UV photomorphogenesis:
Gene expression in cannabis in response to UV exposure

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Science
in
Horticultural Science

at Massey University, Manawatū,
New Zealand.

Reuben Dods
2022

Acknowledgements

Undertaking this masters project has been a deeply valuable learning experience. Conducting a project with BioLumic has provided me an opportunity to get insight into development and commercialisation of a unique agricultural technology. Completing this thesis has allowed me to build an understanding of not only what constitutes robust scientific research but, also about the strategic thinking behind research decisions as they relate to solving business needs.

I want to thank Jason Wargent for his support and guidance as my supervisor. In addition to providing support in undertaking my research Jason has also provided me meaningful support in taking on opportunities outside of my thesis, exploring job opportunities, and in my personal growth and development.

Thank you also to the wider BioLumic team, for supporting directly in my thesis, by coaching me in the lab and providing project guidance as well as making me feel like a welcome member of the group.

Thank you to Callaghan innovation and Horticulture New Zealand, for your financial support during my undertaking of this project.

Finally, a thank you to my family, friends, and my partner Mrinali who have all been there to support in times of need, to take a break with and give words of support and sound advice when they were needed.

Abstract

Globally, changes in legislation have led to a significant increase in large scale commercial cultivation of *Cannabis sativa* (cannabis). For commercial growers, both yield and the concentration of cannabinoids in the harvested flower are key metrics for determining the value of a crop. In cannabis, the concentration of cannabinoids is determined both by underlying cultivar genetics and environmental factors. Ultraviolet (UV) radiation is already known to impact the growth and development of crop plants and has shown potential for use in beneficially controlling agronomically desirable outcomes, including improved disease resistance, increased yields, and greater accumulation of flavonoids in a range of crops. There are few historical studies on the impact of UV light on cannabis, however, preliminary research from BioLumic Limited – a New Zealand biotechnology company, has shown that short duration UV light treatments of young plants could be used to increase the yield and the content of commercially valuable cannabinoids THC and CBD. Little is known about the underlying molecular-level responses of cannabis to UV and extending this knowledge may reveal gene expression diagnostic targets that could be used in increasing the speed and impact of commercial UV light treatment development. In this study, a series of trials were carried out, where young cannabis clonal plants from a range of cannabis genetics were exposed to a number of proprietary UV light treatments. Plants were removed on a timeseries throughout the UV treatment regimens and were assayed via RT-qPCR to provide a timeseries of the expression of genes known to be involved in either UV signalling response, or cannabinoid biosynthesis. UV treatment induced several significant timepoint-based changes in relative expression of UV-response genes *CHS* and *HY5*, but induced limited expression-based change in the cannabinoid biosynthesis genes *CBDAS* and *THCAS*. Study results also indicated that plants displaying (pre-UV treatment) stress symptoms may be primed to respond more strongly to UV treatments, as indicated by higher levels of initial *CHS* gene expression. This study also showed that clone plants originating from different mother plants from the same variety (or ‘strain’) of cannabis, exhibited differences in gene expression when exposed to the same UV treatment. Future work will further explore the response of cannabis to UV treatments, including the expression of genes that may be used as markers for increasing the speed of commercially valuable UV treatment development.

Abbreviations

ANOVA – Analysis of Variance

CBCAS - Cannabichromenic Acid Synthase

CBD - Cannabidiol

CBDAS - Cannabidiolic Acid Synthase

CHS - Chalcone Synthase

COP1 – Constitutive Photomorphogenic 1

C_q – Quantification cycle

CRY – Cryptochrome

DNA - Deoxyribonucleic acid

EC – Electrical conductivity

HC – Head Cheese

HY5 - Elongated Hypocotyl 5

HYH – *HY5* Homolog

LED – Light-emitting-diode

NLS – Nuclear location signal

OLA – Olivetolic Acid

PAR – Photosynthetically active radiation

PVP – Polyvinylpyrrolidone

RB – Resuspension Buffer

RH – Relative humidity

RNA – Ribonucleic acid

RT-qPCR – Reverse Transcriptase quantitative Polymerase Chain Reaction

RUP1/2 – Repressor of UVB Photomorphogenesis 1/2

TBE - Tris-Borate-EDTA

THC - Tetrahydrocannabinol

THCAS - Tetrahydrocannabinolic Acid Synthase

UBQ - Ubiquitin

UV – Ultraviolet

UVR8 – UV-B resistance locus 8

β-Me - 2-mercaptoethanol

Contents

Chapter 1

Introduction

1.1 Cannabis

1.1.1 Cannabis biology and history

Cannabis (*Cannabis sativa* L.) is an annual dicot plant originating from Central Asia that has a long history of cultivation (Andre et al., 2016). Cannabis plants are dioecious with separate male and female plants (Small et al., 2003). Historically, cannabis has been used by people for both plant fibre and the medicinal effects of flowers (Russo et al., 2008; Skoglund et al., 2013; Small et al., 2003). This thesis focuses on cannabis rather than hemp type *Cannabis sativa*. In cannabis, the inflorescences of female plants are the main product. These inflorescences are covered in glandular trichomes which contain bioactive cannabinoid metabolites (Spitzer-Rimon et al., 2019).

1.1.2 Lifting of restrictions and a growing global market

Cannabis has a controversial history, being a criminalised substance in many countries (Bahji & Stephenson, 2019). Due to its illegal status, there has been a lack of research conducted on cannabis production. However, recent changes to legislation mean that cannabis can be produced legally for medicinal, or recreational use in a growing number of countries. cannabis can now be legally grown at commercial scale in countries including the USA, Canada, and New Zealand. (Adams et al., 2021; Bahji & Stephenson, 2019). This lifting of restrictions has seen a significant increase in the legal cultivation of cannabis globally. In 2021 the global legal cannabis market was valued at 17.8 billion USD and has a forecasted compound annual growth rate of 25.3% from 2022 to 2030. The increase in the rate of global cannabis legalisation and a growing acceptance for the use of cannabis in medicine are attributed to this strong growth rate (Grand View Research Inc, 2022).

1.1.3 Cannabinoids

Cannabinoids are a broad class of polyphenols produced by cannabis, found in highest concentration in the mature flowers of female plants (Spitzer-Rimon et al., 2019). The benefit cannabinoids confer to the plant is unknown, but they are hypothesised to have a role as photo protectants, protecting plant from incident UV (Ultraviolet) radiation (Magagnini et al., 2018). The most studied cannabinoids are THC (tetrahydrocannabinol) and CBD (cannabidiol), which are the most abundant cannabinoids present in cannabis flower and the key components of medicinal and recreational drugs derived from the plant (Cavalli & Dutra, 2021). There is also a diverse range of minor cannabinoids present in lower concentrations within cannabis. The presence of these cannabinoids is

believed to primarily be the result of non-enzymatic transformation of the major cannabinoids (Hanuš et al., 2016).

1.1.4 Cannabinoid uses

Many cannabinoids are biologically active and have value for both the medicinal and recreational market. THC and CBD are the two cannabinoids present in cannabis in the highest concentration, and the cannabinoids with the largest body of scientific research. The concentration of THC and CBD is a key determining factor in the financial return a grower will receive for a crop (Freeman et al., 2019). In recreational cannabis products, THC is the key cannabinoid linked to psychoactive effects and therefore its concentration determines product value. THC and CBD-based medicines have shown efficacy for use in treating conditions including migraine pain (Volfe et al., 1985), improving appetite (Kirkham, 2005) and reducing the incidence of epilepsy-induced Seizures (Cortesi & Fusar-Poli, 2006). However, cannabinoids are not effective in treating all pain (Kogan & Mechoulam, 2022). Minor cannabinoids also have medicinal value and are thought to be of particular importance for reducing the incidence of seizures in some patients. The combined interaction of major and minor cannabinoids is thought to confer greater benefit to patients than the sum of actions of any individual cannabinoid, termed 'the entourage effect' by medical practitioners (Russo, 2011).

1.1.5 Cannabinoid biosynthesis

In cannabis, cannabinoids are synthesised though the cannabinoid biosynthesis pathway. This pathway is outlined in figure 1.1. The enzymes involved in the biosynthesis of cannabinoids are GOT (geranylpyrophosphate:olivatolate geranyl transferase), *THCAS* (tetrahydrocannabinolic acid synthase), *CBDAS* (cannabidiolic acid synthase), and *CBCAS* (cannabichromenic acid synthase) (Taura et al., 2009).

1.2 Variation in cannabinoid content

With a higher cannabinoid content leading to a higher price paid for a cannabis product (Freeman et al., 2019), it is important for grower cultivators to have an understanding of what factors causes changes in this. There is significant variation in cannabinoid content in cannabis caused by both underlying cultivar genetics and environmental factors (Llewellyn et al., 2021).

1.2.1 Genetic causes of variation in cannabinoid content

The concentration and ratio of cannabinoids in cannabis flower varies significantly between plants. A significant factor causing this difference is genetic differences between cannabis cultivars (Campbell et al., 2019). For example, 'Head Cheese' is a cultivar of cannabis that has a typical cannabinoid content in dry harvested flower of 26% THC and negligeable CBD (n.d., Leafly), whilst another cultivar 'CBD Charlotte's Angel' has a profile of 1% THC and 15% CBD (n.d., Leafly). Hemp is not a distinct species of cannabis but is simply defined as containing 0.3% or less THC, highlighting the impact of genetics

on cannabinoid content (Campbell et al., 2019). This means that some cultivars produce a more valuable crop due to their cannabinoid content profile. Many cannabis cultivars also have no underlying genetic foundation, so plants that are called the same name may be genetically quite distinct and therefore have different cannabinoid profiles (Schwabe & McGlaughlin, 2019). This means that two cannabis plants called the same name may have drastically different cannabinoid profiles.

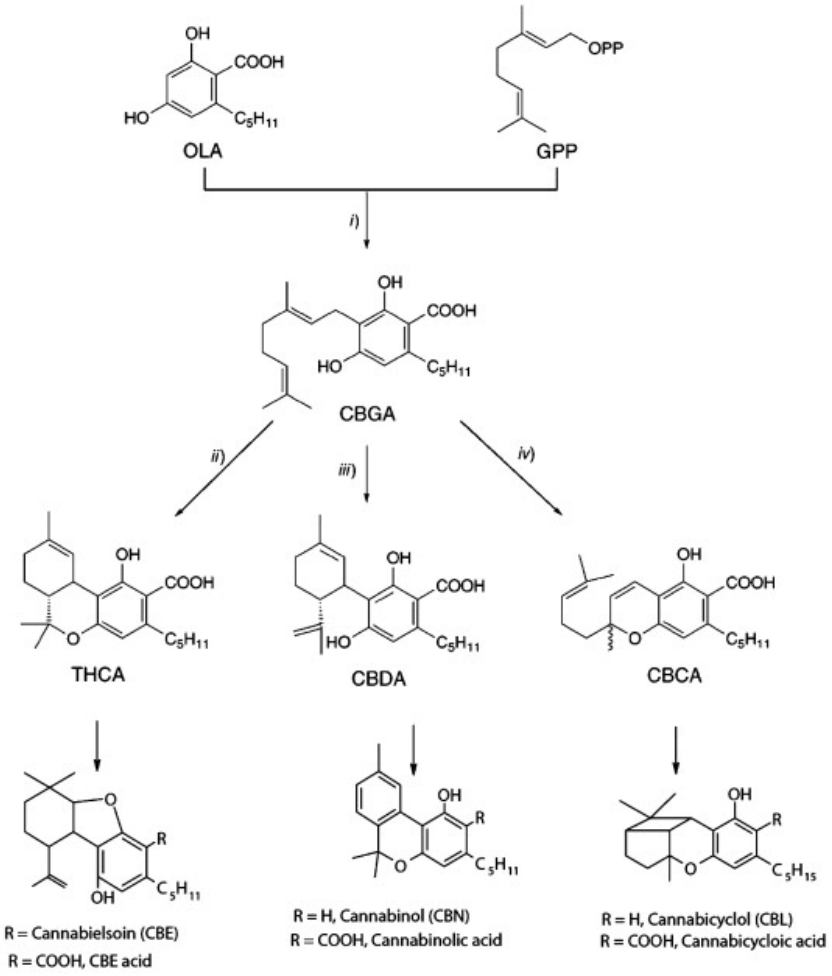

Figure 1.1: Cannabinoid biosynthetic pathway in cannabis. Beginning with olivetolic acid (OLA) and geranyl pyrophosphate (GPP) reaction catalysed through geranylpyrophosphate:olivatolate geranyl transferase (GOT) (i) to form cannabigerolic acid (CBGA). CBGA then further reacts to from one of three cannabinoids, through tetrahydrocannabinolic acid (THCA) synthase (ii) to form THCA, through cannabidiolic acid (CBDA) synthase (iii) to form CBDA or through cannabichromenic acid (CBCA) synthase (iv) to from CBCA. These cannabinoids also react further to produce other variations in cannabinoids and their acid forms, *THCAS* reacts to form cannabielsoin (CBE) or CBE acid, CBDA reacts to form Cannabinol (CBN) or CBN acid and CBCA oxidises to for Cannabicylol (CBL) or CBL acid (Figure taken from Taura et al., 2009).

1.2.2 Environmental causes of variation in cannabinoid content

The concentration of cannabinoids in cannabis flower is not solely caused by underlying genetics, as environmental factors can also significantly impact cannabinoid yield. An investigation into the impacts of natural environmental variation in Colorado on the accumulation of cannabinoids of hemp plants was conducted. The study found that increased irrigation resulted in increased CBD in plants. The study also correlated an increase in CBD with an increase in THC (Campbell et al., 2019). In a study investigating the impact of restricting solar UV radiation on cannabis growth, plants were grown under daylight, shaded daylight, filtered green, blue, and red light, and dark conditions (Mahlberg & Hemphill, 1983). After 33 days gas-liquid chromatography was used to analyse the plants cannabinoid content. The study found that the highest concentration of THC was produced in plants grown under daylight conditions with plants grown under filtered green light and darkness containing significantly lower levels of THC (Mahlberg & Hemphill, 1983). However, the THC content of leaves from plants grown under shaded daylight and filtered red and blue light did not differ significantly from the plants grown in daylight. Plants from all treatments, when subsequently placed under daylight conditions for 66 days, attained levels of cannabinoid synthesis comparable to the daylight controls (Mahlberg & Hemphill, 1983). A second study investigated the impact of supplying additional UV radiation to cannabis plants during growth. The study found that the concentration of THC increased with UV-B dose in both leaf and floral tissues (Lydon et al., 1987). A study into the impact of lighting implements used on cannabis growth found the light environment plays an important role not only in plant size, structure and in the accumulation of cannabinoids. The study found that increasing UV-A/blue radiation could be used to increase the cannabinoid content of plants (Magagnini et al., 2018). A more recent study from Canada treated cannabis plants with supplementary UV radiation and found that a decrease in flower yield and cannabinoid yield was correlated with an increase in the dosage of UV applied (Rodriguez-Morrison et al., 2021).

All the above studies show that by altering the environment there is opportunity to impact cannabinoid concentration and therefore impact cannabis crop value. However, none of the studies show changes to cannabinoid concentration significant enough to warrant the adoption of their environmental changes for improving crop production. However, it has been hypothesised that UV light may be an environmental input that could substantially alter or control cannabis crop performance, which is the focus of this project.

1.3 UV photomorphogenesis

Building an understanding of the impacts of UV on plant growth and development could aid the development of agricultural tools to increase crop productivity. Incident UV radiation causes physical changes in the growth and development of plants (Jenkins, 2009). Plant responses to UV include the

accumulation of flavonoids and anthocyanins (Lois, 1994; Ubi et al., 2006), inhibition of shade avoidance responses such as stem elongation (Sharma et al., 2019) and leaf epidermal cell expansion (Hectors et al., 2010). Several studies have shown that supplementary UV radiation can be used to improve crop performance. UV radiation was shown to cause a 16-fold increase in medicinally valuable flavonoids in Gingko (*Ginkgo biloba*) plants (Zhao et al., 2020). However, this increase in flavonoids was relative to plants grown without any UV present in their growing light spectrum and there was little difference in flavonoid content between Gingko plants grown with increased UV level compared to plants grown with a UV dose equivalent to sunlight (Zhao et al., 2020). This Gingko study shows that UV signalling has a key role in the expression of genes that are required for the synthesis of flavonoids. Studies exposing plants to increased UV radiation have also shown an improvement of performance factors including explanting hardiness (TT & Puthur, 2017), disease resistance and accumulation of flavonoids (McLay et al., 2020). Other studies highlight that not all effects of UV are positive, with a study that screened out solar UV radiation showing improved photosynthetic efficiency and crop yield in wheat (Kataria & Guruprasad, 2015). The impact of UV on plant performance has also been shown to be dose dependant. A study investigating the impact of UV on cannabis growth showed that higher doses of UV lead to commercially unfavourable outcomes, with an increase in plant stress symptoms, decreased yield and decreased cannabinoid content in the cannabis flower produced (Rodriguez-Morrison et al., 2021).

