SHINING THE SPOTLIGHT ON MEDICINAL CANNABIS

From rooting through flowering to specialized metabolites

Wannida Sae-Tang

Propositions

1. Yield in medicinal cannabis primarily depends on intensity and duration of light rather than spectrum of light.
   (this thesis)

2. Cannabis is an excellent model plant for studying high photosynthetic capacity.
   (this thesis)

3. For scientific progress, replication is equally important as novelty.

4. Researchers have a responsibility to report negative results.

5. Legalization of cannabis only works well when there is a high level of social responsibility.

6. A flexible mindset improves productivity, whereas flex desks decrease productivity.

Propositions belonging to the thesis, entitled

Shining the spotlight on medicinal cannabis: From rooting through flowering to specialized metabolites

Wannida Sae-Tang
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Shining the spotlight on medicinal cannabis:

From rooting through flowering to specialized metabolites

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Shining the spotlight on medicinal cannabis:

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Thesis

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To my papa,

whose love and wisdom continue to inspire me.

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Chapter 1

General introduction

1. Medicinal Cannabis

Cannabis (*Cannabis sativa* L.) is an annual herbaceous plant that belongs to the Cannabaceae family (Small, 2017). Cannabis is dioecious, meaning that the male and female flowers occur on separate plants, however some genotypes are monoecious (Raman et al., 2017). The natural origin of cannabis has been suggested to be in low-latitude regions (Zhang et al., 2018) based on genetic and geological distances, and it has been distributed all over the world due to human use. This plant has a long history of utilization as textile fiber, oil, and folk medicine with documented evidence from around the world (Xie et al., 2023). Although the classification in the genus Cannabis is still ambiguous, it is widely considered that there is only one highly polymorphic species of *C. sativa* L., which comprises three subspecies, namely *sativa*, *indica* and *ruderalis* (Small and Cronquist, 1976; Zhang et al., 2018). Differences in plant morphology, origin and even clinical properties are frequently used for classification (McPartland, 2018). Most commercial medicinal cannabis cultivars are hybrids of *indica* and *sativa*. Furthermore, cannabis is often classified based on its chemical profile (Fischedick et al., 2010), such as the chemotype system (Small and Beckstead, 1973). This system includes chemotype I, characterized by high delta-9-tetrahydrocannabinol (THC) (>0.3%) which has a psychoactive effect and low cannabidiol (CBD) (<0.5%); chemotype II, an intermediate chemotype with both THC (>0.3%) and CBD (>0.5%) as dominant; and chemotype III, mainly containing CBD (>0.5%) and very low THC (<0.3%). Subsequently, chemotype IV, characterized by the prevalence of cannabigerol (CBG), and chemotype V, with very low (undetectable) cannabinoids, were introduced (de Meijer et al., 1992; Pacifico et al., 2006).

2. Specialized metabolites in medicinal cannabis

Metabolites in plants can be categorized into primary, essential for basic cell functions such as amino acids and sugars, and secondary, which are unique to specific species though contribute greatly to the molecular diversity. Secondary metabolites are now often called specialized metabolites to avoid any negative connotations and overlap (Tissier et al., 2015). In medicinal cannabis, approximately 565 specialized metabolites, have been identified. These metabolites are synthesized in specialized cell types called disk cells and accumulate in the storage cavities of glandular trichomes (Rodziewicz et al., 2019; Romero et al., 2020). The glandular trichomes are predominantly found on bracts of mature female flowers (Livingston et al., 2020) (Fig 1). Of these, over 120 belong to the group of cannabinoids, which are C21 terpenophenolic compounds

(ElSohly et al., 2017). The biosynthesis of cannabinoids starts with geranyl diphosphate (GPP), derived from the methylerythritol phosphate (MEP) pathway, and olivetolic acid, leading to the formation of the precursor cannabigerolic acid (CBGA) within the plastids of disk cells (Livingston et al., 2022). CBGA serves as the precursor for other cannabinoids, including delta-9-tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), and cannabichromenic acid (CBCA). Decarboxylation subsequently converts these acidic forms into bioactive cannabinoids like CBG, THC, CBD, cannabichromene (CBC), and cannabinol (CBN) (Richins et al., 2018) (Fig 2). Besides, an abundance of terpenoids results in the unique aroma of cannabis (Hanuš and Hod, 2020). Monoterpenoids such as myrcene, limonene, and linalool share a common precursor with cannabinoids, GPP. Sesquiterpenoids such as β-caryophyllene, guaiol, humulene, γ-yudesmol, and β-farnesene originate from farnesyl diphosphate (FPP), synthesized through the mevalonate (MVA) pathway at cytosol of disk cells (Romero et al., 2020).

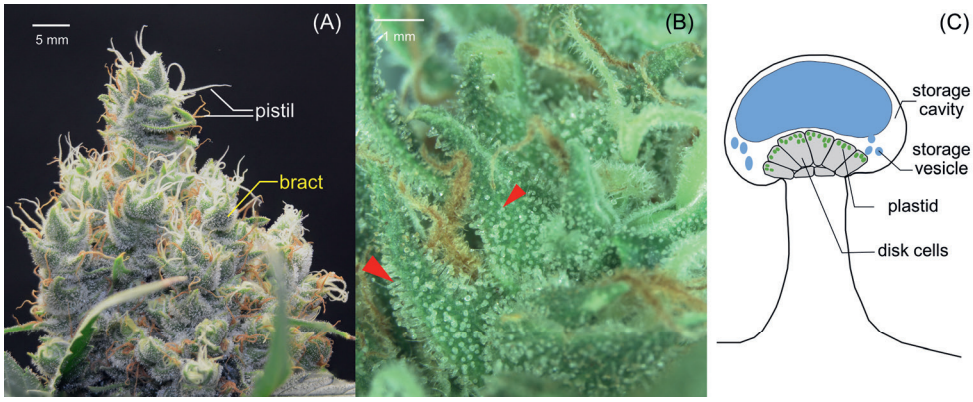

Figure 1. Female inflorescences of medicinal cannabis (A) at 8 weeks after start of the generative phase and their glandular trichomes (red arrow) on bracts (B). Illustration of glandular trichome (C) adapted from Livingston et al. (2022) and Romero et al. (2020).

Each individual compound may have specific medicinal properties, and there is growing interest in the entourage effect, which suggests a potential synergistic property when various compounds are combined (Russo, 2011). This may shift the focus from extracting individual compounds to utilizing the entire raw material for medical purposes. Consequently, the production must consider both the concentrations and variety of compounds present. Due to its psychoactive effect, cannabis has been banned for decades. However, with several studies demonstrating the significant therapeutic

benefits of medicinal cannabis, it is being approved in a number of countries (Caulkins and Kilborn, 2019; Ransing et al., 2022), resulting in a rapidly growing demand. Thus, a high level of cultivation knowledge is required to cultivate cannabis plants with uniform potency and consistent production over time.

