in terms of rooting success and root quality. We also found that a 0.2% indole-3-butyric (IBA) gel was more effective than a 0.2% willow extract gel to stimulate rooting and that position (basal/apical) of stem cutting on the stock plant made little difference.

The physiological responses that may explain these findings were speculated upon; however, further study is required to understand why, for example, leaf number or removal of leaf tips elicits these effects in cannabis stem cuttings. Further, since only one organic rooting hormone, at one concentration, was evaluated, more research is needed to explore organic rooting hormone alternatives for cannabis growers that use organic production methods.

## **Fertilizer and growing substrates**

In chapters three and four of this thesis, it was confirmed that the growing substrate and rate of organic fertilizer had substantial impacts on cannabis growth and quality. Growing substrate requirements varied by growth stage, and optimal fertilizer rates were dependent on the choice of growing substrate. During the vegetative stage, cannabis performed well in both substrates that were evaluated despite the  $\approx 11\%$  difference in CC. During the flowering stage, the substrate with lower CC increased floral dry mass and the concentration and/or yield of some cannabinoids, including THC, compared to the substrate with higher CC.

In the vegetative stage, the optimal organic fertilizer rate was the same for both substrates whereas in the flowering stage, it was different between the substrates; increasing fertilizer rate led to increased growth and yield, but also to a dilution of THC, THCA, and CBGA. The optimal fertilizer rate during the flowering stage may therefore depend on the desired outcome of the grower. In both studies, treatments were tested on a single cultivar of chemotype I, high THCA cannabis; therefore, the effects of growing substrate and fertilizer rate on other cannabinoids of interest, such as CBDA, are still unknown. In fact, this led to our use of a chemotype II cultivar in the subsequent drought stress chapters of this thesis. Chemotype II varieties have significant

quantities of both THCA and CBDA, which allowed assessment of the effects of drought on both medically-relevant cannabinoids. For future research on cannabis production, chemotype II varieties are the recommended subject of evaluation if only a single cultivar can be used.

In both trials, growing substrate pH was monitored to ensure it was within an appropriate range for nutrient uptake. There were no symptoms of nutrient disorder in either trial, suggesting that a growing substrate pH between 5.8 to 7.2 is acceptable during the vegetative stage, and a range of 5.5 to 7.4 is acceptable for the flowering stage. More research is needed, however, to determine the optimal growing substrate pH ranges for cannabis.

The results of the flowering stage fertilizer and growing substrate trial brought about several additional questions which merit further research. The drier (lower CC) growing substrate enhanced floral dry weight as well as cannabinoid content compared to the wetter substrate; however, we can only speculate what caused this effect without further research. The drier substrate required more frequent irrigation to maintain the specified level of root zone moisture and, as a result, more oxygen may have been available in the root zone. It is speculated that cannabis may benefit from high irrigation frequency and/or high root zone oxygen; however, further research is required to control for these variables and to optimize irrigation frequencies and root zone oxygen concentrations for cannabis.

## **Drought stress**

Controlled drought influenced the content of some cannabinoids and terpenoids, as demonstrated in chapters five and six of this thesis. Using leaf wilting as a drought stress indicator, with fertigation triggered at a leaf angle 50% higher than in its turgid state, proved to be effective and easily measured.

There was a notable difference in the effect of drought on cannabinoid content between chapters five and six. In chapter five, drought during week seven increased the content of some cannabinoids compared to the control, whereas there was no increase in the week-seven drought treatment in chapter six. We speculate that differences in flowering-stage pot size between the trials influenced the duration of the drought stress period and therefore led to variable physiological and biochemical responses in the plants. In chapter five, the pots were 11 L in

volume while in chapter six, they were 6 L. A longer, likely more gradual, drought stress period possibly provided by a larger pot may, therefore, be required to increase cannabinoid content in cannabis. This provides another example in which the IRM approach may have been practical. Pot size generally dictates substrate volume which influences a host of interconnected root zone parameters. Attention is required to anticipate how root zone parameters will be affected when one aspect such as pot size is changed. Further research is needed to determine the effects of pot size and duration of water deficit on cannabis secondary metabolism and to explain the physiological changes during drought that bring about these effects.

### **Concluding thoughts**

As the legal cannabis industry is in its infancy there is a need for scientific research on cannabis-specific horticultural management techniques, such as those described within this thesis. Scientists and horticulturalists can provide producers with the tools they need to enhance the safety and quality of their crops while sustaining or increasing yields. Cannabis horticultural research will produce valuable knowledge that will not only help cannabis growers, but also cannabis consumers and the governments that regulate and support this quickly growing industry.

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