1.3.1 UV light perception in plants

Plants have many photoreceptors which allow them to respond to changes in their light environment. Whilst UV makes up a small proportion of natural incident radiation, the perception of and response to UV is an important aspect of plant biology (Jenkins, 2017; Yin & Ulm, 2017). The UV response pathway is functionally conserved across plants, from green algae to more developmentally complex plant species (Fernández et al., 2016). Plants detect UV with UVR8 (UV-B resistance 8) and CRY (Cryptochrome) photoreceptors. These photoreceptors, via a signalling cascade, translate the perception of UV into changes in plant morphology by changing gene expression (Podolec et al., 2021; Yin & Ulm, 2017). Whilst the initial perception of UV radiation is largely understood, how transcriptional changes are regulated following UV perception requires further investigation (Fernández et al., 2016; Liao et al., 2020; Rai et al., 2020).

1.3.2 UV light perception pathway

UV photomorphogenesis is an important function of plants. In plants, the protein UVR8 detects UV-B radiation and forms a complex with the E3 Ubiquitin ligase Constitutively Photomorphogenic 1 (COP1) in the nucleus to promote the transcription factor Elongated Hypocotyl 5 (*HY5*) to control gene expression. Many genes involved in UV responses, including those in the flavonoid biosynthesis and inhibition of hypocotyl extension pathway, are regulated by *HY5*. However, not all changes in gene

expression resulting from exposure to UV radiation can be explained with this pathway. UV exposure is also shown to improve explanting hardiness, disease resistance, but the genetic mechanisms and pathways behind these responses are unknown.

Photoreceptors are a group of proteins used by plants to assess the quality, quantity, direction, and duration of light. Plants use information about their light environment to control growth and development from germination to flowering (Yin & Ulm, 2017). The UV portion of the light spectrum is responsible for promoting significant changes in the development of plants but is also one of the lesser understood light response pathways (Rai et al., 2020; Zhang & Björn, 2009). UV radiation can be sub-divided into three groups UV-A, UV-B and UV-C with wavelength ranges of 315–400 nm, 280–315 nm and 100-280 nm respectively (Rai et al., 2020). The ozone layer blocks UV-C and most of UV-B radiation, whilst UV-A can pass through freely. UV-A is less potentially harmful than the other classes of UV radiation and is detected in plants by a class of photoreceptors called CRYs. UV-A radiation causes general stress responses in plants and UV-B can damage DNA and slow cellular activity. Plants grown in sunlight will adapt to UV-B and initiate measures such as the accumulation of anthocyanins in upper leaf surfaces to reduce UV-B penetration (Jenkins, 2017). UV-B causes more significant photomorphogenic responses than UV-A and is perceived in plants by the UVR8 protein (Hayes et al., 2017; Rai et al., 2020; Zhang & Björn, 2009). UVR8 is a homodimer held together by the electrostatic interaction of charged tryptohans. UV-B radiation disrupts these interactions causing dissociation of inactive UVR8 dimers into active monomers. UVR8 monomers subsequently bind to COP1 (Constitutively Photomorphogenic 1), an E3 Ubiquitin ligase, to initiate the UV-B signalling pathway. This complex is recruited to the nucleus where it is thought to control the activity of the HY5 (Elongated Hypocotyl 5) and HYH (*HY5* Homolog) transcription factors which regulate a subset of UV-B regulated genes. The mechanism by which UVR8 monomers are translocated into the nucleus remains unknown (Yin & Ulm, 2017). There are two working models for the potential mechanism of UVR8 monomer import to the nucleus (Figure 1.2).

In the Import model, UVR8 monomers bind to COP1 in the cytoplasm and the UVR8–COP1 complex enter the nucleus by COP1 Nuclear Location Signal (NLS)-mediated co-import. In the Retention model, UVR8 monomers enter the nucleus through interaction and co-import with a currently unknown protein possessing a NLS signal, or by diffusion of UVR8 monomers into the nucleus independent of a helper protein. However, the interaction of UVR8 with COP1 is required to prevent the exit of UVR8 from the nucleus (Jenkins, 2017; Rai et al., 2020; Yin & Ulm, 2017).

In both proposed models, UVR8 and COP1 interaction in the nucleus leads to stabilisation of HY5and enhanced binding of HY5 to promoter regions of genes responsive to UV-B. RUP1 and RUP2 (Repressor of UV-B Photomorphogenesis 1 and 2) are proteins that facilitate the reversion of UVR8 active monomers to inactive homodimers, likely in the nucleus and cytoplasm (Yin & Ulm, 2017)

Figure 1.2: Two working models for the nuclear import of active UV-B resistance locus 8 (UVR8) monomers. In the Import model UVR8 monomers bind to Constitutively Photomorphogenic 1 (COP1) in the cytoplasm and the UVR8–COP1 complex enters the nucleus by COP1 Nuclear Location Signal (NLS) mediated co-import. In the Retention model UVR8 monomers enter the nucleus through interaction and co-import with a currently unknown protein possessing an NLS signal, or by diffusion of UVR8 monomers into the nucleus independent of a helper protein. The interaction of UVR8 with COP1 is required to prevent the exit of UVR8 from the nucleus (Figure taken from Yin & Ulm, 2017).

UVR8 has been shown to influence gene expression through the promotion of *HY5* and HYH transcription factors which act to change the expression of genetic pathways. *HY5* is known to target many UV response gene such as those encoding enzymes in the flavonoid biosynthesis pathway. However, this model does not account for all genes shown to be regulated by UVR8. Further aspects of the UVR8 response pathway remain to be elucidated. Additionally, a study investigating UV response in Arabidopsis *uvr8* mutants, plants showed some "general injury" plant responses to UV-B, including the upregulation of genes involved in DNA repair. This demonstrates that a UVR8-independent UV-B response pathway also exists (Jenkins, 2017). There are specific and non-specific UV-B responses with differences in incident UV-B radiation leading to a difference in the genes expressed. An increase in UV wavelength, decrease in fluence rate and shorter exposure duration causes an increase in expression of UV-B-specific photomorphogenic genes, relative to non-specific stress genes (Figure 1.3) (Jenkins, 2017).

Figure 1.3: Schematic of specific and non-specific Ultraviolet-B (UV-B) responses showing that incident UV-B radiation causes an increase in expression of UV-B specific photomorphogenic genes, relative to non-specific stress genes, with an increase in UV wavelength, decreased fluence rate and shorter exposure duration. UV-B resistance 8 (UVR8) is a mediator for UV-B-specific responses. (Figure taken from Jenkins, 2017).

UV-B radiation has diverse effects on plant morphology. The best understood responses include increased UV-B tolerance, induction of flavonoid biosynthesis, hypocotyl growth suppression, inhibition of shade-avoidance responses and leaf epidermal cell expansion (Jenkins, 2017; Liao et al., 2020; Yin & Ulm, 2017). Targeted UV treatments have shown some potential for improving crop performance. In lettuce, UV treatments have been shown to increase flavonoid concentration in leaves leading to improved disease resistance against downy mildew (McLay et al., 2020). In ginkgo, UV light has been shown to increase the accumulation of flavonoids with medicinal value in leaves, increasing crop value (Zhao et al., 2020). Whilst the direct mechanism for the increase in flavonoids in lettuce and gingko was not investigated in these studies, the *HY5* transcription factor has been shown to have gene targets in the flavonoid and anthocyanin biosynthesis pathways in Arabidopsis and tomatoes (Nguyen, 2020; Qiu et al., 2019). The cannabinoid biosynthesis pathway is linked to flavonoid biosynthesis so it is hypothesised that *HY5* may have an impact on Cannabinoid biosynthesis in response to UV. In plants there may also be unknown UV response pathways at play. There are a lack of studies investigating a time series of gene expression in plants in response to UV, particular over periods longer than a few hours. By looking at conducting a time series trial observing gene expression in the UV response pathway over days, this study may help to further elucidate plant UV response mechanisms.

1.3.3 Commercial application of UV light treatments

Preliminary research from BioLumic Limited, has shown that UV light treatments can be used to increase total cannabinoid yield in cannabis. These treatments provide an opportunity for growers-cultivators to significantly increase the value of their crop.

BioLumic is developing UV light treatments to improve crop performance across a range of crop types including soy, wheat, canola and has developed several treatments for cannabis. BioLumic treatments include several UV and non-UV factors, but the mechanism lies within the impact of UV on plants growth; in trials with cannabis, clones were treated with proprietary UV light treatments for 1 week before being placed under conventional light conditions for vegetative growth and flowering. Figure 1.4 shows the impact of targeted UV light treatments on CBD Kush, White Cheese, and Charlotte's Angel strains of cannabis. The impact of the UV treatments is significant, resulting in increases of 32% to 54% in flower yield, 27% for THCA concentration and 25% for CBD concentration (Figure 1.4).

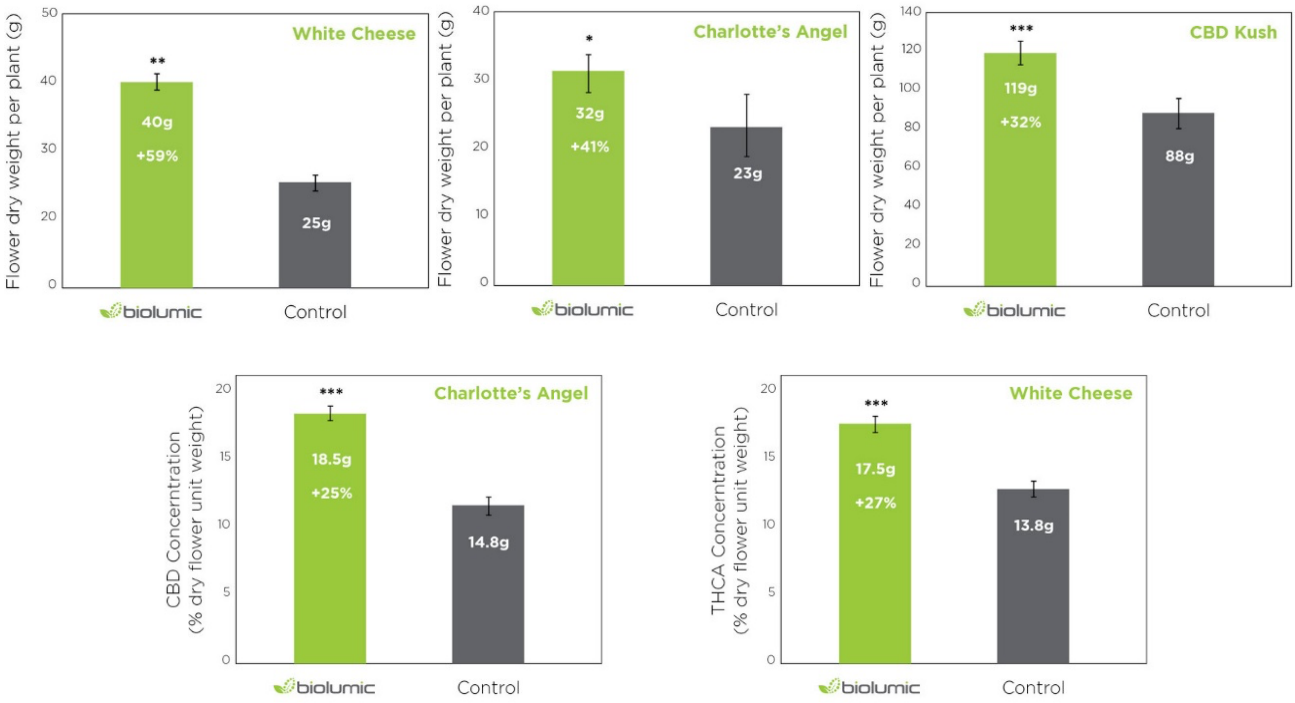

Figure 1.4: BioLumic proprietary ultraviolet (UV) treatments result in an increase in flower dry weight in 'White Cheese', 'Charlotte's Angel' and 'CBD Kush' cultivars of cannabis when compared to control plants. The UV treatments also increase the cannabidiol (CBD) content as a % of flower dry weight in 'Charlotte's Angel' and tetrahydrocannabinolic acid (THCA) content as a % of flower dry weight in 'White Cheese' when compared to control plants. Asterix denotes significant difference at $P<0.05$. (Figure taken from BioLumic Ltd).

Studies in published literature primarily investigate the impact of UV on cannabis growth by exposing plants to higher levels of UV radiation for the total duration of their growth. The UV treatments used by BioLumic to achieve the results in Figure 1.4 differ to UV treatments described in the literature in that they are acclimation type treatments (Wargent et al., 2015). Under BioLumic recipes plants are treated with UV for a short duration, generating changes that result in an increased plant responsiveness to UV over their lifespan. Acclimated plants still require presence of UV in their growing light spectrum to display an acclimation response (Jansen et al., 1998).

Other UV light treatments for commercial cannabis production have also been developed, but these aim to improve crop performance through using UV for crop sanitation to reduce disease pressure, killing surface microbes with UV light. A treatment that exposed cannabis plants to UV-C light daily resulted in a 45.2% decrease in powdery mildew incidence (Scott & Punja, 2021).

Whilst preliminary work from BioLumic in cannabis is promising, it is yet to be published and peer reviewed. Additionally, there is still a lack of understanding behind the molecular basis of the response to UV that results in an increase in cannabinoids. No studies on the impact of UV or other environmental factors have investigated the genetic mechanisms behind changes in cannabinoid concentration. For UV induced increase in yield and cannabinoid accumulation, it is hypothesised that analysing the expression of genes in the UV response and cannabinoid biosynthesis pathways will contribute to a greater understanding of these responses of cannabis to BioLumic UV treatments. If environmental stimuli could be utilised to increase expression of the cannabinoid biosynthesis pathway, this would offer potential to significantly increase crop value.

1.3.4 UV light impacts on cannabis metabolism

The genes encoding the enzyme or enzymes for cannabinoid biosynthesis that are upregulated by exposure to UV-B radiation are unknown. There are limited studies on cannabis production published. However, the polyketide synthase gene that catalyses the synthesis of OLA (olivetolic acid) has strong sequence homology with *CHS* (Chalcone synthase). OLA is a precursor to THC and CBD in the cannabinoid biosynthesis pathway. *CHS* is a key gene in the flavonoid biosynthesis pathway, catalysing the first dedicated step, and is upregulated by the *HY5* transcription factor (Zhang & Björn, 2009). The enzyme that catalyses the synthesis of olivetolic acid may be responsible for increased THC and CBD biosynthesis in response to UV. By conducting gene expression analysis in cannabis under UV light exposure, further insights into total yield and cannabinoid increases can be generated and their association with UVR8 – *HY5* regulated genes and the non-UVR8 response pathway can be better understood. This will allow further optimisation of UV treatments to further increase flower and cannabinoid yield and to identify cannabis cultivars more responsive to UV treatments.

1.4 Research aims

The study aims to build upon the understanding of the response of cannabis to UV light, by quantifying gene expression in cannabis plants under UV treatment. The study aims to build an understanding of the expression of genes in the UV response and the cannabinoid biosynthesis pathway.

Selection of the target genes for this study was made from a list of genes related to the UV-response and cannabinoid biosynthesis pathways which was constructed based on hypothesised involvement in the increase in cannabinoid accumulation in cannabis under UV treatment. The genes selected for expression analysis were cannabidiolic acid synthase (*CBDAS*), tetrahydrocannabinolic acid synthase (*THCAS*), chalcone synthase (*CHS*) and elongated hypocotyl 5 (*HY5*).

1.4.1 Cannabis resources

Due to the history of cannabis an illegal substance in many countries, there is limited scientific research on the production of crop and the impact of environmental stimuli. There are a handful of studies that investigate gene expression in cannabis and some studies that investigate the response of cannabis to UV light. However, there are not studies that investigated the impact of UV on gene expression in cannabis. To aid in the selection of genes for study, there is a partially annotated cannabis genome available (Van Bakel., et al, 2011).

For the study of gene expression patterns in the UV response pathway, there are greater resources available. However, there are still gaps in understanding in this field. There are few studies that look at the expression of genes over a time series in response to UV light, particularly over periods longer than a few hours.

Additionally, cannabis is a crop with significant genetic variation and high heterozygosity. This means that trials of UV treatments on one cultivar will likely not be representative of the impact that the treatments would have on other cultivars. UV treatments may have to be designed to be cultivar-specific. Additionally, cannabis cultivars largely do not have an underlying genetic basis, with significant genetic variation shown in plants given the same name (Schwabe & McGlaughlin, 2019).

1.5 The commercial opportunity for UV treatments

Cannabis production has been legalised in a several regions leading to an increase in the number of growers and the amount of cannabis cultivated globally. With a significant amount of cannabis produced indoors and growing industry competition, there is a clear and present opportunity to use UV

treatments to improve cannabis crop performance. grower cultivators want more out of their crop, produce higher value crops. To increase return on investment and be competitive, cannabis growers want to maximise crop yields and to produce cannabis flowers with high cannabinoid content. UV treatments, such as those developed by BioLumic, offer the potential to increase yields and cannabinoid content of cannabis crops for grower cultivators (BioLumic).