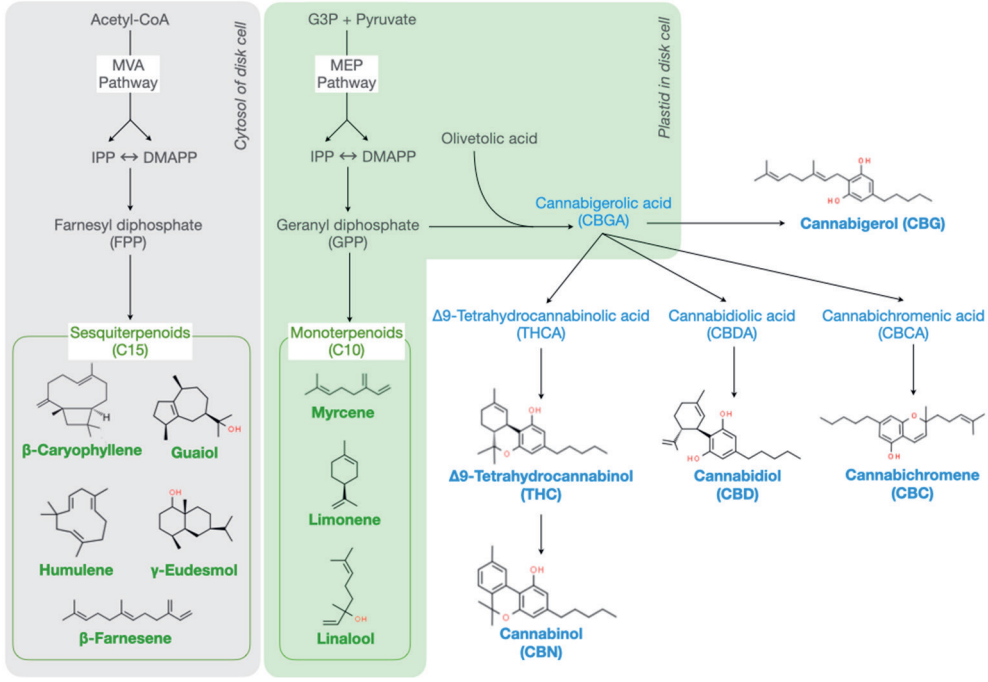

Figure 2. General scheme of biosynthetic pathways of terpenoids and cannabinoids adapted from Deguchi et al. (2020) and Romero et al. (2020) . The chemical structures are obtained from ChemSpider (Royal Society of Chemistry, 2024).

3. Cultivation cycle of medicinal cannabis

Medicinal cannabis cultivation involves three main phases: propagation, vegetative phase, and generative phase, until the harvest of inflorescences (Fig 3). Each phase requires specific conditions to ensure optimal plant development.

3.1. Plant propagation

Stem cuttings, also called “cloning” by growers, is a widely-used method of asexual propagation because it enables to produce large numbers of uniform, genetically identical plants with desired characteristics (Potter, 2014) at relatively low cost. Adventitious root formation is a complex process, which leads to roots at the base of the stem cuttings. It is affected by climatic conditions such as air and substrate humidity, temperature and light, as well as endogenous factors including phytohormones and

carbohydrate status of the cuttings (Ruedell et al., 2015). In practice, exogenous auxin (e.g. indole-3-butryic acid, IBA) is applied to enhance rooting (Caplan et al., 2018). The stem cuttings are rooted in a closed chamber to ensure high humidity. This phase usually takes 14-24 days, depending on cultivar. During this stage, light should be provided with a long photoperiod (≥ 16 h per day) to prevent flowering.

3.2. Vegetative growth

Once rooted cuttings are obtained, they can be transplanted and enter the "vegetative phase", a term commonly used by cannabis growers. During this phase, the vegetative organs, including roots, leaves and stems are developed (Moher et al., 2022). This phase in cannabis is vital to gain the desirable plant size, according to preference of growers, and its duration can vary from 10 days to one month (Saloner et al., 2019). Pruning is often performed during this phase to modify plant architecture which is beneficial for the later stage. For instance, creating an open architecture increases yield by improving microclimate and light interception at the bottom of the canopy (Danziger and Bernstein, 2021). A long photoperiod (recommended ≥16 h of light per day) is necessary to maintain vegetative growth while preventing flowering (Chandra et al., 2017a; Potter, 2014).

3.3. Flowering

Cannabis is identified as a shortday plant, naturally flowering in early autumn when the photoperiod becomes shorter and the nights longer than the critical dark period threshold (Dowling et al., 2021). Some studies suggest that cannabis flower initiation is age-dependent, as solitary flowers occur under both long and short photoperiods (Spitzer-Rimon et al., 2022, 2019). Nonetheless, short photoperiods are essential for flower maturation and branching at the apex, leading to the development of dense inflorescences (Duchin et al., 2020; Spitzer-Rimon et al., 2019).

In cannabis production, the vegetative phase is followed by the "flowering phase", referred to as the "generative phase" in this thesis. During this phase, the plants are exposed to a short photoperiod, typically 12 h of light per day, to initiate flowering. This phase lasts for 8-10 weeks until the inflorescences are mature and ready to be harvested (Chandra et al., 2017). Determining the optimal harvest timing to maximize inflorescence yield and cannabinoid content is subjective and can depend on factors such as genotype and growing conditions. Growers often base their decision on the coloration of glandular trichomes (Fig 1A-B) (Punja et al., 2023) and pistils (Fig 1A), firmness of inflorescences, and aroma (reviewed by Jin et al., 2019).