Furthering the understanding of gene expression patterns in the UV response and cannabinoid biosynthesis pathways and how expression correlates with changes in flower yield and cannabinoid content at the point of harvest in cannabis would provide the opportunity for gene expression patterns to be used as markers in the screening of future UV treatments during treatment development. At present the screening of new potential treatments takes 9 weeks to determine the impact on the treatment of final yield and cannabinoid profile. cannabis plants must be grown to harvest maturity to determine the efficacy of treatments in improving yield and cannabinoid content. Building a greater understanding of the corelation of gene expression patterns, in the UV response and cannabinoid biosynthesis pathways, at the point of time under treatment and how these gene expression patterns are linked to final yield and cannabinoid content, would allow for these patterns could be used to screen new UV treatments much faster, with cannabis plants no longer needing to be grown to yield for treatment screening. Building an understanding of these expression patterns means that fewer UV treatments would need to be taken to yield to determine their impact on crop performance. This will increase the speed of screening and development of commercially beneficial UV treatments that increase yield and cannabinoid content. Results from building an understanding of the UV response and cannabinoid biosynthesis pathway in cannabis could also contribute to breeding efforts in the crop, providing potential targets for marker-assisted breeding.

Chapter 2

Materials and methods

The experimental strategy of this project was to build a *de novo*, time course-based understanding of gene expression response to commercial UV light treatments, in a selection of cannabis genotypes. Briefly, in three trials, cannabis clones were grown until roots had visibly developed, before being subjected to either, one of three proprietary commercial UV light treatments, or a control treatment. Cannabis plants were removed in timeseries from treatment before being destructively harvested, with RNA extracted from the shoot tissue. This RNA was used in Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) to quantify the expression of a group of genes, that are be hypothesised to be involved in the UV-cannabis response. Additionally, a leaf clip sensor was used to non-destructively measure relative flavonoid content of the leaves of each of the UV-treated and control plants immediately prior to destructive RNA harvest. All trialling and laboratory work was carried out at BioLumic Limited's Ministry of Health licenced cannabis R&D facility in Palmerston North (MoH Medicinal cannabis Licence no: 113672).

2.1 Cannabis cloning and plant husbandry

Cuttings were taken from cannabis mother plants, from a collection of cannabis genetics held by BioLumic. Mothers are cannabis plants that have been grown for an extended period in a vegetative growth habit until they have sufficient plant tissue available to make cuttings to be used in clonal propagation. For trials, the cannabis cultivar 'Head Cheese' (HC) was used. Clones were taken from two mothers denoted HC1 and HC2.

For Trial 1 and 2, cuttings were taken to be 15cm in length and placed in a jug of water. Cuttings were then removed from the water, and the top third of leaves removed by cutting with a pair of secateurs. Stem cutting bases were then cut at a 45-degree angle using a scalpel and the cut end dipped into rooting hormone [Yates Clonex Rooting Hormone Gel, NZ] deep enough to cover 12-15 mm of the stem base for a duration of 15 seconds. Cuttings treated with hormone were plugged into pre-soaked rockwool cubes deep enough to cover the dipped section of the stem. Rockwool cubes were soaked in a nutrient solution [Goliath Veg Accelerator, NZ] with a pH of 5.8 and EC of 0.5 for 30 minutes.

For Trial 3, clones were taken following the above methodology with the following differences: Cuttings taken for clones had at least 3 fully expanded leaves. Cuttings were cut to a length of 15 cm but were taken so that there was 1 cm-1.5 cm stem below the bottom node, with the axillary buds removed from the node. The leaves on cuttings for Trial 3 were not cut to reduce leaf area.

Once a tray was filled with plugged cuttings, this was covered with a humidity dome and vents closed. Clones were watered daily by adding water or pH 5.8 and EC 0.5 feed solution [Goliath Veg Accelerator, NZ] to the bottom of the clone trays. The foliage of clones was sprayed 1-2 times daily with clone spray [FloraMax Clone Spray, AUS] for the first 8 days. Clones were kept under an 18 hr light (5.45 am-11.45 pm), 6 hr dark photoperiod receiving an average photosynthetically active radiation (PAR) of 100 µmol/m^2/s at the canopy top supplied by ELIXIA lights [Heliospectra, U.S]. Domed clone trays were kept in a room style growth chamber with a room temperature of 22-24 °C and humidity of 65-70%. The humidity and feeding regime of the clones was managed as shown in Table 2.1 during the 14 days after plugging.

Table 2.1: Humidity as % relative humidity (RH) and feeding regime for care of cannabis clones. Room temperature maintained at 22-24 °C. A dash in the Feed column indicates no feed, just base irrigation. EC denotes electrical conductivity. ”Removed” in the Dome Use column indicates that domes were removed.

Day	Feed	Humidity (%RH)	Dome Use
0-4	-	100	Vents closed
5	-	85-90	Vents ½ open
6	-	85-90	Vents open
7-9	-	80	Removed
10	EC 0.8, pH 5.8	80	Removed
10-14	-	75	Removed

2.2 Treatment application

2.2.1 Light treatments

Light treatments were applied to cannabis clones approximately 14 days after clonal propagation took place. The readiness of clones for light treatment was phenotypically assessed as by when roots were visibly growing out from the sides of the rockwool cubes. Light treatments were applied using LED-based, software-controlled light treatment systems developed by BioLumic Ltd, which were in the form of plant ‘shelves’, with LED units situated above each shelf, where plants were placed for treatment. Trial clones received either a Control treatment, where LED units only delivered PAR, or one of three treatments with the same PAR as per control, and supplementary UV dosages, which are named UV1, UV2 and UV3. The light regimen details of control, UV1, UV2, and UV3 conditions are protected trade secrets, and property of BioLumic Ltd, therefore cannot be disclosed in detail here. However, broadly indicative published information on BioLumic light regimens can be accessed here: https://patents.google.com/patent/US10517225B2. An ILT950 Spectroradiometer

[SpectriLight, USA] was used to calibrate and measure the lighting treatments. The BioLumic lighting system was situated in a whole-room growth chamber, with the UV treatments provided as their only source of light for 7 days; with plants screened off from any other light, with air/humidity exchange promoted with the rest of the room. The growth chamber was kept at 65% RH with a daytime temp of 24 °C and overnight temperature of 22 °C. A sample of plants from each treatment group were removed for destructive harvest at 10:30am as a timeseries over the treatment. Day 0 samples were also taken at approximately 8am and retained under non-treatment lighting before destructive harvest at 10:30 am. For each trial, the number of clones, mother identify, treatment types and sampling regime is described in Table 2.2. Due to an error in sample collection, samples of cannabis plants from two mothers were taken in Trial 2. It was intended only for plants from one cannabis mother to be included in the sampling. This error led to two biological replicates for each treatment and time point from two mothers instead of four biological replicates for each treatment and time point from one mother.

Table 2.2: Summary of the mother identity, number of clones included, treatment groups included and days of sampling for each of the trials in the study. Plants used are of the cultivar Head Cheese (HC). Four clones from each mother were placed into each treatment in Trial 2, with each biological replicate taken consisting of two plants from each mother. BioLumic Ltd staff aided in the production of cannabis clones for Trial 1 and provided cannabis clones for Trials 2 and 3. In the treatment group columns, a dash indicates no plants were placed into that treatment group.

Trial no.	Mothers used	Plants in treatment group				Biological replicates	Sampling days
		Control	UV1	UV2	UV3		
1	HC2	18	15	15	-	3	0 (Control only), 1,2,3,5,7
2	HC1, HC2	8	8	8	8	4	1,3
3	HC2	12	12	12	12	3	1,3,5,7

2.2.2 Flavonoid measurement

After the removal of cannabis plants from treatment, a Dualex leaf clip meter [ForceA, FR] was used to measure the relative flavonoid content of plant leaves in a similar manner to that which has been used previously (Wargent et al., 2015). Two leaves were measured per clone, with only the upper unshaded leaves measured. Unshaded leaves are those that did not have higher leaves shading them from light during treatment. Clones were destructively harvested immediately after flavonoid measurement.

2.2.3 Destructive harvest

Cannabis clones were cut with sterilised scissors removing only the unshaded leaves and connecting stem. After cutting, these top sections of each clone were immediately placed into individual labelled 15 mL or 50 mL falcon tubes and placed into liquid nitrogen to be rapidly cooled. Tubes were then

placed into a labelled box in a -80 °C freezer for storage. For each sampling day, clones from all treatment groups were removed from the treatment rack at the same time. Clones were measured with a leaf clip meter and destructively harvested in an order alternating by treatment group to reduce any difference in gene expression that may be caused by longer exposure to the fluorescent lighting in the processing room.

2.3 Quantifying gene expression

2.3.1 Gene selection

To select genes for analysis, a list of genes related to the UV-response and cannabinoid biosynthesis pathways was constructed based on hypothesised involvement in the pathway responsible for an increase in cannabinoid accumulation in cannabis under UV treatment. RNA-seq data previously produced by BioLumic was used to support the selection of four genes from this list for analysis. The RNA-seq data, generated from whole shoot tissue samples taken on day 7 of treatment, showed the difference in gene expression in cannabis plants treated with a proprietary UV recipe and plants treated with a control treatment, containing only PAR. Four genes were selected for analysis, on the basis of hypothesised roles in UV-cannabis plant response. The genes selected for analysis were cannabidiolic acid synthase (*CBDAS*), tetrahydrocannabinolic acid synthase (*THCAS*), chalcone synthase (*CHS*) and elongated hypocotyl 5 (*HY5*). ubiquitin (*UBQ*) was used as a house-keeping gene, showing a baseline of gene expression, to compare the expression levels of other genes. *UBQ* was selected as baseline for gene expression based on RNA-seq analysis conducted by BioLumic on leaf tissue from cannabis plants that were treated with UV.

2.3.2 Tissue grinding

Clone whole shoot tissue samples were removed from the freezer and kept on ice or liquid nitrogen until ready for processing. Individual samples were placed into a sterilised mortar and pestle. Liquid nitrogen was added to the mortar and samples ground to a fine powder without any distinguishable lumps. Liquid nitrogen was added as needed to prevent samples from thawing. 50-70 mg of each sample was scooped with a cooled small metal spoon into a cooled, 1.5 mL microcentrifuge tube with the remaining tissue scooped into a cooled 15 mL falcon tube. Both tubes were kept in liquid nitrogen before being stored in the -80 °C freezer.

2.3.3 RNA extraction

The E.Z.N.A.® Plant RNA Kit [Omega Bio-Tek, USA] was used for RNA extraction. The 50-70 mg ground plant tissue samples were removed from the -80 °C freezer and kept on an ice rack or in liquid nitrogen until ready for use. 500 µL resuspension buffer (RB) buffer was prepared for each of the tissue samples, with 20 µl 2-mercaptoethanol (β-Me) [Sigma-Aldrich, China] added per 1 mL of

RB buffer [Omega Bio-Tek]. For Trial 3 samples, 15 mg/mL of Polyvinylpyrrolidone (PVP) [Sigma-Aldrich, China] was added to the RB buffer master mix, shaken until dissolved and left until bubble dissipated. This addition aided significantly in improving the quality of RNA extracted. RNA wash buffer II [Omega Bio-Tek] was prepared with ethanol added according to directions on the bottle.

500 µl of RB buffer was added to each of the samples immediately after their removal from the cooling rack. After the addition of the buffer each of the samples were vortexed to homogenise. Once vortexed samples were centrifuged for 3 minutes at maximum speed (15,000 x g). For each sample, a Homogeniser Mini Column [Omega Bio-Tek, USA] was inserted into a 2 mL collection tube. Cleared lysate from each centrifuged sample was a transferred onto the Homogeniser Mini Column without disturbing the pellet. The lysate was then centrifuged for 1 minute at maximum speed. The Homogeniser Mini Columns were discarded and 500 µL 70% ethanol was added to the flow through of each of the samples and mixed with a pipette. For each of the samples a HiBind® RNA Mini Column [Omega Bio-Tek, USA] was inserted into a fresh 2 mL collection tube. 700 µl of each sample and ethanol mix was transferred onto the HiBind® columns and centrifuged at maximum speed for 1 minute. The filtrate was discarded, and collection tube reused. This centrifuging of the samples and discarding of lysate was repeated until all the sample has been transferred to the HiBind® column.

A DNase digestion was carried out to remove DNA from the samples. A DNase digestion mix was prepared consisting of 89 µL nuclease free water, 10 µl buffer [New England BioLabs, USA], and 1 µL DNase enzyme per sample [New England BioLabs, USA]. 100 µL of the DNase digestion mixture was added directly onto the surface of the membrane of the HiBind® columns for each of the samples. Samples were incubated at 37 °C and covered with aluminium foil for 10 minutes. After incubation, 500 µL of RNA Wash Buffer I [Omega Bio-Tek, USA] was added to each of the tubes. Tubes were then centrifuged at maximum speed for 30 seconds with the filtrate discarded and the collection tube reused. 700 µL of RNA wash Buffer II [Omega Bio-Tek, USA] was added to each of the samples before they were centrifuged at maximum speed for 30 seconds. The filtrate is discarded, and the collection tube is reused. This wash step with 700 µl of RNA wash buffer II a centrifuge at maximum speed for 30 seconds, with the filtrate discarded and collection tube reused was repeated twice more for a total of 3 washes with RNA wash buffer II. After the wash is completed, the empty HiBind® tubes, sitting in empty collection tubes, are centrifuged at maximum speed for 3 minutes. The HiBind columns are then transferred into clean 1.5 mL microcentrifuge tubes. 70 µL of nuclease free water was added directly onto the membrane of each of the HiBind® columns and allowed to sit for 3 minutes, after which the tubes were centrifuged at maximum speed for 30 seconds. The flowthrough of is then added directly back to the membrane of each of the HiBind® columns and allowed to sit for 3 minutes before the tubes are again spun in the centrifuge at maximum speed for 30 seconds. The 70 µL of eluted RNA solution was aliquoted, labelled and stored at -80 °C.

2.3.4 RNA quality assessment

RNA Quality was assessed through agar gel electrophoresis. Tris-Borate-EDTA (TBE) buffer, was prepared from 5x TBE Concentrate [Sigma-Aldrich, USA] and deionised water. An agar gel was prepared with 60 mL 0.5 x TBE buffer, 0.9 g of Agarose powder [Sigma-Aldrich, USA] and 3 µL RedSafe™ Nucleic Acid Staining Solution (20,000x) [iNtRON Biotechnology, KR]. TBE buffer and agar were mixed and heated in a microwave to dissolve. RedSafe™ was added after removal from the microwave after the mixture had cooled. Once the mixture had cooled to handling temperature it was poured into a gel mould and allowed to set. Once set the gel was placed in an electrophoresis box and covered with 0.5 x TBE buffer. 5 µL of 100 bp ladder and purple loading dye mix [New England BioLabs, USA] was loaded into the first and last loading wells of the gel. For RNA samples, 1 µl of purple loading dye [New England BioLabs, USA] was pipetted onto a piece of tape on the lab bench. 3 µL of RNA sample was pipetted into this drop of dye and mixed. 3 µL of the resulting mix was pipetted into an empty loading well in the gel. This was repeated for all samples. The gel was run at a constant voltage for 25 minutes. The device was set to 120 V, 700 mA and 150 Watts. Once run, the gel was removed from the box and imaged to assess RNA quality.

2.3.5 RT-qPCR

The Luna® Universal One-Step RT-qPCR Kit [New England BioLabs, USA] was used to carry out RT-qPCR analysis. For each 20 µL reaction a master mix was made of 10 µL of Luna One-Step Reaction mix, 1 µL Luna WarmStart® RT Enzyme Mix, 0.8 µl 10 µM forward primer, 0.8 µl 10 µM reverse primer [Macrogen, KR] and 5.4 µl of nuclease-free water. Table 2.3 shows a list of the primer pairs used in RT-qPCR. Master mix was made in bulk for each primer pair with an extra allowance of 10% made. 18 µL of master mix was added to each reaction tube with 2 µL of sample RNA. Reaction tubes were placed in a thermocycler [MyGoPro, USA] with the reaction profile set to 55 °C for 10minutes, then 95 °C for 60 seconds, then 45 cycles of 60 °C (30second hold) to 90 °C (10second hold), and a final melt from 60 °C to 97 °C at 0.1 °C / second. MyGoPro version 3.5 software was used to run the PCRs. RNA from each individual biological sample was run in technical triplicates. For each of the primer pairs a non-template control reaction was run with 2 µL of nuclease-free water added instead of 2 µL of sample RNA. If any non-template control reaction tube returned a C_q value withing 5 cycles of any positive sample well, the reaction repeated after eliminating the cause of contamination.

Table 2.3: Forward and reverse primer pair sequences used for the amplification of target Ribonucleic acid (RNA) sequences during Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). RNA was extracted from the leaves of cannabis (*C. sativa*) cultivar Head Cheese plants following UV treatment. The genes of interest were cannabidiolic acid synthase (*CBDAS*), tetrahydrocannabinolic acid synthase (*THCAS*), chalcone synthase (*CHS*) and elongated hypocotyl 5 (*HY5*). Ubiquitin (*UBQ*) was used to establish a baseline of gene expression.