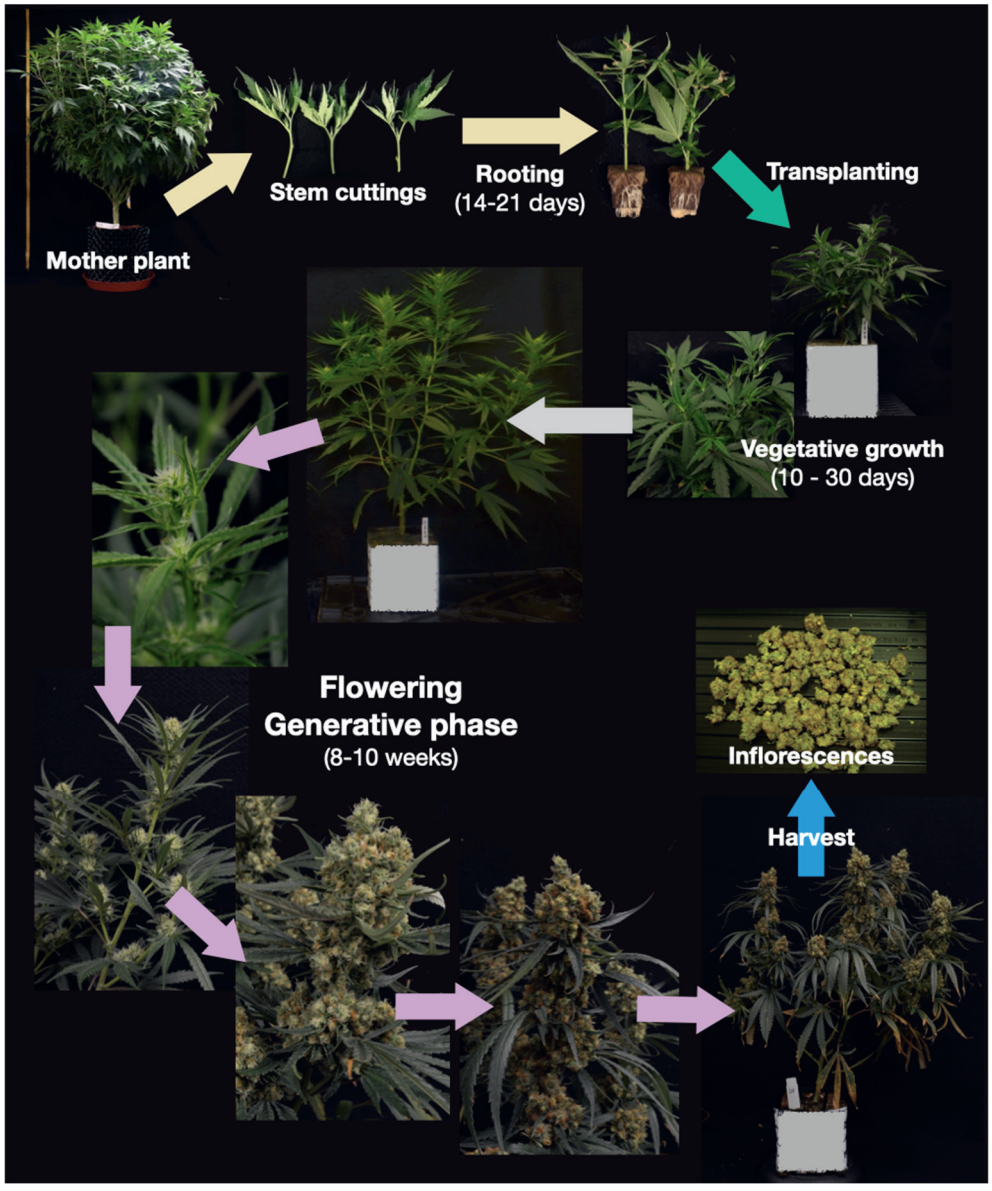

Figure 3. Cultivation cycle of medicinal cannabis.

4. Indoor cultivation using artificial light for medicinal cannabis

Medicinal cannabis is often cultivated indoors, both at large-scale farms as well as home-scale growers (Hammersvik et al., 2012). Indoor cultivation with artificial light provides favorable growing conditions, including temperature, air humidity, carbon dioxide

concentration, and light, to maximize yield and to potentially achieve a consistent specialized metabolite production (SharathKumar et al., 2020). This comes along with a high standard of safety and consistency of medical grade materials in which the use of pesticides is not allowed. Despite the high investment and operational costs of indoor cultivation, the potential for significant returns from medicinal cannabis production can offset these costs (Seaborn, 2020; Vanhove et al., 2012), making it an appealing business opportunity.

Artificial lighting is a major cost in indoor cultivation (Kozai and Sasaki, 2013). Recently, traditional lighting sources like fluorescent and high-pressure sodium (HPS) lamps have been replaced by light-emitting diodes (LEDs) (Bantis et al., 2018). LEDs offer several advantages, including high efficacy, a variety of light colors, compact size, and less heat emission (Kozai, 2016; Kusuma et al., 2020). The adjustable lighting aligns perfectly with the requirements of medicinal cannabis cultivation, where light conditions—particularly light intensity and photoperiod—must vary according to each growth phase to optimize plant development and achieve the desired yield. This allows for more cultivation cycles per year, regardless of natural seasons. Moreover, the homogeneous control of light could control the quantity and quality of production, compared to open-field cultivation.

5. Role of light in plant growth and development

Light is an essential source of energy (light intensity or daily light integral (DLI)) to drive photosynthesis which determines plant growth. Besides, light spectrum and photoperiod provides signals, perceived by photoreceptors influencing plant physiology and morphological development. Additionally, effects of light intensity, light spectrum and photoperiod may interact with one another.

5.1. Photosynthesis

The light employed in photosynthesis is referred to as photosynthetic photon flux density (PPFD), which ranges from 400 to 700 nm and is also known as photosynthetically active radiation (PAR) (McCree, 1971). This light is captured by plants through photosynthetic pigments, such as chlorophyll and carotenoids (Simkin et al., 2022), and converted into chemical energy. In many horticultural crops, every 1% increase in light intensity results in a 0.7%-1% increase in harvestable biomass (Marcelis et al., 2006). Studies in cannabis have shown that the leaf photosynthetic rate increases significantly with increasing light intensity, up to 1,500-2,000 μmol $m^{-2}s^{-1}$, and it is not fully light-saturated

(Chandra et al., 2015, 2008). Furthermore, an increase in inflorescence yield with an increase in light intensity was observed in cannabis (Eaves et al., 2020; Rodriguez-Morrison et al., 2021a).

The light spectrum also influences photosynthesis. **Red** (600-700 nm) was found to have the highest spectral quantum efficiency (Evans, 1987; McCree, 1971). However, sole red light causes red light syndrome, characterized by low chlorophyll fluorescence (F_v/F_m), unresponsive stomata, and impaired plant growth (Hogewoning et al., 2010; Trouwborst et al., 2016). Adding **blue** (400-500 nm) to red is necessary to maintain regular plant architecture and photosynthesis capacity. The presence of blue enhances stomatal conductance and chloroplast relocation which improve photosynthetic efficiency (Boccalandro et al., 2012; Kagawa, 2003). **Green** (500-600 nm) is less absorbed by leaves but penetrates deeper through the leaf and canopy, potentially improving canopy photosynthesis (Smith et al., 2017). However, a meta-analysis showed that green photons are equally effective as red and blue photons for biomass production (Chen et al., 2024). Although **far-red** (700-800 nm) is outside the PAR range, it has recently been reported that its photons between 700 and 750 nm are equally efficient in driving canopy photosynthesis than PAR, when provided together with PAR (Zhen and Bugbee, 2020). Light spectrum can also indirectly enhance plant photosynthesis by influencing plant architecture and hence light interception.

5.2. Photomorphogenesis

In addition to PAR, a broader range of wavelengths from UV-B to far-red can be also sensed by several plant photoreceptors which provide signals to mediate photomorphogenesis (Kami et al., 2010). Blue light affects the plant photoreceptor, phototropin, which regulates phototropism and leaf expansion (Briggs and Christie, 2002). Blue also triggers cryptochrome, leading to reduced internode length and compact plant (Cope et al., 2014; Wollaeger and Runkle, 2015). Red and far-red are perceived by phytochromes. Far-red reduces phytochrome photostationary state (PSS), which is the ratio between the amount of active phytochrome (Pfr) and the total amount of phytochrome (sum of active (Pfr) and inactive (Pr) phytochromes) (Sager et al., 1988). Far-red light induces shade avoidance responses such as elongation of stem and leaf, by converting phytochromes from their active to inactive form, which increased endogenous auxin accumulation (Franklin, 2008; Mroue et al., 2018; Tao et al., 2008). As far-red could enhance auxin accumulation, this was found to improve rooting in stem cuttings of chrysanthemum (Christiaens et al., 2019). Adding far-red to a red/blue

background light increased tomato plant dry mass and fruit production. This was due to increased total light absorption, accelerated flowering, and increased dry matter partitioning to fruits (Ji et al., 2020; Kalaitzoglou et al., 2019). Furthermore, bud outgrowth or branching is inhibited by far-red in rose (Demotes-Mainard et al., 2016). Compared to HPS lamps which has low blue light, cannabis under blue-rich LEDs showed shorter and more horizontally oriented branch growth (Magagnini et al., 2018; Namdar et al., 2019).

Flowering of many plants is photoperiod dependent. In the shortday model plant chrysanthemum, flowering is controlled by *FLOWERING LOCUS T (FT)-like* genes (*CsFTL*) and antiflorigenic FT/TFL family protein genes (*CsAFT*) (Higuchi, 2018). Repeated shortday cycles are needed, which diminishes *CsAFT* expression and continuously upregulates *CsFTL3* expression, to obtain flower development until anthesis (Nakano et al., 2019). Shortday results in a reduced DLI when PPFD remains unchanged, thereby limiting crop photosynthesis (Eichhorn Bilodeau et al., 2019). Photoperiod extension with blue (i.e., a longday consisting of shortday followed by 4 h of blue light) has been studied in chrysanthemum and also several other shortday species to increase DLI during shortdays, without negative effects on flower bud formation (Jeong et al., 2014; SharathKumar et al., 2024). In some shortday, plants a single long night is enough to initiate flowering, for example cockle bur (*Xanthium strumarium* L.) and Japanese morning glory (*Pharbitis [Ipomea] nil*.) (Thomas and Vince-Prue, 1996). Conversely, in soybean, another shortday plant, the flowering process can be reversed when switching from shortday to longday (Han et al., 2006; Wu et al., 2006). However, the response of cannabis flowering to photoperiod extension and its effects when applied after flower initiation are not clear.

5.3. Biosynthesis of specialized metabolites

Plants require sufficient light for photosynthesis, which supplies the necessary carbon source for specialized metabolite biosynthesis (Darko et al., 2014; Li et al., 2020). An increase in light intensity increases concentrations of these metabolites in mint (*Mentha arvensis* L.), *Glechoma longituba* (Nakai) Kupr., and some leafy vegetables (de Souza et al., 2015; Thoma et al., 2020; Zhang et al., 2015).

Specialized metabolites are often produced in response to environmental stress, including high light stress, and these metabolites serve as photo-protectants or antioxidants (Ouzounis et al., 2015; Ramakrishna and Ravishankar, 2011). Furthermore, some enzymes in the biosynthesis pathways are regulated by light. For

example, in carotenoid biosynthesis, phytoene synthase is up-regulated by elongated hypocotyl 5 (HY5) as a consequence of a high red:far-red ratio (reviewed by Contreras-Avilés et al., 2024). **UV** (280-350 nm), with its short wavelength and high frequency, induces the production of specialized metabolites like polyphenols, terpenoids, and alkaloids. Blue, located next to UV in the spectrum, serves a similar role in stimulating the production of these metabolites (Thoma et al., 2020; Zhang et al., 2021). In cannabis, Bernstein et al. (2019) reported that cannabinoids including THC, CBD, CBG and CBC accumulated more in inflorescences at higher positions and was greater at the top which may refer to a UV protection property of cannabinoids. Compared to HPS lamps, plants grown under blue-enriched LEDs (blue:red 4:1) produced 40% less total inflorescence mass. However, these inflorescences exhibited a 66% higher total cannabinoid content and an increase in cytotoxicity to cancer cells (Namdar et al., 2019). The positive effect of blue from LEDs was also found on CBD and CBG content in inflorescence compared to low blue from HPS lamps (Magagnini et al., 2018). On the other hand, a study by Westmoreland et al. (2021) did not find an effect of blue on cannabinoids.

Many studies have established the impact of light on commercial crops, such as leafy vegetables, leading to the formulation of optimal lighting strategies used in indoor production (Neo et al., 2022). Nevertheless, research on cannabis is still relatively limited, and the effects of light are not consistently reported.

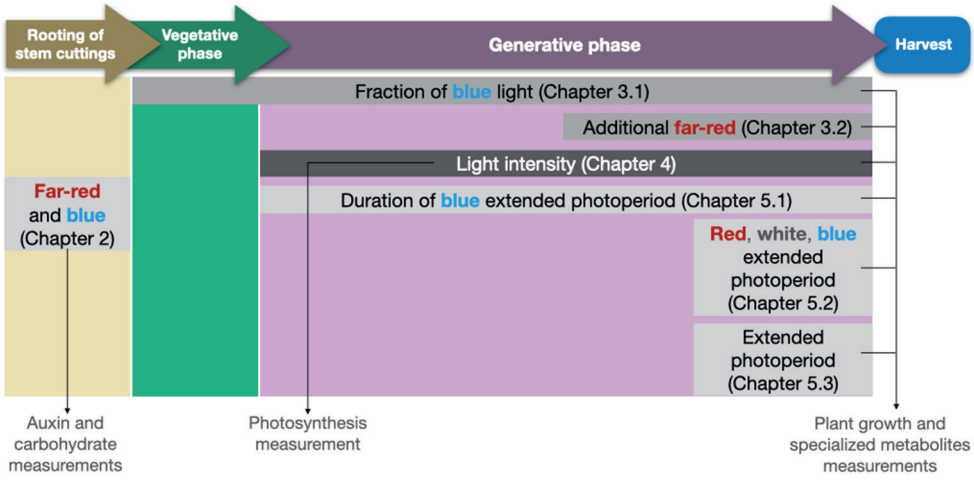

Figure 4. Schematic representation of the research reported in this thesis. Chapters are aligned with the growing phases of medicinal cannabis.

4. Aim of research and thesis outline

The overall aim of this research is to understand the physiological and morphological responses of medicinal cannabis to light spectrum, light intensity, and photoperiod. Light effects are investigated for crucial processes in different developmental stages, i.e., rooting of cuttings, plant architecture during the vegetative stage, flower induction and development and accumulation of specialized metabolites during the generative phase (Fig 4). The roles of light found in this research aim to support the optimization of light strategies for indoor medicinal cannabis production to achieve a high yield and quality based on scientific evidence.

Four research questions are formulated according to each experimental chapter (Chapter 2-5):

- Do far-red light and blue light increase rooting in stem cuttings via accumulation of auxin and carbohydrates?
- Do fraction of blue light and additional far-red light influence concentrations of specialized metabolites and inflorescence yield?
    - Does high blue light increase concentrations of specialized metabolites but decrease inflorescence yield due to compact plant?
    - Does additional far-red light during the latter part of the generative phase increase concentrations of specialized metabolites, inflorescence yield, and inflorescence elongation?
- Does high light intensity increase inflorescence yield and concentrations of specialized metabolites, and which underlying components are responsible for this increase?
- Does extending the photoperiod after flower initiation still impair flowering? If not, does the extended photoperiod increase dry matter production and influence specialized metabolites?

In **Chapter 2**, I examined the impact of light spectrum on the rooting of stem cuttings. The hypothesis was that the presence of far-red and the absence of blue promotes adventitious rooting, which is mediated by the accumulations of auxin and carbohydrates. Adventitious rooting on stem cuttings was studied for conditions where far-red was added to either red:blue or sole red. Cuttings were treated with and without

exogenous auxin powder. Auxin and carbohydrate levels in leaves and stem tissues were analyzed to investigate correlations with rooting under these light spectra.

In Chapter 3, I focused on light spectra applied during the vegetative and generative phases to observe the responses of plant morphology, dry matter production, and concentrations of specialized metabolites. This chapter consists of two experiments. In one experiment, different fractions of blue in white light were applied throughout vegetative and generative phases. It was hypothesized that low blue increases inflorescence yield as a result of less compact plants while high blue increases concentrations of specialized metabolites. The other experiment studied additional far-red during the last half of the generative phase compared to normal white light. It was hypothesized that far-red causes elongation of the inflorescence and increases inflorescence yield, and that far-red also increases the concentration of specialized metabolites.

In Chapter 4, I examined the effects of light intensity, ranging from 600 to 1000 µmol $m^{-2}s^{-1}$ PPFD during the generative phase on dry matter production and specialized metabolites. It was hypothesized that high light intensity increases concentrations of specialized metabolites, and high light intensity also increases dry matter production due to increased photosynthesis rate. Photosynthesis light response curves were measured along the treatment period. Furthermore, the underlying components explaining responses of yield to light intensity were studied using yield component analysis.