Gene	Primer Sequences
CBDAS	Forward 5'- AAGCACGTATTTGGGGTGAG -3'
	Reverse 5'- GTGGGATGCTTTGTTCGTTT -3'
CHS	Forward 5'- GCCCATTTTTGAATTGGTCT -3'
	Reverse 5'- CCCAGGAACATCTTTAAGCAA -3'
HY5	Forward 5'- ATACAACGGCAAGTCGGAGA -3'
	Reverse 5'- CCTCTTAGAGAAACTAACCATCTCG -3'
THCAS	Forward 5'- TCCAAGATTGGCGTATCTCA -3'
	Reverse 5'- TTTCACCCCAAATACGTGCT -3'
UBQ	Forward 5'- TACTGCGCCAGCTAACAAACC -3'
	Reverse 5'- CACCCGTCTGACCTGAATC -3'

2.3.6 Primer efficiency testing

Cannabis RNA known to be of good quality, provided by BioLumic Ltd, was used to test the efficiency of the 5 primer pairs to be used in RT-qPCR for gene expression analysis: a forward and reverse primer for each of the genes of interest, *CBDAS*, *THCAS*, *CHS* and *HY5*, and a primer pair for the house keeping gene to be used as a baseline for gene expression, UBQ. Serial dilutions of the RNA were carried out for each of the primer pairs to create a dilution series. RNA was diluted 5-fold 7 times to give a final solution with 6.4 x 10^{-5} RNA content of the initial RNA concentration. RT-qPCR was carried out for each of the primer pairs with the dilution series of RNA. For each of the primer pairs tested, a slope was calculated for the plot of C_q values against the dilution series. All primer pairs tested had a slope with approximate values of 2 meaning they were appropriate for use in the gene expression analysis.

2.4 Data analysis

Data was exported from MyGoPro version 3.5 and analysed in Microsoft Excel 365 version 2205. To analyse data, C_q values from the PCR were transformed into a $\log_2$ of fold change in expression of genes against control.

Statistical tests were run using Microsoft Excel 365 version 2205. To determine significance between means of normally distributed data, statistical tests including ANOVA (Analysis of Variance) and t-tests were run.

Chapter 3

Results

Three trials were completed to investigate the impact of three different UV light treatments on cannabis against each other and control treatment. Cannabis plants were removed in timeseries from treatment before RNA was extracted and used in RT-qPCR to quantify the expression of a group of genes, that are hypothesised to be involved in the UV-cannabis plant response. Flavonoid and chlorophyll content of the leaves of each of the plants was measured after their removal from treatment. There were significant differences in cannabis clone plant health across the three trials which was a clear underpinning factor in several project results.

3.1 Cannabis clones displaying a stress phenotype have an impacted UV response

Cannabis clones were prepared for each of the growth trials by taking cuttings from mother plants and rooting them in rock wool cubes. After sufficient rooting, approximately two weeks after cuttings were taken, clones were placed into UV treatment. In Trials 1 and 2, cannabis clones displayed significant stress phenotypes including leaf yellowing and browning, leaf tip curling and leaf necrosis (Figure 3.1). Cannabis clones from Trial 3 displayed stress responses in much fewer plants, with less frequent leaf tip curling and less pronounced yellowing than in clones from Trials 1 and 2 (Figure 3.1). Cannabis clones in Trial 3 were also a more vibrant green and had significantly higher chlorophyl content in upper leaves than plants in Trials 1 and 2 (Figure 3.2). Poor nutrient and irrigation management leading to nutrient and water stress may have been the cause of these symptoms. Leaves displaying significant yellowing or browning were excluded from destructive harvest samples that were RNA-extracted for gene expression analysis.

3.1.1 Initial flavonoid accumulation is higher in cannabis clones displaying a stress phenotype

After the removal of cannabis clones from UV treatment, a Dualex leaf clip meter was used to measure the flavonoid content of leaves. Two leaves were measured per clone. To compare flavonoid accumulation between trials, the measured values were averaged across treatments for each trial (Figure 3.3). Trial 3 plants had a lower initial flavonoid accumulation and show a clear increase in flavonoid content over treatment. Plants from Trials 1 and 2, where a clearly observable stress phenotype was present, had a higher initial flavonoid accumulation and no clear trend in flavonoid accumulation over treatment. On day 1 of treatment, plants from all trials had significantly different levels of flavonoid accumulation, Trial 1 plants had the greatest accumulation and Trial 3 plants had the least (Figure 3.3;

P<0.05). From day 3 onwards, there was no significant difference in flavonoid content between plants from different trials.

Figure 3.1: Stress phenotypes in cannabis (*C. sativa*) cultivar Head Cheese clones from UV treatment Trials 1-3. More pronounced leaf yellowing and browning on cannabis clones from Trials 1 & 2. Photos taken after plant removal from treatment. Labels 1-3 denote the respective trials that pictured clones are from.

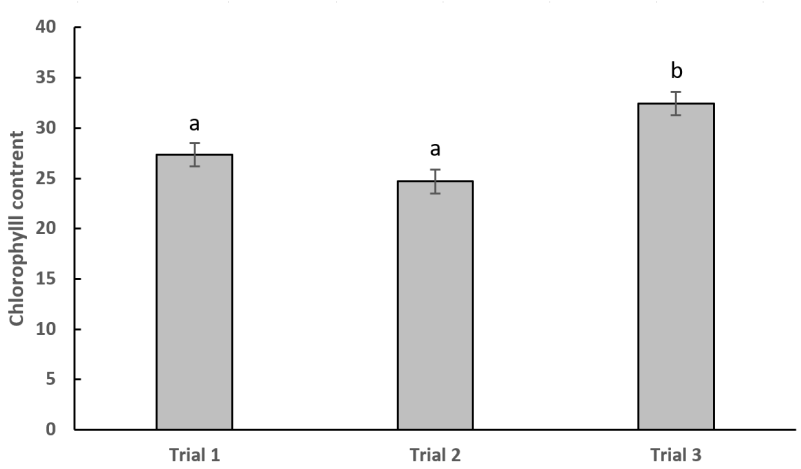

Figure 3.2: Chlorophyll accumulation in leaves of cannabis (*C. sativa*) cultivar Head Cheese plants following UV treatment. Chlorophyll accumulation averaged across all plants in each trial irrespective of treatment group. Cannabis (C. sativa) cv. Head Cheese plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested for RNA extraction throughout treatment, and 1 day after treatment

(day 7). Chlorophyll content of leaves was measured after plant removal from treatment. Significant differences in chlorophyll content in plants between trials is denoted by letter groups where different letters indicate a significant difference at P<0.05 according to t-tests. Error bars indicate 1 S.E.

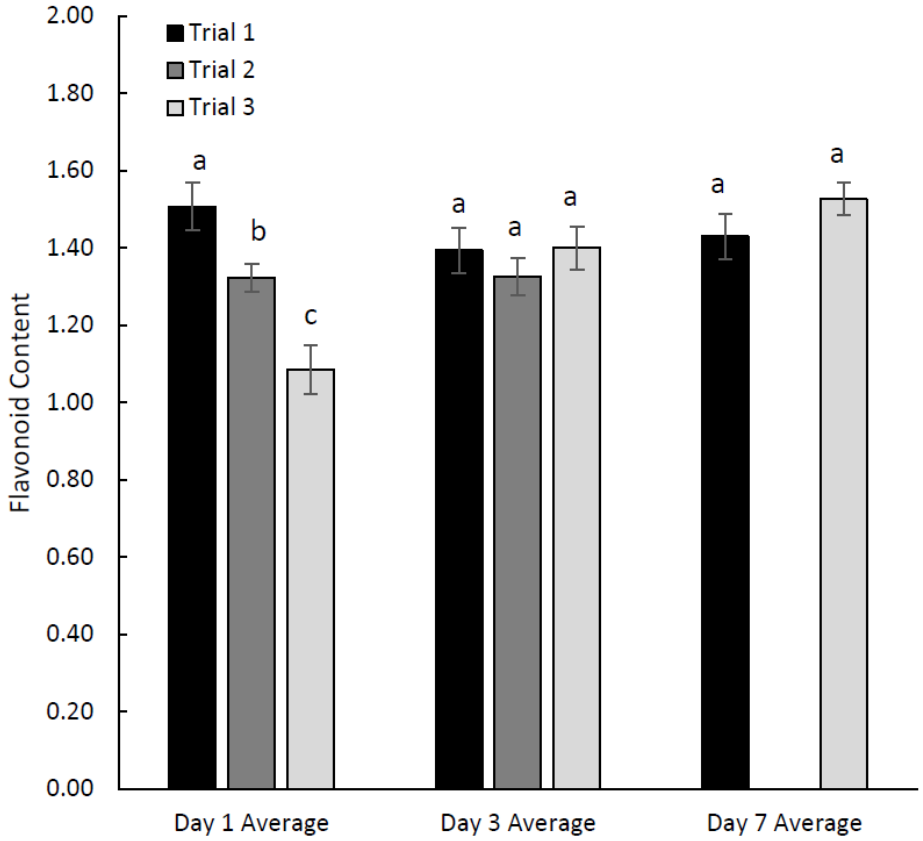

Figure 3.3: Flavonoid accumulation in leaves of cannabis (*C. sativa*) cultivar Head Cheese plants following UV treatment. Flavonoid accumulation averaged across all treatments in cannabis (*C. sativa*) cv. Head Cheese. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested for RNA extraction throughout treatment, and 1 day after treatment (day 7). Flavonoid content of leaves was measured after plant removal from treatment. Significant differences in flavonoid content between trials for the same day is denoted by letter groups where different letters indicate a significant difference at P<0.05 according to t-tests. Error bars indicate 1 S.E.

3.2 Impact of UV on flavonoid accumulation in cannabis clones was inconsistent across trials

3.2.1 Cannabis plants displaying a stress phenotype showed no increase in UV-induced flavonoid accumulation: Trial 1 and Trial 2

In Trial 1, leaf clip measurements taken from clones after their removal from treatment for destructive harvest, showed plants from the control treatment had higher flavonoid content on day 1 of treatment and plants from UV1 and UV2 treatment groups had higher flavonoid content by day 5 and 7 (Figure 3.4). The flavonoid content in day 5 and day 7 control plants was significantly lower than day

1 control plants (Figure 3.4; day 1 vs day 5 P = 0.0079, day 1 vs day 7 P = 0.0184). UV1 and UV2 did not have a significant difference in flavonoid accumulation over treatment. There was no significant difference in flavonoid content between treatments on the same day of leaf tissue harvest.

In Trial 2, leaf clip measurements show plants from UV3 treatment had lower flavonoid content on day 1 than the Control, UV1 and UV2 treatment groups. On day 3 the plants from the control treatment group had higher flavonoid content than UV1, UV2 and UV3 treatment groups. On day 3, the flavonoid content of UV1 treated plants was significantly lower than day 1 UV1 treated plants (Figure 3.5; P = 0.0201). Plants from Control, UV2 and UV3 treatments do not have a significant difference in flavonoid content between days 1 and 3. On day 1, the flavonoid content of plant from UV3 treatment was significantly lower than plants from all other treatment groups (Figure 3.5; P = 0.0012). There was no other significant difference in flavonoid content between treatment groups on the same day of harvest.

3.2.2 Plants that did not display a stress phenotype did show an increase in UV-induced flavonoid accumulation: Trial 3

In Trial 3, plants from all treatment groups showed an increase in flavonoid content from day 1 to day 7, with the most notable jump from 1 to day 3 (Figure 3.6). On day 1, plants from UV2 and UV3 treatment groups had lower flavonoid content than plants from the control and UV1 treatment groups. On Day 3 plants from UV2 and UV3 treatment groups have a lower flavonoid content than plant from the control or UV3 treatment groups. The increase in flavonoid content from day 1 to day 7 was significant for all treatment groups expect for the control (Figure 3.6; P value range: 0.0033 to 0.0125). There was no significant change in flavonoid content over treatment for plants in the control group. There was no significant difference in flavonoid content between treatments on the same day of harvest.

3.2.3 Summary of flavonoid fold changes by trial and treatment

For each trial, the fold change in flavonoid content from the first to last day of harvest was calculated. Across Trials 1 and 2, there was a negligible or negative fold change in the accumulation of flavonoids between the first and last days of clone harvest (Table 3.1). In Trial 3 there was a positive fold change in the accumulation of flavonoids for all treatment groups with the greatest increase seen in plants that received UV treatment.

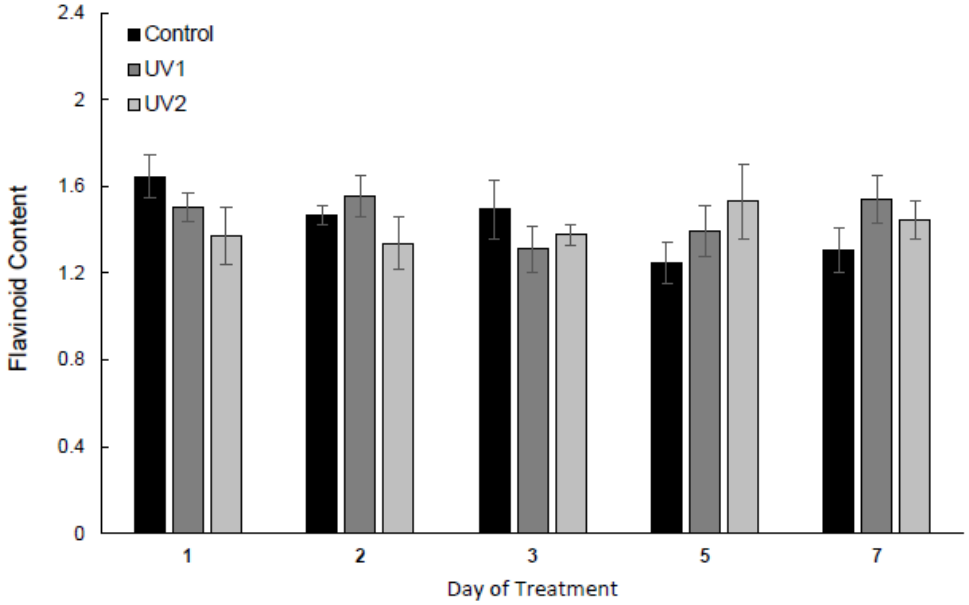

Figure 3.4: Flavonoid accumulation in leaves of cannabis (*C. sativa*) cultivar Head Cheese plants following UV treatment.in Trial 1. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Flavonoid content of leaves was measured after plant removal from treatment. There were no significant differences in flavonoid content between treatments for the same day at $P<0.05$ according to ANOVA tests. Error bars indicate 1 S.E.

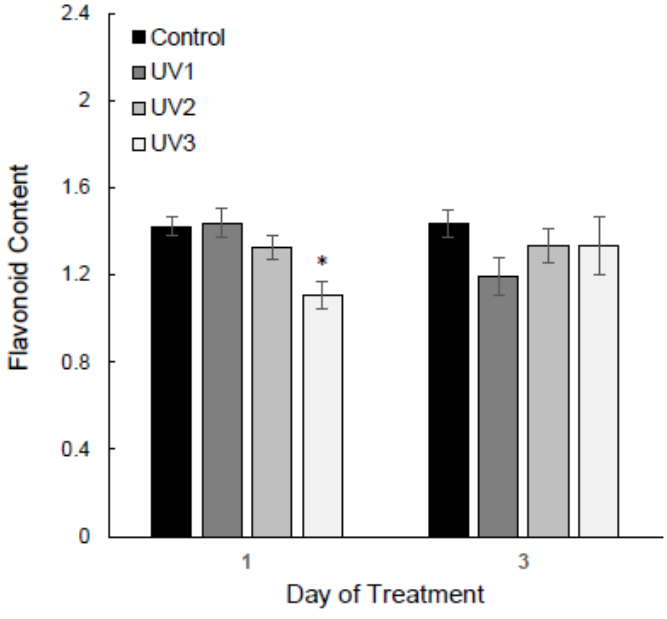

Figure 3.5: Flavonoid accumulation in leaves of cannabis (*C. sativa*) cultivar Head Cheese plants following UV treatment.in Trial 2. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested on day 1 and 3 of treatment. Flavonoid content of leaves was measured after plant removal from treatment. Significant differences in flavonoid content between treatment groups is denoted with an asterisk where *=$P<0.05$ according to t-tests. Error bars indicate 1 S.E.