In Chapter 5, I focused on flowering regulated by photoperiod to explore the possibility of increasing inflorescence yield and cannabinoids by an extended photoperiod after flower initiation. It was hypothesized that an extended photoperiod by blue light does not impair flower development. Hence, extending the photoperiod by blue light would increases inflorescence yield due to higher cumulative light interception and would increase concentration of cannabinoids due to high fraction of blue light. For this, a series of experiments was conducted with various strategies of extending photoperiod, including various durations, light spectra and light intensities.

In Chapter 6, the findings of this thesis are discussed in relation to relevant literature. Future research was proposed to address knowledge gaps found in cannabis studies on light, as well as the possibility of expanding a cannabis study to other crops. Lastly, based on this thesis, recommendations for lighting use in cannabis indoor cultivation are provided.

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Chapter 2

Effect of far-red and blue light on rooting in medicinal cannabis cuttings and related changes in endogenous auxin and carbohydrates

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Abstract

Stem cuttings are used in the commercial cultivation of many crops, including medicinal cannabis, to produce large numbers of uniform and genetically identical plants. Light is an important environmental factor determining the success of the rooting of stem cuttings. The aim of this study was to investigate the influence of different fractions of far-red and blue during the adventitious rooting phase of medicinal cannabis stem cuttings on rooting and whether these effects are related to changes in endogenous auxin and/or carbohydrates. Two experiments were conducted in climate chambers with sole LEDs lighting (blue, red, far-red) using two cannabis cultivars. In Experiment 1, four light treatments were applied: 100 µmol $m^{-2}s^{-1}$ red:blue (88:12) with additional 0, 50 or 100 µmol $m^{-2}s^{-1}$ far-red and a fourth treatment with 100 µmol $m^{-2}s^{-1}$ sole red with additional 50 µmol $m^{-2}s^{-1}$ far-red. In Experiment 2, the following four light treatments were applied: 90 µmol $m^{-2}s^{-1}$ red:blue (45:45) with additional 0 or 45 µmol $m^{-2}s^{-1}$ far-red, a third treatment with 45 µmol $m^{-2}s^{-1}$ sole red with additional 45 µmol $m^{-2}s^{-1}$ far-red, and a fourth dynamic treatment which was 45 µmol $m^{-2}s^{-1}$ sole red with additional 45 µmol $m^{-2}s^{-1}$ far-red for 7 days followed by 90 µmol $m^{-2}s^{-1}$ red:blue (45:45) for 14 days. The effects on rooting in both experiments were measured after 21 days of light treatments. In Experiment 2, periodic measurements of auxin and carbohydrates were performed. Far-red improved adventitious rooting only in Experiment 2, where both cultivars responded similarly. Adding far-red only during the initial stage (7 days) of rooting was sufficient to improve rooting, while it did not result in excessive stem elongation. The presence or absence of blue did not significantly affect rooting. Although the positive effects of far-red on auxin and carbohydrate concentrations in stem cuttings are a likely explanation for the observed effects of far-red on rooting, we did not find a correlation between auxin or carbohydrates and rooting.

Keywords: light spectrum; rooting; cannabis; IAA; sugar; starch

1. Introduction

Cannabis (*Cannabis sativa* L.) has a long history of usage as medicine, oil, fiber, and textile. This plant species contains a number of specialized metabolites, including cannabinoids, terpenes, and flavonoids, with their contents depending on genotype (Andre et al., 2016). These specialized metabolites are synthesized and accumulated in glandular trichomes located densely on mature female inflorescences (Livingston et al., 2020). The dominant cannabinoids are delta-9-tetrahydrocannabinol (THC), which has psychoactive effects, and cannabidiol (CBD) which has therapeutic effects for a variety of chronic diseases, particularly those involving the neurological system (Andre et al., 2016; Richins et al., 2018). A synergistic effect of several cannabinoids and terpenes is known as the entourage effect, which has been hypothesized to lead to enhanced therapeutic benefits (Grotenhermen, 2003; Russo, 2011). Since its medical benefits have been demonstrated, the legalization of medicinal use cannabis occurs in a number of countries (Caulkins and Kilborn, 2019; Rehm and Fischer, 2015), resulting in rapidly growing demand. Hence, a high level of cultivation knowledge is needed to produce cannabis plants with uniform potency and stable production over time.

In commercial cannabis cultivation, young plants are vegetatively propagated from stem cuttings to produce large numbers of uniform and genetically identical plants at relatively low cost (Potter, 2014). The rooting of stem cuttings is a crucial process. Endogenous auxin may induce adventitious roots (Pacurar et al., 2014). Indole-3-acetic acid (IAA) is the most abundant natural auxin (Kerr and Bennett, 2007); it is produced in the shoot apical meristem or young leaves and then polarly transported to the base by cell-to-cell transport or through phloem (Muday and DeLong, 2001; Swarup et al., 2001). The auxin accumulation at the root zone initiates cell division and adventitious root formation (Vanneste and Friml, 2009). In addition, rooting cofactors such as amino acids, vitamins, microelements, polyphenols, and phenolics may be required as auxin-synergists during root initiation (Foong and Barnes, 1981; Heuser, 1976; Jarvis, 1986). In practice, synthetic auxins such as indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) are often applied to enhance root formation from cuttings in several horticultural plants, including cannabis (Blythe et al., 2007; Caplan et al., 2018; Chandra et al., 2017b). Nevertheless, the use of synthetic chemicals may no longer be allowed in medicinal cannabis production due to food and drug safety (Lenton et al., 2018; Taylor and Birkett, 2020).

Light is one of the environmental factors determining the success of rooting by being a source of energy in photosynthesis and a signal to control photomorphogenesis. Nevertheless, high radiation may cause auxin breakdown (Jarvis and Shaheed, 1987). Especially in some woody species, stem cuttings rooted better in the dark than in light (Druart et al., 1982; Fett-Neto et al., 2001). Far-red light (700-800 nm) increased endogenous auxin accumulation via phytochromes and triggered shade avoidance responses in *Arabidopsis* (Mroue et al., 2018; Tao et al., 2008). The higher expression of auxin synthesis genes was consistent with increased accumulation of auxin and stem elongation in far-red enriched light (Gommers et al., 2018). Hence, this increase in auxin by far-red could have a positive effect on rooting. In several species, such as *Chrysanthemum*, *Rhododendron*, Chinese *Thuja*, and *Leucothoe*, the rooting success rate of cuttings was greater under a lower red: far-red ratio (Christiaens et al., 2019, 2016; Park et al., 2022). Blue light (400-500 nm) generally inhibits plant elongation, mediated by cryptochromes (Ahmad et al., 2002; Pedmale et al., 2016), depending on the species and fraction of blue (Kong et al., 2018; Larsen et al., 2020). As the effects of blue on elongation might be opposite to those of far-red, blue might counteract the effects of far-red on rooting.

Besides auxin, carbohydrates also affect rooting. Carbohydrates are a substrate for root formation, and supply energy for roots (Corrêa et al., 2005). In some cases, a high accumulation of carbohydrates at the basal part improves the root formation of cuttings, as reviewed by da Costa et al. (2013). Far-red is reported to influence carbohydrate status in plants. Adding far-red to shorter-wavelength photons can enhance photosynthesis (Zhen and Bugbee, 2020). In soybean seedlings far-red increased photosynthetic rate, resulting in an increase in carbohydrate content (Yang et al., 2020). In tomato fruits and leaves, far-red increased soluble sugar content by regulating starch breakdown (Courbier et al., 2020; Ji et al., 2020). Thus, far-red may enhance rooting by altering the levels of carbohydrates.

Although it is known that light spectra influence the metabolism of auxin and carbohydrates, the consequences for rooting are less known and could be species-dependent. The role of light spectra in medicinal cannabis is still unrevealed. Therefore, this study aims to investigate the influence of different fractions of far-red and blue light during the adventitious rooting of medicinal cannabis stem cuttings. We investigated whether this potential influence is related to a change in auxin and/or carbohydrate accumulation, based on the hypothesis that presence of far-red and absence of blue

results in accumulation of auxin and carbohydrates, leading to an enhancement of rooting.

2. Materials and methods

2.1. Cutting specification and rooting condition

Two experiments on the effects of light spectra on the rooting of stem cuttings of medicinal cannabis (*Cannabis sativa* L.) cultivars 'White Russian' ('WR') and 'Critical CBD' ('CCBD') were conducted. The 'WR' is a chemotype I (a high THC/CBD ratio; >1) while the 'CCDB' is a chemotype II (an intermediate THC/CBD ratio; 0.5-0.3) (de Meijer et al., 1992; Pacifico et al., 2006). Stem cuttings were cut from 3-6 months old mother plants grown in a glasshouse (Wageningen University and Research, Greenhouse Horticulture, Bleiswijk, the Netherlands). The cuttings were collected from different batches of mother plants, taken in May 2019 (replicate 1 of Exp 1), June 2019 (replicate 2 of Exp 1), July 2019 (replicate 3 of Exp 1), April 2020 (replicate 4 of Exp 1), June 2020 (replicate 5 of Exp 1), October 2020 (replicate 1 of Exp 2), and May 2021 (replicate 2 of Exp 2). The averages of percentage of rooted cuttings from all cultivars and treatments in each replicate were 53.6%, 52.9%, 66.1%, 92.4%, and 93.9% in Exp 1 and 75.7%, and 59.9% in Exp 2. The climate conditions in the glasshouse were 24-25 °C, 65%-70% relative humidity (RH) and 550-750 µmol CO_2 mol^{-1} under a 16-h (replicate 4 of Exp 1) or an 18-h photoperiod (all other replicates) of solar light, supplemented with about 400 µmol $m^{-2}s^{-1}$ of either HPS lamps (SONT Philips, Eindhoven, the Netherlands) for Exp 1 or LEDs, consisting of 86% red, 6% green and 8% blue for Exp 2 (Green Power DRW LB, Philips, Eindhoven, the Netherlands). The cuttings were excised from the top shoots of the mother plants, 9±1 cm long and 0.3±0.1 cm stem diameter, with 2-3 fully expanded leaves. The cuttings were taken in the morning, placed in closed plastic bags, and transferred to a climate-controlled room where light treatments were applied from the same day onwards.

For the first 7 days, the cuttings were under a transparent plastic to maintain high humidity. The light transmission of the transparent plastic without condensation was about 95%. The temperature and RH in the growth chamber were set at 25 °C and 80% (Exp 1) or 28 °C and 85% (Exp 2), without supplemental carbon dioxide. The realized temperature was 25.0±0.1 °C and RH was 78.7±1.9% (Exp 1), or 27.8±0.8 °C and 84±0.6% (Exp 2), measured at plant level. The cuttings were irrigated with a nutrient solution with EC 1.5 dS m^{-1}, pH 5.0, 1.25 mM NH_4^+; 6.2 mM K^+; 1.9 mM Ca^{2+}; 0.9 mM

Mg^{2+}; 10.5 mM NO_3^-; 0.85 mM SO_4^{2-}; 0.85 mM PO_4^{3-}; 60 µM Fe^{2+}; 20 µM Mn^{2+}; 3 µM Zn^{2+}; 20 µM B^{2+}; 0.5 µM Cu^{2+}; 0.5 µM Mo^{2+}, at 2-4 days interval depending on the wetness of the substrate. Light was provided during an 18-h photoperiod by LEDs with adjustable spectrum (Green Power Dynamic 2.0 LED research modules, with a GrowWise Control System, Philips, Eindhoven, the Netherlands). The room was divided into 4 compartments separated by white plastic sheets to arrange 4 light treatments (Table 1). The light spectrum and intensity were measured using a spectroradiometer (Specbos 1211, JETI, Jena, Germany) for 9 points m^{-2} without the transparent plastic covering, at 15 cm height from the table (approximately at plant height); the distance from the plant height to the lamps was 165 cm, and the results are shown in Table S1.

Table 1. Overview of light treatments in Exp 1 and 2 with their spectral distribution. R is red light (600-700 nm), B is blue light (400-500 nm), and FR is far-red light (700-800 nm). Phytochrome stationary state (PSS) is calculated according to Sager et al. (1988). PPFD means photosynthetic photon flux density (400-700 nm; µmol $m^{-2}s^{-1}$). PFD means photon flux density (400-800 nm; µmol $m^{-2}s^{-1}$)

Light treatments		PFD			PSS	PPFD	PFD
		R	B	FR			
Exp 1	R88B12	88	12	0	0.88	100	100
	R88B12+FR50	88	12	50	0.77	100	150
	R88B12+FR100	88	12	100	0.68	100	200
	R100+FR50	100	0	50	0.78	100	150
Exp 2	R45B45	45	45	0	0.86	90	90
	R45B45+FR45	45	45	45	0.69	90	135
	R45+FR45	45	0	45	0.70	45	90
	Dynamic -Day 1-7 (R45+FR45)	45	0	45	0.70	45	90
	-Day 8-21 (R45B45)	45	45	0	0.86	90	90

Experiment 1

Cuttings were inserted into fine river sand in black plastic pots (8×8×13 cm, width×length×height) with one cutting per pot. The sand allowed for the observation of the roots at the end of the experiment. Before insertion into the sand, for half of the cuttings, the lower end of the stem was dipped in a synthetic auxin powder, 0.25% indole-3-butyric acid (IBA) (Rhizopon, the Netherlands). Four light treatments were applied, consisting of (1) 100 µmol $m^{-2}s^{-1}$ red:blue (88:12) without far-red, R88B12; (2) with 50

µmol $m^{-2}s^{-1}$ far-red, R88B12+FR50; (3) with 100 µmol $m^{-2}s^{-1}$ far-red, R88B12+FR100; or (4) 100 µmol $m^{-2}s^{-1}$ sole red with 50 µmol $m^{-2}s^{-1}$ far-red, R100+FR50 (Table 1, Table S1).

Final rooting assessment, after washing out the sand, was performed twenty-one days after start of treatments. Cuttings with at least one root longer than 0.5 cm were considered as rooted cuttings, when calculating the percentage of rooted cuttings. The roots were dried in a ventilated oven at 105°C for 48 h to obtain the dry weight. The height of rooted cuttings was measured from top internode to stem end.