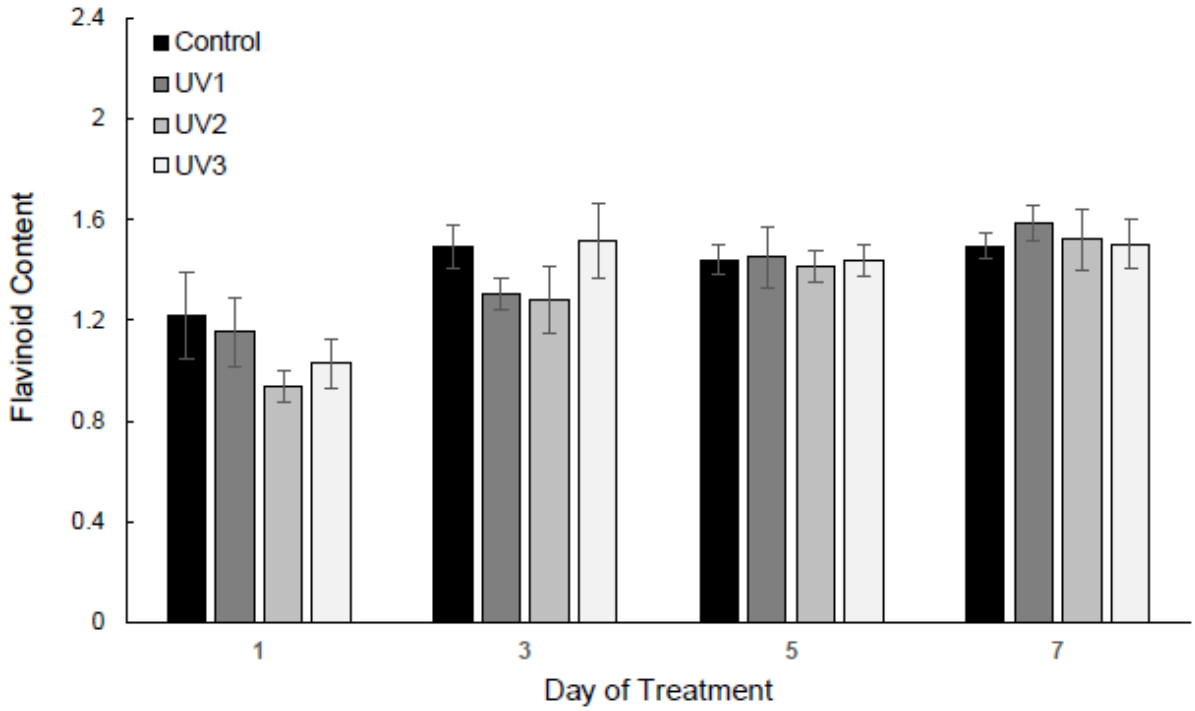

Figure 3.6: Flavonoid accumulation in leaves of cannabis (*C. sativa*) cultivar Head Cheese plants following UV treatment.in Trial 3. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Flavonoid content of leaves was measured after plant removal from treatment. There were no significant differences in flavonoid content between treatments for the same day at $P<0.05$ according to ANOVA tests. Error bars indicate 1 S.E.

Table 3.1: Fold change of flavonoid content in in leaves of cannabis (*C. sativa*) cultivar Head Cheese plants following UV treatment.. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Flavonoid content of leaves was measured after plant removal from treatment. For Trial 2, plants were only harvested on days 1 and 3 of treatment, so the fold change in flavonoids presented is between these two days.

Trial No.	**Control**	**UV1**	**UV2**	**UV3**
Trial 1 day 1 - 7	-0.21	0.02	0.05	-
Trial 2 day 1 - 3	0.01	-0.17	0.01	0.21
Trial 3 day 1 - 7	0.23	0.38	0.62	0.46

3.3 Impact of UV treatment on gene expression in cannabis

Clones were removed from UV treatment in a timeseries and destructively harvested. The top, unshaded leaves of clones were removed and frozen before being ground and RNA extracted. This RNA was used in RT-qPCR with primer pairs to quantify the difference in expression of four genes of interest: *CBDAS*, *THCAS*, *CHS*, and *HY5*, in UV-treated and untreated cannabis plants. A summary of the cannabis mothers and sampling timepoints for each of the growth trials is presented in Table 3.2. The delta-delta Ct calculation (Livak & Schittgen, 2001) was used to quantify differences in gene expression in UV-treated and untreated plants. *UBQ* expression was quantified for all plants harvested in UV treatment trials and used as the housekeeping gene for delta-delta Ct calculations.

Table 3.2: Summary of cannabis mothers, UV treatment groups and sampling timepoints in each of the growth trials in which cannabis clones were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment and 1 day after treatment (day 7). Flavonoid and chlorophyll of sample leaves was measured before the Ribonucleic acid (RNA)was extracted from plants samples and used in Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) to quantify the relative expression of candidate genes.

Trial No.	Mothers Used	UV treatments Included	Sampling days
1	HC2	UV1, UV2	1,2,3,5,7
2	HC1, HC2	UV1, UV2, UV3	1,3
3	HC2	UV1, UV2, UV3	1,3,5,7

3.3.1 *CBDAS* expression was largely unresponsive to UV treatment

There was a up and down cycling in expression of *CBDAS* in treated plants compared to the control evident in Trials 1 and 3 (Figure 3.7) This cycling pattern was not evident in Trial 2 due to plant samples only being taken at two timepoints. In Trial 1, *CBDAS* expression was lower on average in treated plants compared to the control over the treatment. In Trial 3, there was a cycling of *CBDAS* expression with average expression over the whole treatment similar in treated and control plants.

In terms of specific treatments over time, *CBDAS* expression in UV3 day 1, Trial 3, was significantly increased compared to control plants (Figure 3.7; Log_2 fold = 0.72; $P < 0.05$). There were no other significant differences in *CBDAS* expression in any UV treatment against control. Within each of the trials, *CBDAS* expression had a consistent pattern across UV treatment groups. There was no significant difference in expression between UV treatments according to ANOVA and t-tests (Figure 3.7, $P < 0.05$).

3.3.2 *THCAS* expression is largely unresponsive to UV treatment

There was a up and down cycling in expression of *THCAS* in treated plants compared to the control evident in Trials 1 and 3 (Figure 3.8). This cycling pattern was not seen in Trial 2 due to plant samples only being taken at two timepoints. In Trial 1 average *THCAS* expression in treated plants and control plants over treatment was similar. In Trial 3 *THCAS* expression was lower on average in treated plants compared to control over the treatment.

In terms of specific treatments over time, *THCAS* expression in UV1 day 5, Trial 3, was significantly decreased compared to control plants (Figure 3.8; Log_2 fold = -1.18; $P < 0.05$). There were no other significant differences in *CBDAS* expression in any UV treatment against control. Within each of the trials, *THCAS* expression has a consistent pattern across UV treatment groups. There was no significant difference in expression between UV treatments according to ANOVA and t-tests (Figure 3.8, $P < 0.05$).

3.3.3 *CHS* expression was increased in the first 24 hours of UV treatment of cannabis plants

There was increased *CHS* expression in Trials 1 and 2 on day 1, followed by flat expression with little fold change in expression against the control from day 3 onwards (Figure 3.9). In Trial 3 there was a downwards expression trend of *CHS* expression in treatments compared to the control.

The increase in day 1 *CHS* expression in Trials 1 and 2 was statistically significant for all UV treatments compared to the control (Figure 3.9, Log_2 fold 0.59 to 2.43, $P < 0.05$). In Trial 3, *CHS* expression was significantly different to control on days 5 and 7 under UV1 and on day 7 under UV3 (Figure 3.9, Log_2 fold -0.63 to -1.27, $P < 0.05$). There were no other significant differences in *CHS* expression between UV-treated and control plants.

On Days 1 and 3 in Trial 1, UV2 treated plants had significantly higher expression of *CHS* than UV1 treated plants. Otherwise, there was no significant difference in *CHS* expression between different UV treatments in the same trial according to ANOVA and t-tests (Figure 3.9, $P < 0.05$).

3.3.4 *HY5* has the greatest variation of expression in UV-treated plants

There was increased *HY5* expression in Trials 1 and 2 on day 1 (Figure 3.10). In Trial 1 this was followed by general smaller positive increased expression in *HY5* compared to the control. In Trial 2, there was a strong decrease in *HY5* expression in treated plants against the control on day 3 (Figure 3.10). In Trial 3, the change in *HY5* expression was smaller and a decrease in expression against control was seen over the treatment.

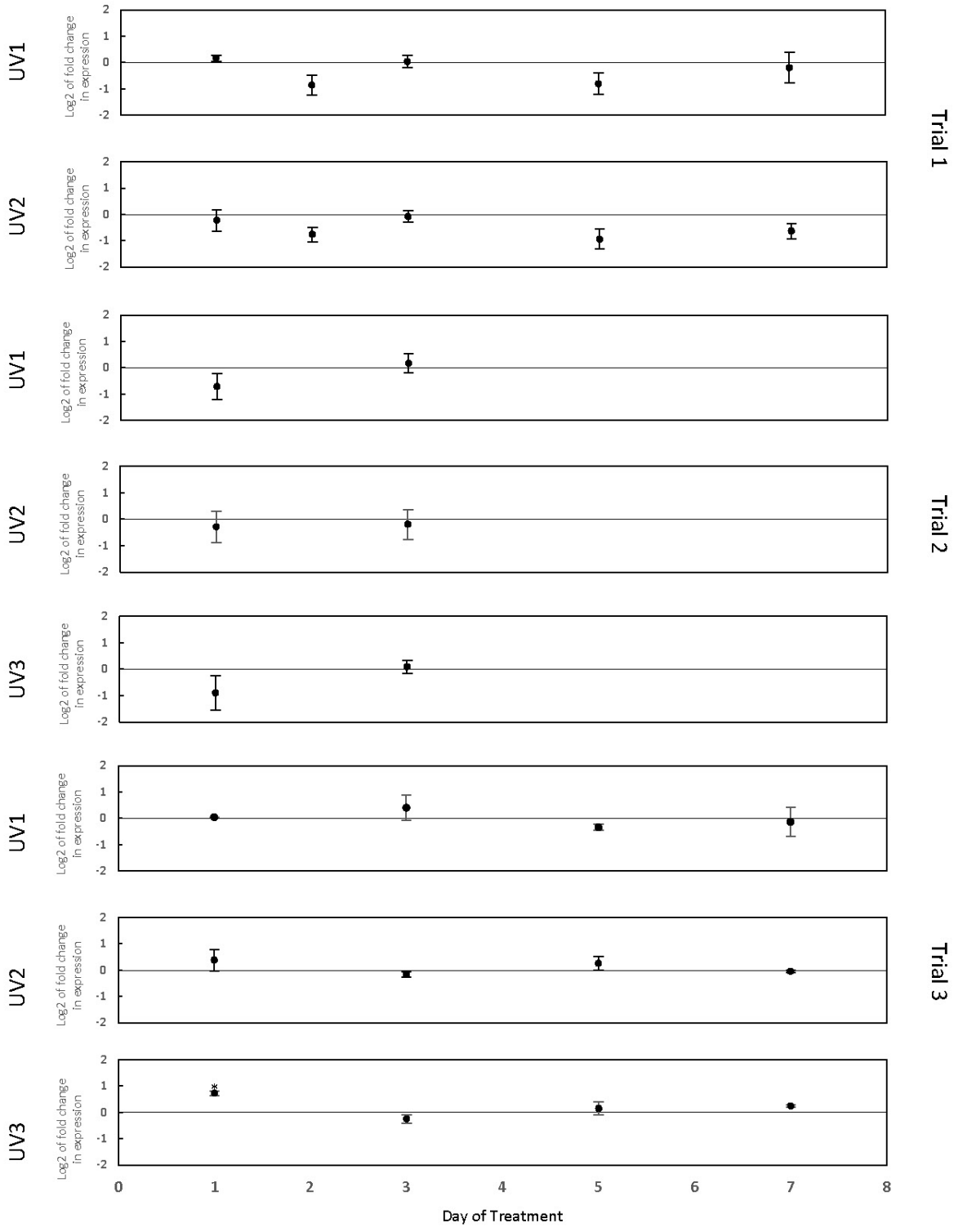

Figure 3.7: Log_2 of fold change in cannabidiolic acid synthase (*CBDAS*) gene expression against control in leaves of cannabis (*C. sativa*) cv. Head Cheese following UV treatment. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). RNA was extracted and gene expression quantified through Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). Significant differences in gene expression between treated plants and control (x-axis) are denoted with an asterisk where *=P<0.05 according to t-tests. Error bars indicate 1 S.E.

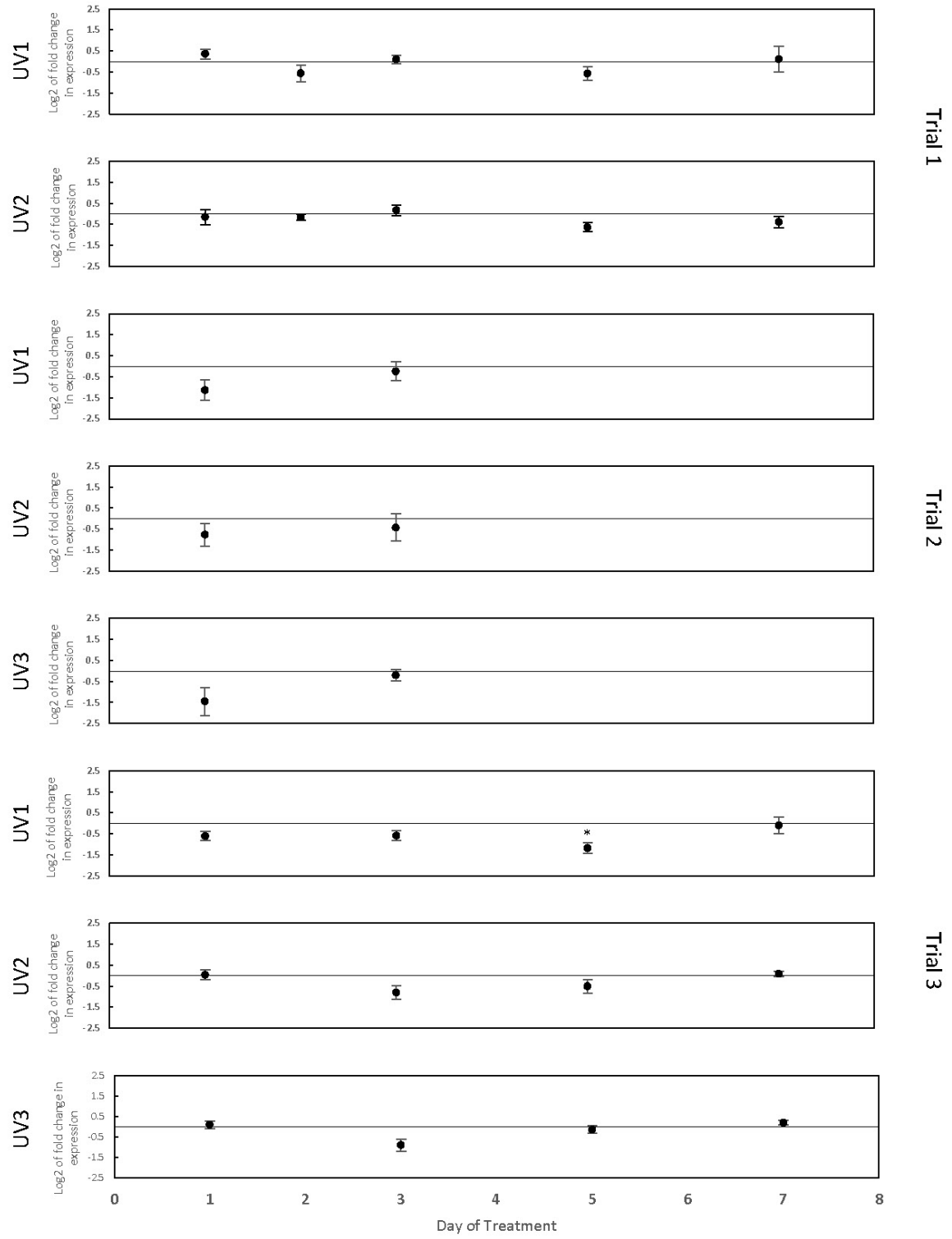

Figure 3.8: Log_2 of fold change in tetrahydrocannabinolic acid synthase (*THCAS*) gene expression against control in leaves of cannabis (*C. sativa*) cv. Head Cheese following UV treatment. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). RNA was extracted and gene expression quantified through Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). Significant differences in gene expression between treated plants and control (x-axis) are denoted with an asterisk where *=P<0.05 according to t-tests. Error bars indicate 1 S.E.

At 16 of 28 timepoints sampled across all trials, *HY5* expression was significantly different in UV-treated plants to control plants (Figure 3.10, Log_2 fold -4.32 to 0.79, $P < 0.05$). This is compared to 8 timepoints for *CHS* (Figure 3.7) and 1 timepoint each for both *CBDAS* (Figure 3.8) and *THCAS* (Figure 3.9) where gene expression in UV-treated plants was significantly different to the control. There was no significant difference in *HY5* expression between different UV treatments in the same trial according to ANOVA and t-tests (Figure 3.10, $P < 0.05$).

3.4 Clones from each cannabis mother exhibited different gene expression responses to UV

In Trial 2, cannabis clones from two different mothers, HC1 and HC2, were used. Both mothers are the of the same cultivar 'Head Cheese' but were the progeny of different seeds. At each time point in Trial 4 plants were sampled for each treatment, with two plants originating from each mother. Gene expression data for this trial has been segregated by mother.

3.4.1 *CBDAS* and *THCAS* expression in clones from different mothers is different under UV

In HC1 clones, there was little change in *CBDAS* and *THCAS* expression in under UV treatments against control (Figure 3.11, Figure 3.12). In HC2, on day 1 there was decreased expression of *CBDAS* and *THCAS* against the control (Figure 3.11, Figure 3.12). This was a statistically significant difference in *CBDAS* and *THCAS* expression to HC1 for UV3 (Figure 3.11, Log_2 fold 0.24 vs -2.03, $P < 0.05$; Figure 3.12, Log_2 fold -0.32 cs -2.59, $P < 0.05$). There was no other significant difference in *CBDAS* or *THCAS* expression between HC1 and HC2 according to t-tests (Figure 3.11 $P < 0.05$; Figure 3.12 $P < 0.05$).