Experiment 2

Cuttings were inserted into 3.6×3.6×4 cm stonewool plugs (Grodan, the Netherlands), placed in a 7x11-hole plastic tray. Four light treatments were applied, consisting of (1) 90 µmol $m^{-2}s^{-1}$ red:blue (45:45) without far-red, R45B45; (2) with 45 µmol $m^{-2}s^{-1}$ far-red, R45B45+FR45; (3) 45 µmol $m^{-2}s^{-1}$ sole red with 45 µmol $m^{-2}s^{-1}$ far-red, R45+FR45; and (4) a dynamic treatment in which the cuttings were under R45FR45 for 7 days followed by R45B45 for 14 days (Table 1, Table S1). No external synthetic auxin was applied.

Final rooting assessment was performed twenty-one days after start of treatments, same as for Experiment 1. Rooting was scored after vertically cutting the stonewool plug into two halves using the following criteria: 1= no browning, no thickening, no callus, and no rooting, 2= browning and thickening stem, 3= callus (larger than 1 mm^3), 4= root tips , 5= few roots longer than 0.5 cm inside plug, 6= few roots outside plug, 7= roots covered half of the stonewool plug, 8= roots fully covering the stonewool plug. The cuttings with a score of 4 or higher were considered as rooted cuttings. The height of rooted cuttings was measured as explained for Experiment 1.

2.2. IAA extraction and analysis

In Experiment 2, the youngest fully expanded leaf per cutting on day 5, 10 and 21 (final assessment) after start of treatments was collected in the middle of photoperiod to analyze the IAA content. The leaflet tissue was immediately frozen using liquid nitrogen and stored at -80°C. Three leaflets from 3 different cuttings were pooled into one sample, in total there were 6 samples of each light treatment. The samples were ground by a ball mill at 80 Hz for 20 s, after which samples of 0.01 g FW were extracted with 1 mL of ice-cold methanol (MeOH) containing [phenyl $^{13}C_6$]-IAA (0.1 nmol mL^{-1}) as an internal standard in a 2-mL Eppendorf tube. The tubes were vortexed and sonicated for

10 min in an ultrasonic water bath (Branson 3510, Branson Ultrasonics, Eemnes, the Netherlands) and placed overnight in an orbital shaker at 4°C. Next, samples were centrifuged for 10 min at 11,500 rpm in a centrifuge (Heraeus Fresco 17, Thermo Fisher Scientific, Waltham, USA) at 4°C, after which the organic phase was loaded on a 100 mg 1.5 mL^{-1} Extra-Clean SPE Amino cartridge (S*Pure Pte. Ltd., Singapore). The cartridge was equilibrated prior to sample loading, and it was subsequently washed and eluted as previously described (Ruyter-Spira et al., 2011). The MeOH was evaporated in a speed vacuum system (SPD121P, Thermo Savant, Hastings, UK) at room temperature and the residue was resuspended in 100 µL acetonitrile:water:formic acid (20:80:0.1, v/v/v). The samples were filtered through a 0.45 µm filter (Minisart SRP4, Sartorius, Goettingen, Germany) and measured on the same day. IAA was analysed using a Waters Xevo TQs tandem quadruple mass spectrometer as previously described (Schiessl et al., 2019).

2.3. Carbohydrate analysis

In Experiment 2, carbohydrates were measured according to Larsen et al. (2022). The youngest fully expanded leaf and lower 3 cm from the base of stem tissue were sampled in the middle of photoperiod on day 10 and 21 after start of treatments, freeze dried, and ground in a ball mill. 0.015 g DW of tissue powder was extracted with 5 mL of 80% ethanol at 80°C for 20 min in a shaking water bath. Then the extracts were centrifuged for 5 min at 8,500 rcf (Universal 320R, Hettich). 1 mL of supernatant was transferred to a 2-mL Eppendorf tube and dried in a vacuum centrifuge (Savant SpeedVac SPD2010, Thermo Fisher Scientific, Waltham, USA) at a setting of 50°C and 5.1 mbar for 120 min. The pellet with the remaining supernatant was stored for starch measurement at -20°C. The dried samples in Eppendorf tube were resuspended in 1 mL Milli-Q water and sonicated in an ultrasonic water bath (Branson 2800, Branson Ultrasonics, Eemnes, The Netherlands) for 10 min. The solutions were centrifuged at 21,100 rcf for 10 min (Sorvall Legend Micro 21R, Thermo Fisher Scientific). After 10 times of dilution with Milli-Q water, glucose, fructose and sucrose were quantified using a High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD; Dionex ICS500. Thermo Fisher Scientific) equipped with a CarboPac PA1 column (250x2 mm) (Thermo Fisher Scientific) eluted with 100 mM NaOH at a flow rate of 0.25 mL min^{-1} at 25°C. Chromeleon 7.2 (Thermo Fisher Scientific) was used for data analysis. Total soluble sugar was calculated as the sum of glucose, fructose, and sucrose.

The stored pellet was used for starch analysis. After washing three times with 80% ethanol, the pellet was dried in a vacuum centrifuge (Savant SpeedVac SPD2010, Thermo Fisher Scientific, Waltham, USA) at 55°C and 5.1 mbar for 25 min, then resuspended in 2 mL of 1 g L^{-1} thermostable alpha-amylase (SERVA Electrophoresis GmbH) in Milli-Q water and incubated for 30 min at 90°C in a shaking water bath. Then 1 ml of 0.5g L^{-1} amyloglucosidase (Sigma 10115) in 50mM citrate buffer (pH 4.6) was added and incubated at 60°C for 10 min in a shaking water bath. After centrifugation at 21,100 rcf for 10 min and 20-50 times of dilution with Milli-Q water, glucose was quantified using HPEAC-PAD as described above.

2.4. Statistical design and analysis

Experiment 1 consisted of 5 replicate trials that were executed consecutively, each time with new randomization, representing 5 blocks. Per replicate 15-25 cuttings were used per treatment with split-split-plot design; the main factor being light, the subfactor was with/without IBA and the sub-subfactor was cultivar. Experiment 2 consisted of 2 replicate trials, one was conducted in a commercial facility while the other was conducted at Wageningen University and Research. In trial 1 and 2, 40 and 70 cuttings were used per treatment with a split-plot design; the main factor was light and the subfactor was cultivar. The exact numbers of cuttings in each trial are shown in Table S2. IAA and carbohydrate contents were determined in trial 2 of Experiment 2, where 6 pooled samples per treatment were analysed and considered as replicates. Analysis of variance (ANOVA) was conducted followed by mean separation by Fisher's protected LSD test at P=0.05 in Genstat 19th edition (VSN International, London, UK). The normality and homogeneity of the residuals were examined with Shapiro-Wilk and Bartlett's tests, and in all cases both assumptions were met, except for the residuals of rooting parameters in Experiment 2, where homogeneity was assumed as it could not be tested because of the small number of replicates (n=2).

3. Results

3.1. Rooting

Adding far-red to a background of red:blue (R88B12) did not significantly affect the rooting of cannabis stem cuttings in any of the two cultivars in Experiment 1 where cuttings were placed in sand substrate (Fig 1A-F). Changing the fraction of blue light from

12% (R88B12+FR50) to 0% (R100+FR50) did not affect the rooting. The application of IBA enhanced the percentage of rooting and root dry weight which did not interact with light nor cultivar (Table S3). Plant height tended to increase with far-red intensity, although this was not statistically significant (Fig 1C,F). The cultivar 'WR' rooted better than 'CCBD'; on average, the percentage of rooted cuttings was about 12% higher in 'WR' than in 'CCBD'.