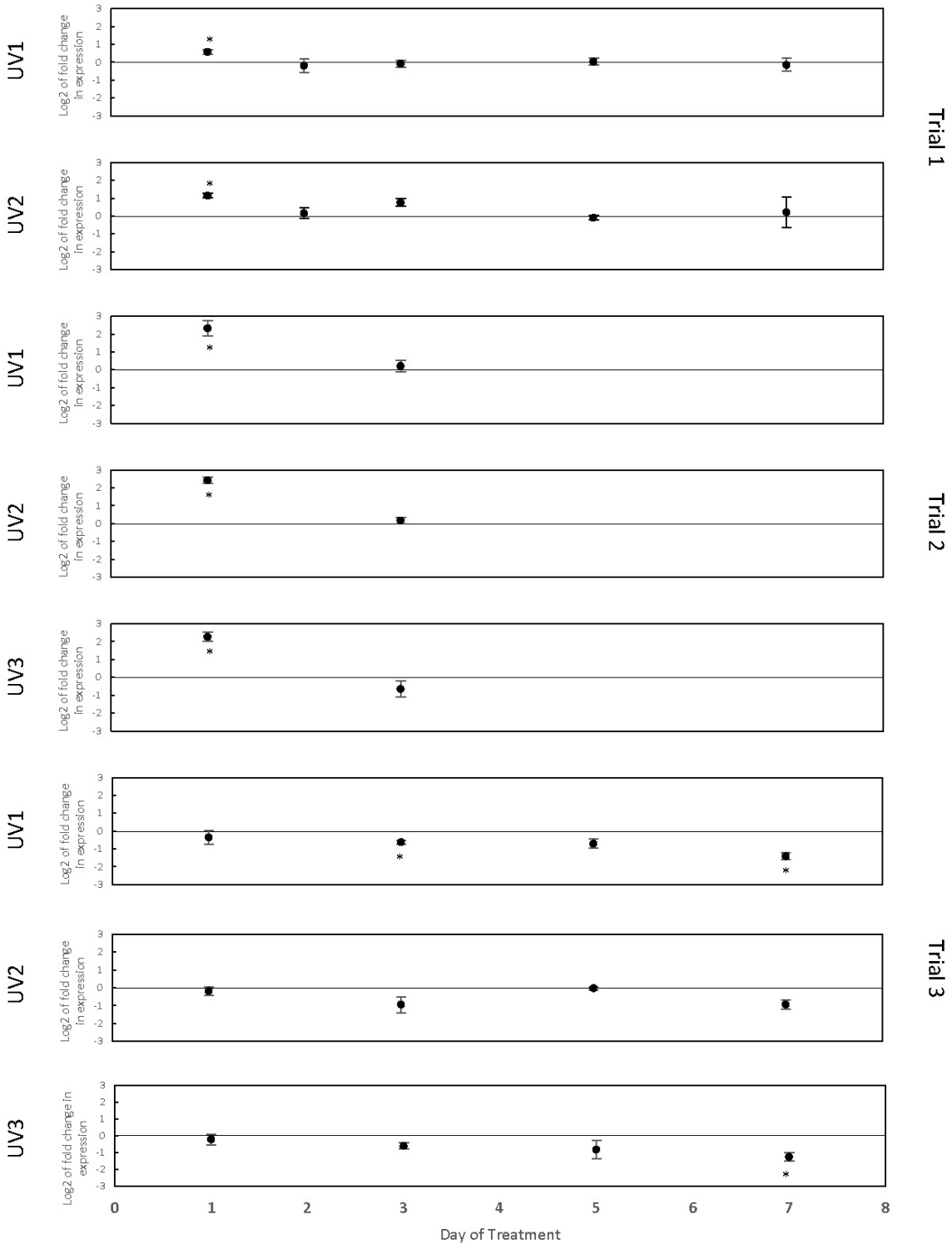

Figure 3.9: Log_2 of fold change in chalcone synthase (*CHS*) gene expression against control in leaves of cannabis (*C. sativa*) cv. Head Cheese following UV treatment. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Ribonucleic acid (RNA) was extracted and gene expression quantified through Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). Significant differences in gene expression between treated plants and control (x-axis) are denoted with an asterisk where *=P<0.05 according to t-tests. Error bars indicate 1 S.E.

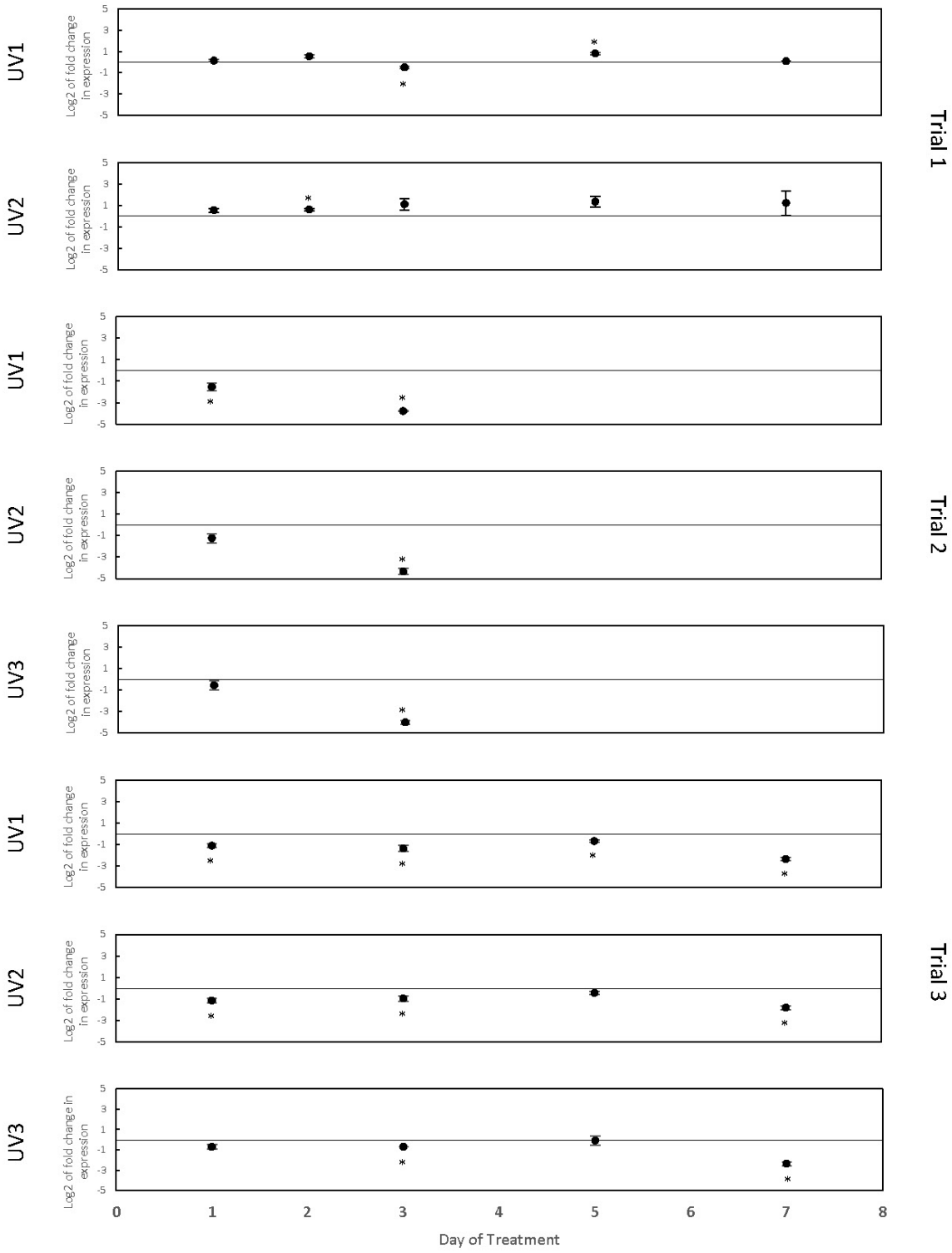

Figure 3.10: Log_2 of fold change in elongated hypocotyl5 (*HY5*) gene expression against control in leaves of cannabis (*C. sativa*) cv. Head Cheese following UV treatment. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Ribonucleic acid (RNA) was extracted and gene expression quantified through Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). Significant differences in gene expression between treated plants and control (x-axis) are denoted with an asterisk where *=$P<0.05$ according to t-tests. Error bars indicate 1 S.E.

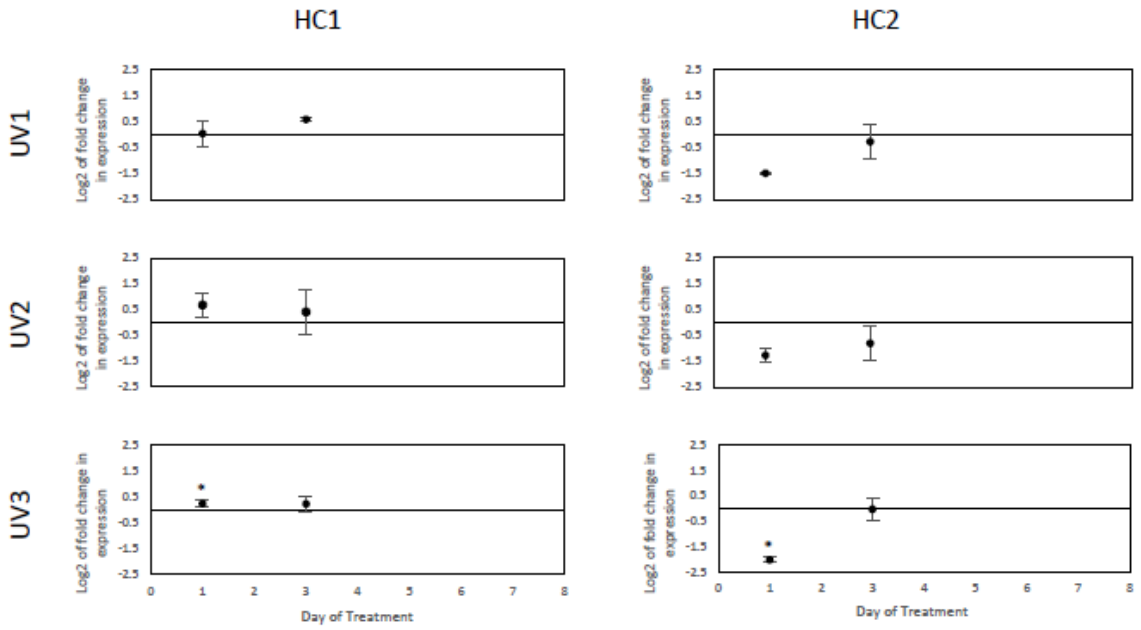

Figure 3.11: Log_2 of fold change in cannabidiolic acid synthase (*CBDAS*) gene expression against control in leaves of cannabis (*C. sativa*) cv. Head Cheese following UV treatment. Clones were taken from two different mothers (HC1 and HC2), with data segregated by mother plants. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Ribonucleic acid (RNA) was extracted and gene expression quantified through Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). Significant differences in gene expression between plants from the same treatment and timepoint but different mothers are denoted with an asterisk where *=P<0.05 according to t-tests. Error bars indicate 1 S.E.

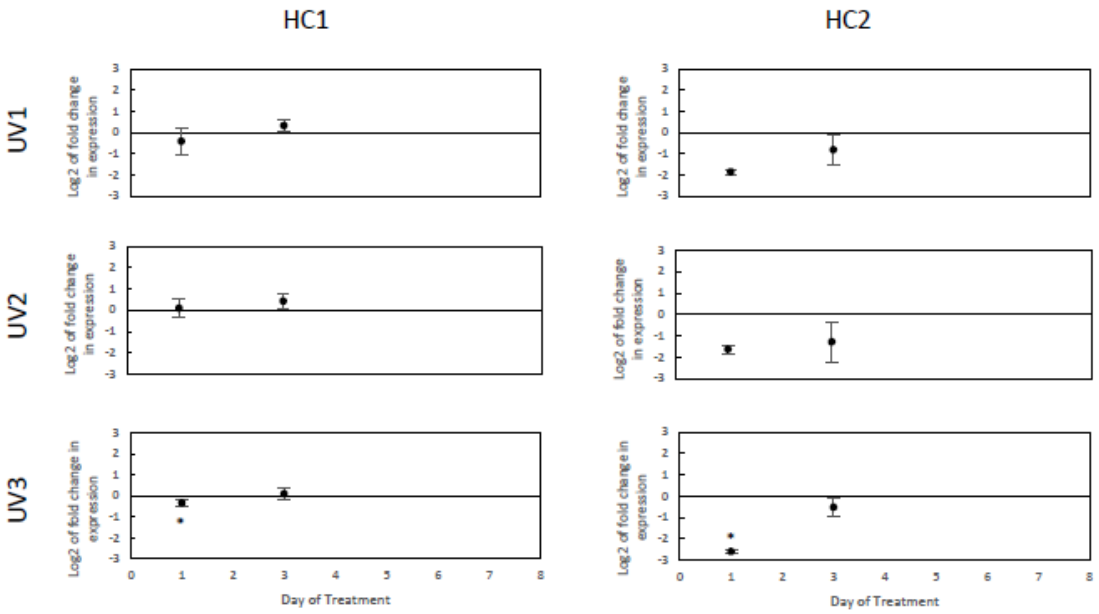

Figure 3.12: Log_2 of fold change in tetrahydrocannabinolic acid synthase (*THCAS*) gene expression against control in leaves of cannabis (*C. sativa*) cv. Head Cheese following UV treatment. Clones were taken from two different mothers (HC1 and HC2), with data egregated by mother plants. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Ribonucleic acid (RNA) was extracted and gene expression quantified through Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). Significant differences in gene expression between plants from the same treatment and timepoint but different mothers are denoted with an asterisk where *=P<0.05 according to t-tests. Error bars indicate 1 S.E.

3.4.2 *CHS* and *HY5* expression in clones from different mothers is consistent under UV

In Trial 2, a similar expression level and pattern is seen in both HC1 and HC2 clones for **the** *CHS* and *HY5* genes (Figure 3.13, Figure 3.14). There was no significant difference in *CHS* or *HY5* expression between HC1 and HC2 clones according to t-tests (Figure 3.13 $P < 0.05$, Figure 3.14 $P < 0.05$).

3.5 Gene expression deviation from control differs by trial and treatment

To analyse the deviation of gene expression from the control, the mean of the absolute values of $\log_2$ fold change in expression for each treatment and trial was taken. Trial 2 had the greatest deviation in gene expression from the control for all genes, while Trial 1 and 3 had similar deviation of expression (Figure 3.15, Figure 3.16, Figure 3.17, Figure 3.18). In Trial 1, *CHS* and *HY5* gene expression had greater deviation from the control under UV2 than UV1 (Figure 3.17, Figure 3.18). In Trial 3, *THCAS* had greater deviation from the control under UV1 than UV2 and UV3 (Figure 3.16). Otherwise, there was little difference in deviation from control between different treatments for the same gene and trial. *HY5* had the greatest deviation in gene expression from the mean followed by *CHS*. *CBDAS* and *THCAS* had a similar level of deviation in expression from control and less than the deviation of *CHS*.

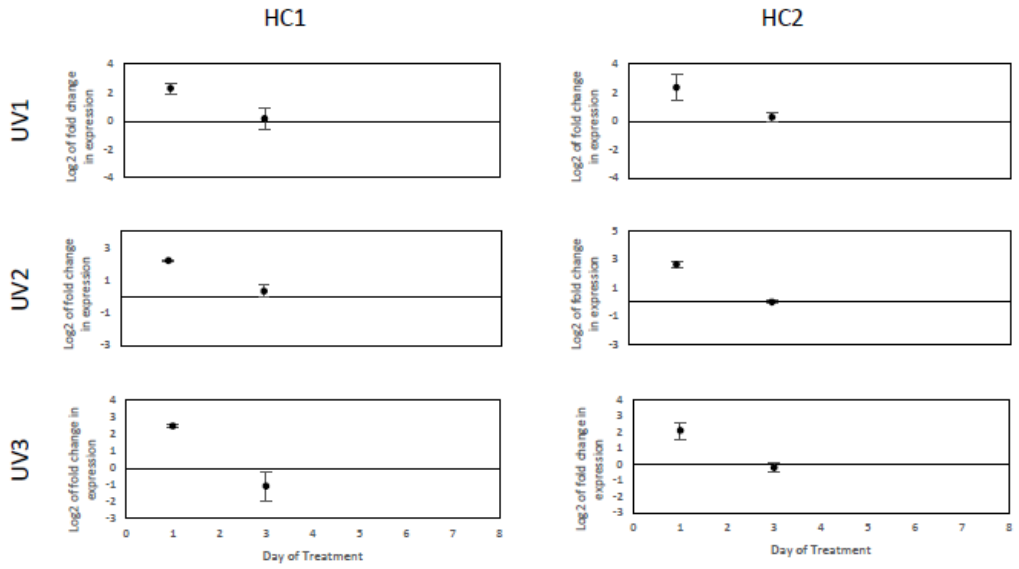

Figure 3.13: $\log_2$ of fold change in chalcone synthase (*CHS*) gene expression against control in leaves of cannabis (*C. sativa*) cv. Head Cheese. Clones were taken from two different mothers (HC1 and HC2), with data segregated by mother plants. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Ribonucleic acid (RNA) was extracted and gene expression quantified through Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). Significant differences in gene expression between plants from the same treatment and timepoint but different mothers are denoted with an asterisk where *=P<0.05 according to t-tests. Error bars indicate 1 S.E.

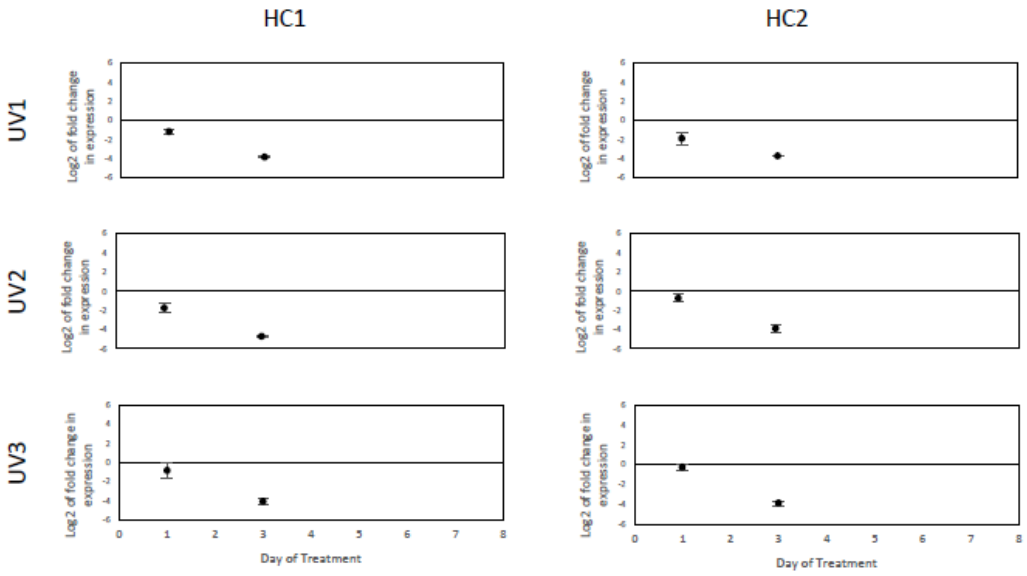

Figure 3.14: Log_2 of fold change in elongated hypocotyl5 (*HY5*) gene expression against control in leaves of cannabis (*C. sativa*) cv. Head Cheese. Clones were taken from two different mothers (HC1 and HC2), with data segregated by mother plants. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Ribonucleic acid (RNA) was extracted and gene expression quantified through Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). Significant differences in gene expression between plants from the same treatment and timepoint but different mothers are denoted with an asterisk where *=P<0.05 according to t-tests. Error bars indicate 1 S.E.

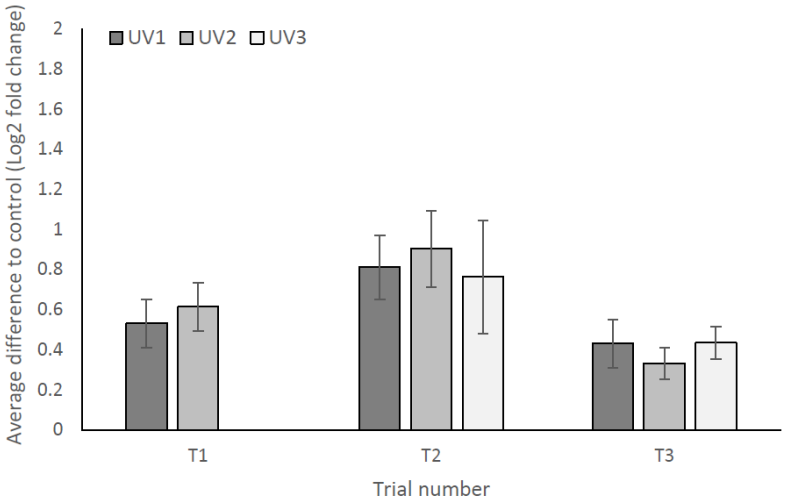

Figure 3.15: Means of the absolute values of Log_2 fold change in cannabidiolic acid synthase (*CBDAS*) gene expression against control in leaves of cannabis (*C. sativa*) cv. Head Cheese. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Ribonucleic acid (RNA) was extracted and gene expression quantified through Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). For each trial, the mean of the absolute values of Log_2 fold change in gene expression was taken to give an average of the difference to gene expression in control plants. Error bars indicate 1 S.E.

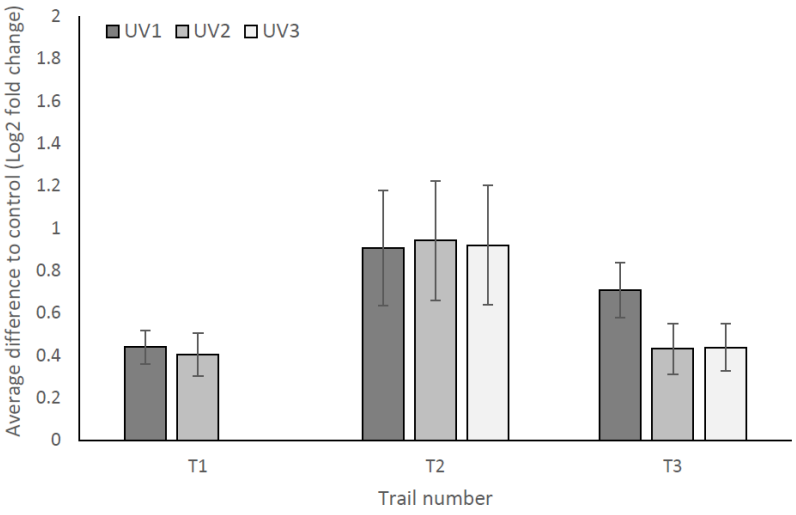

Figure 3.16: Means of the absolute values of Log_2 fold change in tetrahydrocannabinolic acid (*THCAS*) gene expression against control in leaves of cannabis (*C. sativa*) cv. Head Cheese. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Ribonucleic acid (RNA) was extracted and gene expression quantified through Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). For each trial, the mean of the absolute values of Log_2 fold change in gene expression was taken to give an average of the difference to gene expression in control plants. Error bars indicate 1 S.E.

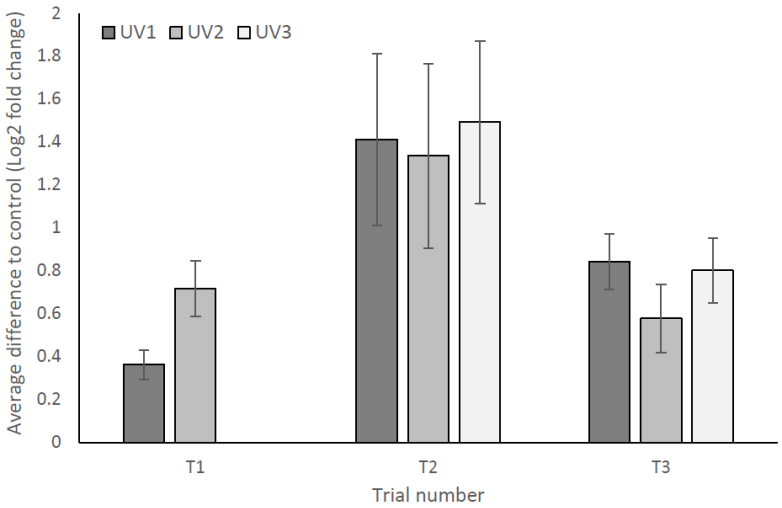

Figure 3.17: Means of the absolute values of Log_2 fold change in chalcone synthase (*CHS*) gene expression against control in leaves of cannabis (*C. sativa*) cv. Head Cheese. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Ribonucleic acid (RNA) was extracted and gene expression quantified through Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). For each trial, the mean of the absolute values of Log_2 fold change in gene expression was taken to give an average of the difference to gene expression in control plants. Error bars indicate 1 S.E.

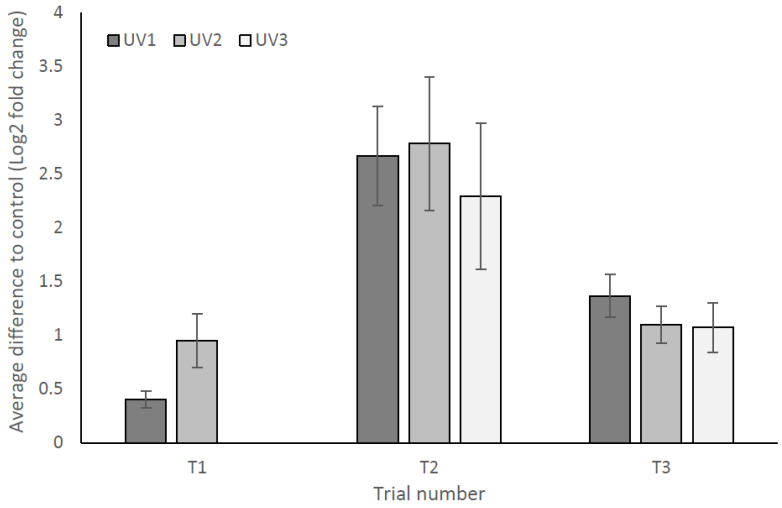

Figure 3.18: Means of the absolute values of Log_2 fold change in elongated hypocotly5 (*HY5*) gene expression against control in leaves of cannabis (*C. sativa*) cv. Head Cheese. Cannabis plants were treated with a proprietary UV treatment (1-3) or control light treatment for a duration of 6 days. Samples of treatment groups were taken and destructively harvested throughout treatment, and 1 day after treatment (day 7). Ribonucleic acid (RNA) was extracted and gene expression quantified through Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR). For each trial, the mean of the absolute values of Log_2 fold change in gene expression was taken to give an average of the difference to gene expression in control plants. Error bars indicate 1 S.E.

Chapter 4
Discussion

This study is one of the first to investigate gene expression in cannabis in response to UV exposure. The study aimed to further the understanding of the response of cannabis to UV light by quantifying the expression of genes in both UV response and cannabinoid biosynthesis pathways. The genes selected for expression were *CBDAS*, *THCAS*, *CHS* and *HY5*. In three trials, cannabis clones were treated with proprietary UV recipes developed by BioLumic. Clones were removed from treatment in timeseries with RNA extracted and used in RT-qPCR for gene expression analysis. Dualex leaf clip meters were then used to measure the relative flavonoid and chlorophyll content of leaves in cannabis plants after their removal from the UV treatment regime. Expression of the four genes of interest in UV-treated plants was then compared to plants that received a control treatment containing only PAR light.

4.1 Impact of plants displaying stress symptoms on UV-mediated response

In Trial 1 and 2, cannabis clones displayed a strong stress phenotype, showing significant leaf yellowing and browning (Figure 3.1). Trial 3 plants showed a much reduced stress phenotype and were a more vibrant green than Trial 1 and 2 plants, containing significant higher levels of chlorophyll in upper leaves (Figure 3.2). It is believed that poor management of clones lead to nutrient, water or other stresses leading to the stress phenotype. Trial 1 and 2 plants had a significantly higher flavonoid accumulation than Trial 3 plants on day 1 of UV treatment across all treatment groups including the control (Figure 3.4, Figure 3.5, Figure 3.6). Plant stress factors including accumulation in response to nitrogen/phosphorus depletion (Lillo et al., 2008; Olsen et al., 2009) and drought stress (Nakabayashi et al., 2014) have been shown to cause an increase in flavonoid accumulation. In Trial 3 plants there was a clear increase in flavonoid content over treatment in UV-treated plants (Table 3.1). There was no clear trend in flavonoid accumulation in Trial 1 and 2 plants. The high initial flavonoid accumulation in Trial 1 and 2 plants obscured any trends in accumulation induced by UV treatment (Figure 3.4, Figure 3.5). There was a decline in flavonoid accumulation shown for control plants in Trial 1 and UV1 treated plants in Trial 2. However, the flavonoid measurements were not repeat measures of the same plants so this decline could be due to chance variation in flavonoid content between clones. Future trials could benefit from repeat flavonoid measurements of the same plant over treatment to build a better understanding of the impact of UV treatments on flavonoid accumulation.

In Trial 1 and 2 UV-treated plants *CHS* expression was increased significantly on day 1 of treatment relative to the control (Figure 3.9, Log_2 fold 0.59 to 2.43, $P < 0.05$). There was no significant change in expression relative to the control in *CHS* on day 1 in Trial 3 UV-treated plants. The significant increase in *CHS* expression relative to control only in Trial 1 and 2 plants suggests that the plant nutrient

or water stress may have primed them for greater response to other abiotic stressors such the UV radiation the plants were exposed to during UV treatment. The induction of *CHS* expression is well documented as a response to environmental stressors (Zhang & Björn, 2009) but there is little research that shows that plant nutrient or water stress primes a response to UV exposure. The peak *CHS* expression on day 1 of UV treatment in Trials 1 and 2 suggests that *CHS* could be a useful marker for determining stress in cannabis plants entering UV treatment.

In Trial 1 and 2 higher initial flavonoid accumulation is likely to solely be a result of the nutrient or water stress prior to the start of treatment. The day 1 flavonoid measurements were taken after plants had only 2 hours of exposure to UV treatment, and in Trial 1 and 2, flavonoid accumulation was consistently higher than Trial 3 plants across all treatment groups including the control. In future trials, cannabis flavonoid content should be measured prior to plants receiving UV treatment so that the impact of UV treatments on flavonoid accumulation can be better understood.

The increased flavonoid accumulation and significantly increased *CHS* expression suggest that plant stress was a confounding variable in Trials 1 and 2. Trial 3 plants, with less visible stress phenotypes (Figure 3.1) and leaves containing a higher chlorophyll content (Figure 3.2), wrre a more accurate representation of cannabis plants that are produced in a commercial setting. Due to improvements made to cannabis clone care, plant stress was not a confounding variable in Trial 3, making it more reliable for drawing conclusions about the response of cannabis to UV treatments both in flavonoid accumulation and gene expression. In future trials, careful attention should be paid to the management of clones to ensure plant stress is not a confounding factor.

Individual plant health records were not kept during any of the trials, so it can not be determined if plant stress phenotypes were uniformly distributed across treatment groups. Individual plant health records should be kept in future trials so that gene expression data can be analysed in context of the health of individual cannabis clones.

4.2 *CHS* and *HY5* expression shows a greater UV-mediated response than *CBDAS* and *THCAS*

Across all trials, there was greater variation between UV-treated and control plants, in the expression of *CHS* and *HY5* than *CBDAS* and *THCAS*. *THCAS* and *CBDAS* had only one timepoint each across all three trials that had gene expression that was significantly different to control plants (Figure 3.7, Figure 3.8). *CHS* had 8 timepoints (Figure 3.9) and HY5 had 16 timepoints (Figure 3.10) where gene expression was significantly different to control across all trials. The average variation in gene expression between UV-treated and control plants was also greater in *CHS* and *HY5* than *CBDAS* and *THCAS* across all trials and treatment groups, with *HY5* also having greater variation than *CHS* (Figure 3.15, Figure 3.16, Figure 3.17, Figure 3.18). In the UVR8 detection model of UV light, *HY5* is

a transcription factor that regulates the expression of many genes in responses linked to UV (Yin & Ulm, 2017). *HY5* is directly involved in the beginning UV light perception signalling pathway. *CHS* catalyses the first dedicated step in the flavonoid biosynthesis pathway (Zhang & Björn, 2009). Flavonoid accumulation is a well-documented response in plants to UV exposure (Yin & Ulm, 2017) and is consistent with Trial 3 results showing an increase in flavonoid accumulation over treatment (Table 3.1). *THCAS* and *CBDAS* sit in the cannabinoid biosynthesis pathway, which is preceded by the flavonoid biosynthesis pathway. *CBDAS* and *THCAS* are from a pathway position perspective, further away from the perception of UV light than *CHS* which is in turn, further than *HY5* to UV perception. The greater difference of the genetic pathway may be the reason for smaller variation in the expression of *CBDAS* and *THCAS* genes than *CHS* and *HY5*. This greater pathway distance could lead to a dilution of the UV signal, resulting in a lesser impact of the UV treatment on gene expression.

The significant change in expression of *HY5* in plants under UV treatment suggests that the response of the cannabis plants to the UV treatments is mediated through the UVR8 pathway. UVR8 activity could be investigated to confirm the involvement of the UVR8 perception pathway in the response of cannabis plants to BioLumic UV treatments. This would build a greater understanding the response of cannabis to UV treatments but would require the use of a protein immunoprecipitation assay to compare the levels of UVR8 monomerisation between UV-treated and control plants. The use of an immunoprecipitation assay was outside of the scope of this study and would be costly to conduct.

4.3 Gene expression differences in clones originating from separate mother genotypes

In Trial 2, two different mother plants provided material to produce cannabis clones that underwent UV treatment. The two mothers were the same cultivar, Head Cheese, but had different genotypes as they were the progeny of different seeds. The Clones sourced from each mother made up half of the plants in each treatment group, and for each sample taken, two plants from each mother were taken as replicates. This allowed for the comparison of the gene expression in cannabis plants of different genotypes under the same UV treatment for each sampling timepoint. There was no significant difference in *CHS* and *HY5* expression between cannabis clones from different mother genotypes (Figure 3.13, Figure 3.15). There was a clear difference in *CBDAS* and *THCAS* expression between cannabis clones from different mothers, with HC1 clones having significantly greater expression of these two genes than HC2 clones, on day one of treatment under UV3 (Figure 3.11, Figure 3.12). There was also greater expression of *CBDAS* and *THCAS* in HC1 clones than HC2 on day one of UV1 and UV2 treatment, but this difference was not statistically significant according to t-tests. The lack of significance may be due to the small sample size, with only two plants per mother per time point in each UV treatment. If future trials were repeated with larger sample sizes, the difference in expression

of *CBDAS* and *THCAS* in HC1 and HC2 may be statistically significant. Future trials investigating differences in gene expression between cannabis clones sourced from different mother genotypes should use large sample sizes.

The results showed that *CHS* and *HY5* expression was consistent across clones from the two different mother plants whilst *CBDAS* and *THCAS* expression was different. As discussed in section 4.2, the position of *CHS* and *HY5* in the UV perception and response pathway is much closer to initial UV perception than *CBDAS* and *THCAS*. One hypothesis for the variation in expression of *CBDAS* and *THCAS* between clones from each of the two genotypes is that there are genetic differences in the pathway from UV perception to induction of *CBDAS* and *THCAS* expression and that the cumulative impact of these differences across the pathway results in differences between clones from the two mothers in *CBDAS* and *THCAS* expression under the same UV treatment. *CHS* and *HY5* sit much closer to the perception of UV so there will be less cumulative differences in this genetic pathway by the time the expression of these genes is regulated in response to UV perception. Alternatively, there may be differences in a few specific genes in either the UV perception or cannabinoid biosynthesis pathway that were responsible for the differences in expression of *CBDAS* and *THCAS* between clones from mothers of different genotypes under the same UV treatment.

4.4 Industry relevance of study findings

Whilst informing a greater understanding of the responses of cannabis to UV, this study also has relevance to the commercial cultivation of cannabis. As discussed in section 1.5, to increase their return on investment, cannabis growers want to maximise yields and flower cannabinoid content. UV treatments, developed by BioLumic, have the potential to increase yields and cannabinoid content of cannabis.

In developing new UV treatments BioLumic screens recipes to determine their efficacy in improving cannabis crop yield and the cannabinoid profile of flower harvested. To determine the impact of a UV treatment, cannabis plants must be grown to yield maturity, which is approximately 9 weeks after UV treatment for most cultivars. Marker genes, which are correlated with an improvement in yield and cannabinoid profile would offer the opportunity to screen UV treatments by looking at gene expression in UV-treated cannabis plants during and 1 day after treatment. This would reduce the time to screen UV treatments by 9 weeks. It would allow for the screening of more UV treatments and therefore increase the speed of development of UV treatments effective at improving crop performance. Marker genes useful for the screening of UV treatments must have a unique expression pattern under UV treatments that confers a yield and/or cannabinoid profile benefit. Expression of these marker genes must be able to be distinguished between cannabis plants receiving a UV treatment that confers a commercial benefit by improving crop yield and cannabinoid content, and UV treatment that does not

confer this benefit. The expression pattern of multiple marker genes may be required to effectively screen UV treatments. In all trials in this study, there was no significant difference in the expression of *CBDAS*, *THCAS*, *CHS* or *HY5* between different UV treatments in the same trial. This suggest that these genes would not be effective markers for new recipe development, as there was no significant difference in expression between treatments. However, as none of the trials had plants that were taken to harvest maturity, it unknown whether there would be a significant difference in the yield and cannabinoid profile outcomes caused my different UV treatments.

None of the trials were taken to harvest in this study, therefore, only limited conclusions about the genes effectiveness as marker genes can be drawn. In future trials, gene expression analysis should be conducted in plants that are taken to yield maturity. Destructive harvest of clones could be avoided through using leaf tip cuttings for gene expression analysis. This would allow gene expression in plants at the stage of UV treatment to be directly compared to the yield and cannabinoid profile outcomes from that plant.

The difference in gene expression of *CBDAS* and *THCAS* between clones from different genotypes from the same cultivar under the same UV treatment discussed in section 4.3 highlights that there are differences in the response of different cannabis genotypes to the same UV treatment. This suggests that different cultivars of cannabis or different genotypes within the same cultivar of Cannabis may have different yield and cannabinoid profile outcomes under the same UV treatment. This means that UV treatments may have to be designed for specific cannabis cultivars to confer the greatest positive impact on yield and cannabinoid profile for growers. Further trials should be conducted with different cannabis cultivars.

4.5 Conclusions

This study aimed to further the understanding of the response of cannabis to UV light. The UV treatments in the project caused significant changes in expression of *CHS* and *HY5* but little change in *CBDAS* and *THCAS*. Plants displaying stress symptoms may be primed to respond more strongly to UV treatments, having higher initial *CHS* expression. Clones originating from separate mother genotypes of the same cultivar had difference in gene expression under the same UV treatment. UV treatments have significant potential for use as a tool in commercial cannabis cultivation to improve yield and cannabinoid profile. Future work will further explore the response of cannabis to UV treatments, including the expression of genes that may be used as markers for increasing the speed of new effective UV treatment development.

Bibliography

Adams, P. J., Rychert, M., & Wilkins, C. (2021). Policy influence and the legalized Cannabis industry: learnings from other addictive consumption industries. *Addiction, 116*(11), 2939-2946.

Andre, C. M., Hausman, J.-F., & Guerriero, G. (2016). *Cannabis sativa*: the plant of the thousand and one molecules. *Frontiers in plant science, 7*, 19.

Bahji, A., & Stephenson, C. (2019). International perspectives on the implications of Cannabis legalization: a systematic review & thematic analysis. *International journal of environmental research and public health, 16*(17), 3095.

BioLumic Ltd. *Growing Better Cannabis.* Retrived 30/07/22. https://www.BioLumic.com/Cannabis

Campbell, B. J., Berrada, A. F., Hudalla, C., Amaducci, S., & McKay, J. K. (2019). Genotype× environment interactions of industrial hemp cultivars highlight diverse responses to environmental factors. *Agrosystems, geosciences & environment, 2*(1), 1-11.

Cavalli, J., & Dutra, R. C. (2021). A closer look at cannabimimetic terpenes, polyphenols, and flavonoids: a promising road forward. *Neural regeneration research, 16*(7), 1433.

Cortesi, M., & Fusar-Poli, P. (2006). Potential therapeutical effects of cannabidiol in children with pharmacoresistant epilepsy. *Medical hypotheses, 68(4)*, 920-921.

Freeman, T. P., Groshkova, T., Cunningham, A., Sedefov, R., Griffiths, P., & Lynskey, M. T. (2019). Increasing potency and price of Cannabis in Europe, 2006–16. *Addiction, 114*(6), 1015-1023.

Fernández, M. B., Tossi, V., Lamattina, L., & Cassia, R. (2016). A comprehensive phylogeny reveals functional conservation of the UV-B photoreceptor *UVR8* from green algae to higher plants. *Frontiers in plant science, 7*, 1698.

Grand View Research Inc. (2022). *Legal Cannabis Market Size, Share & Trends Analysis Report By Source (Marijuana, Hemp), By Derivative (CBD, THC), By End Use (Medical Use, Recreational Use, Industrial Use), By Region, And Segment Forecasts, 2022 - 2030*. https://www.grandviewresearch.com/industry-analysis/legal-Cannabis-market

Hanuš, L. O., Meyer, S. M., Muñoz, E., Taglialatela-Scafati, O., & Appendino, G. (2016). Phytocannabinoids: a unified critical inventory. *Natural product reports, 33*(12), 1357-1392.

Hayes, S., Sharma, A., Fraser, D. P., Trevisan, M., Cragg-Barber, C. K., Tavridou, E., Fankhauser, C., Jenkins, G. I., & Franklin, K. A. (2017). UV-B perceived by the *UVR8* photoreceptor inhibits plant thermomorphogenesis. *Current biology, 27*(1), 120-127.

Hectors, K., Jacques, E., Prinsen, E., Guisez, Y., Verbelen, J.-P., Jansen, M. A., & Vissenberg, K. (2010). UV radiation reduces epidermal cell expansion in leaves of Arabidopsis thaliana. *Journal of experimental botany, 61*(15), 4339-4349.

Jansen, M. A., Gaba, V., & Greenberg, B. M. (1998). Higher plants and UV-B radiation: balancing damage, repair and acclimation. *Trends in plant science, 3*(4), 131-135.

Jenkins, G. I. (2009). Signal transduction in responses to UV-B radiation. *Annual review of plant biology, 60*, 407-431.

Jenkins, G. I. (2017). Photomorphogenic responses to ultraviolet-B light. *Plant, cell & environment, 40*(11), 2544-2557.

Kataria, S., & Guruprasad, K. (2015). Exclusion of solar UV radiation improves photosynthetic performance and yield of wheat varieties. *Plant physiology and biochemistry, 97*, 400-411.

Kirkham, T. (2005). Endocannabinoids in the regulation of appetite and body weight. *Behavioural pharmacology, 16*(5-6), 297-313.

Kogan, N. M., & Mechoulam, R. (2007). Cannabinoids in health and disease. *Dialogues in clinical neuroscience, 9*(4), 413.

Leafly. *CBD Charlotte's Angel*. Retrived 29/08/22. https://www.leafly.com/strains/cbd-charlottes-angel

Leafly. *Head Cheese*. Retrived 29/08/22. https://www.leafly.com/strains/head-cheese

Liao, X., Liu, W., Yang, H. Q., & Jenkins, G. I. (2020). A dynamic model of UVR8 photoreceptor signalling in UV-B-acclimated Arabidopsis. *New phytologist, 227*(3), 857-866.

Lillo, C., Lea, U. S., & Ruoff, P. (2008). Nutrient depletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway. *Plant, cell & environment, 31*(5), 587-601

Llewellyn, D., Golem, S., Foley, E., Dinka, S., Jones, M., & Zheng, Y. (2021). Cannabis yield increased proportionally with light intensity, but additional ultraviolet radiation did not affect yield or cannabinoid content. *doi: 10.20944/preprints202103.0327.v1*

Lois, R. (1994). Accumulation of UV-absorbing flavonoids induced by UV-B radiation in Arabidopsis thaliana L. *Planta, 194*(4), 498-503.

Lydon, J., Teramura, A. H., & Coffman, C. B. (1987). UV-B radiation effects on photosynthesis, growth and cannabinoid production of two *Cannabis sativa* chemotypes. *Photochemistry and photobiology, 46*(2), 201-206.

Magagnini, G., Grassi, G., & Kotiranta, S. (2018). The effect of light spectrum on the morphology and cannabinoid content of *Cannabis sativa* L. *Medical cannabis and cannabinoids, 1*(1), 19-27.

Mahlberg, P. G., & Hemphill, J. K. (1983). Effect of light quality on cannabinoid content of *Cannabis sativa* L.(Cannabaceae). *Botanical gazette, 144*(1), 43-48.

McLay, E. R., Pontaroli, A. C., & Wargent, J. J. (2020). UV-B induced flavonoids contribute to reduced biotrophic disease susceptibility in lettuce seedlings. *Frontiers in plant science, 11*, 594681.

Nakabayashi, R., Mori, T., & Saito, K. (2014). Alternation of flavonoid accumulation under drought stress in Arabidopsis thaliana. *Plant signaling & behavior, 9*(8), e29518.

Nguyen, N. H. (2020). HY5, an integrator of light and temperature signals in the regulation of anthocyanins biosynthesis in Arabidopsis. *AIMS molecular science, 7*(2), 70-81.

Olsen, K. M., Slimestad, R., Lea, U. S., Brede, C., Løvdal, T., Ruoff, P., Verheul, M., & Lillo, C. (2009). Temperature and nitrogen effects on regulators and products of the flavonoid pathway: experimental and kinetic model studies. *Plant, cell & environment, 32*(3), 286-299.

Podolec, R., Lau, K., Wagnon, T. B., Hothorn, M., & Ulm, R. (2021). A constitutively monomeric *UVR8* photoreceptor confers enhanced UV-B photomorphogenesis. *Proceedings of the national academy of sciences, 118*(6), e2017284118.

Qiu, Z., Wang, H., Li, D., Yu, B., Hui, Q., Yan, S., Huang, Z., Cui, X., & Cao, B. (2019). Identification of candidate HY5-dependent and-independent regulators of anthocyanin biosynthesis in tomato. *Plant and cell physiology, 60*(3), 643-656.

Rai, N., O'Hara, A., Farkas, D., Safronov, O., Ratanasopa, K., Wang, F., Lindfors, A. V., Jenkins, G. I., Lehto, T., & Salojärvi, J. (2020). The photoreceptor UVR8 mediates the perception of both UV-B and UV-A wavelengths up to 350 nm of sunlight with responsivity moderated by cryptochromes. *Plant, cell & environment, 43*(6), 1513-1527.

Rodriguez-Morrison, V., Llewellyn, D., & Zheng, Y. (2021). Cannabis inflorescence yield and cannabinoid concentration are not increased with exposure to short-wavelength ultraviolet-B radiation. *Frontiers in plant science, 12*.

Russo, E. B. (2011). Taming THC: potential Cannabis synergy and phytocannabinoid-terpenoid entourage effects. *British journal of pharmacology, 163*(7), 1344-1364.

Russo, E. B., Jiang, H.-E., Li, X., Sutton, A., Carboni, A., Del Bianco, F., Mandolino, G., Potter, D. J., Zhao, Y.-X., & Bera, S. (2008). Phytochemical and genetic analyses of ancient Cannabis from Central Asia. *Journal of experimental botany, 59*(15), 4171-4182.

Schwabe, A. L., & McGlaughlin, M. E. (2019). Genetic tools weed out misconceptions of strain reliability in *Cannabis sativa*: implications for a budding industry. *Journal of cannabis research, 1*(1), 1-16.

Scott, C., & Punja, Z. K. (2021). Evaluation of disease management approaches for powdery mildew on *Cannabis sativa* L. (marijuana) plants. *Canadian journal of plant pathology, 43*(3), 394-412.

Sharma, A., Sharma, B., Hayes, S., Kerner, K., Hoecker, U., Jenkins, G. I., & Franklin, K. A. (2019). UVR8 disrupts stabilisation of PIF5 by COP1 to inhibit plant stem elongation in sunlight. *Nature communications, 10*(1), 1-10.

Skoglund, G., Nockert, M., & Holst, B. (2013). Viking and early Middle Ages northern Scandinavian textiles proven to be made with hemp. *Scientific reports, 3*(1), 1-6.

Small, E., Pocock, T., & Cavers, P. (2003). The biology of Canadian weeds. 119. *Cannabis sativa* L. *Canadian journal of plant science, 83*(1), 217-237.

Spitzer-Rimon, B., Duchin, S., Bernstein, N., & Kamenetsky, R. (2019). Architecture and florogenesis in female *Cannabis sativa* plants. *Frontiers in plant science, 10*, 350.

Taura, F., Sirikantaramas, S., Shoyama, Y., & Morimoto, S. (2009). Phytocannabinoids in *Cannabis sativa*: recent studies on biosynthetic enzymes. *Cannabinoids in nature and medicine. Wiley-VHCA AG*, 51-65.

TT, D. T., & Puthur, J. T. (2017). UV radiation priming: a means of amplifying the inherent potential for abiotic stress tolerance in crop plants. *Environmental and experimental botany, 138*, 57-66.

Ubi, B. E., Honda, C., Bessho, H., Kondo, S., Wada, M., Kobayashi, S., & Moriguchi, T. (2006). Expression analysis of anthocyanin biosynthetic genes in apple skin: effect of UV-B and temperature. *Plant science, 170*(3), 571-578.

Van Bakel, H., Stout, J. M., Cote, A. G., Tallon, C. M., Sharpe, A. G., Hughes, T. R., & Page, J. E. (2011). The draft genome and transcriptome of *Cannabis sativa*. *Genome biology, 12*(10), 1-18.

Volfe, Z., Dvilansky, A., & Nathan, I. (1985). Cannabinoids block release of serotonin from platelets induced by plasma from migraine patients. International jounral of clinical pharmacolology research, *5*(4), 243-246.

Wargent, J. J., Nelson, B. C. W., McGhie, T. K., & Barnes, P. W. (2015). Acclimation to UV-B radiation and visible light in *Lactuca sativa* involves up-regulation of photosynthetic performance and orchestration of metabolome-wide responses. *Plant, cell & environment, 38*(5), 929-940.

Yin, R., & Ulm, R. (2017). How plants cope with UV-B: from perception to response. *Current opinion in plant biology, 37*, 42-48.

Zhang, W. J., & Björn, L. O. (2009). The effect of ultraviolet radiation on the accumulation of medicinal compounds in plants. *Fitoterapia, 80*(4), 207-218.

Zhao, B., Wang, L., Pang, S., Jia, Z., Wang, L., Li, W., & Jin, B. (2020). UV-B promotes flavonoid synthesis in *Ginkgo biloba* leaves. *Industrial crops and products, 151*, 112483.