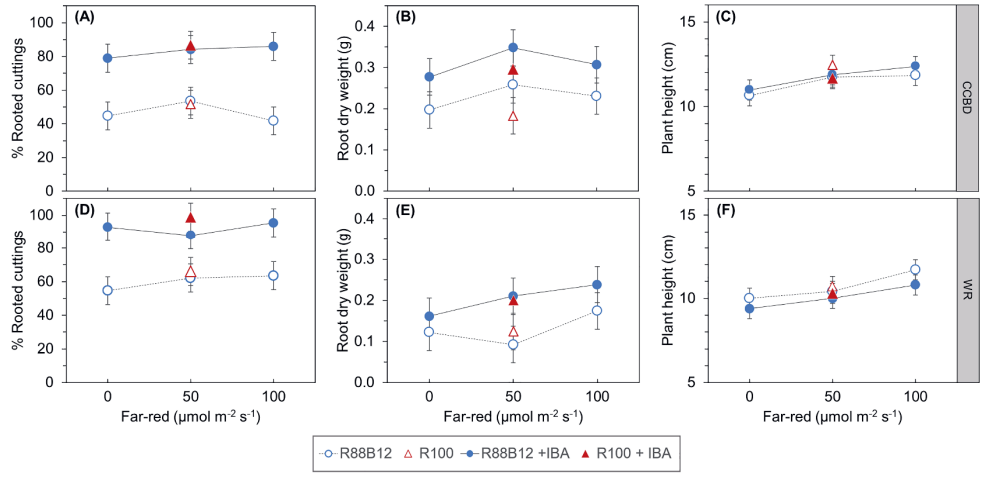

Figure 1. Effect of far-red on percentage of rooted cuttings (A,D), root dry weight (B,E) and rooted plant height (C,F) of cultivars 'CCBD' (A-C) and 'WR' (D-F) with (closed symbol) or without IBA application (open symbol) in Experiment 1. Different intensities of far-red light were added to a background of red:blue light (R88B12, 88 µmol $m^{-2}s^{-1}$ red + 12 µmol $m^{-2}s^{-1}$ blue) (blue circles) and at the intermediate far-red level the red:blue was compared with sole red background light (R100, 100 µmol $m^{-2}s^{-1}$ red) (red triangle). Data were obtained 21 days after start of treatments. All data are the means of 5 trials. The error bars indicate ± standard error of means. There is no significant effect of light on all parameters for each cultivar and each IBA application separately (n=5, P=0.05). F-Probability values are shown in Table S3.

Adding far-red to either a background of red:blue (R45B45) or a sole red of 45 µmol $m^{-2}s^{-1}$ (R45) significantly increased the percentage of rooted cuttings in both cultivars, in Experiment 2 where cuttings were placed in stonewool substrate (Fig 2A). Blue light did not affect the rooting. Light treatments did not affect the rooting scores of rooted cuttings, which on average had few roots that appeared outside the plug (Fig 2B). Plant height was strongly increased by adding far-red to either red:blue (R45B45) or sole red light (R45) (Fig 2C). For the dynamic treatment, cuttings were exposed to far-red added to sole red light for the first 7 days, followed by equal red:blue without far-red. This

dynamic treatment significantly increased the percentage of rooted cuttings, while the plant height was not statistically significantly different from the cuttings not receiving far-red. No significant difference between cultivars was shown, and no interaction between light spectrum and cultivar was found (Table S4).

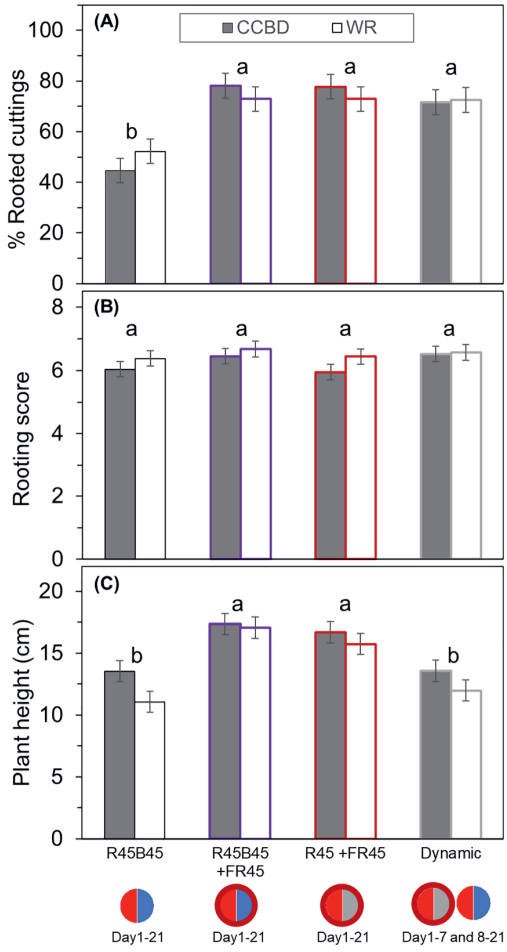

Figure 2. Effect of light spectrum on percentage of rooted cuttings (A), rooting score of rooted cuttings (higher score means more roots) (B) and height (C) of 'CCBD' and 'WR' cuttings in Experiment 2. Far-red (FR45) was added to red:blue (R45B45) and sole red (R45). The dynamic treatment means the cuttings were exposed to R45FR45 until day 7, followed by R45B45 on day 8-21. External auxin was not applied in any of the treatments. Data were obtained 21 days after start of treatments. All data are the means of 2 independent trials. The error bars indicate ± standard error of means. Since there was no cultivar effect, and no interaction between light spectrum and cultivar, the different letters indicate the significant differences of the means of both cultivars according to Fisher's protected LSD test (n=2, P=0.05). F-Probability values are shown in Table S4.

3.2. IAA content

To investigate whether the effects of far-red on rooting are related to endogenous auxin, IAA content was measured in the young leaf of cuttings at 5, 10 and 21 days after start of treatments. In the early phase (5 days after start of treatments), the IAA content in the leaf of cuttings grown under far-red was lower than for the control (no far-red), though this effect was only statistically significant in cultivar 'CCBD' (Fig 3A,D). Also at 10 days after start of treatments, far-red decreased leaf IAA content; however, this time the reduction was not statistically significant in 'CCBD'. The lowest leaf IAA content was found when far-red was added to sole red in the 'WR' cuttings (Fig 3B,E). Leaf IAA content at the end of the experiment (21 days after start of treatments) was not significantly affected by light treatments (Fig 3C,F).

3.3. Carbohydrate content

The soluble sugar and starch contents in the young leaf and stem of cuttings at 10 and 21 days after start of treatments were measured to determine whether far-red improved rooting by changes in carbohydrates accumulations. Total soluble sugar content in the young leaf was not influenced by light treatments (Fig 4A,B,E), except for cultivar 'WR' at 21 days after start of treatments, where sugar was decreased by exposure to additional far-red (Fig 4F). Total soluble sugar in the stem of cultivar 'CCBD' was significantly increased by additional far-red at 10 days (Fig 4C), but later (21 days after start of treatments) there was no effect of light (Fig 4D). In contrast, the stem of 'WR' cuttings under additional far-red had higher sugar content at 21 days after start of treatments (Fig 4H). Among all four light treatments, cuttings placed under red:blue with additional far-red showed the highest starch content in both leaf and stem at 10 days after start of treatments (Fig 5A,E,C,G). At 21 days after start of treatments, additional far-red tended to reduce starch in stem but not in dynamic treatment (Fig 5D,H).

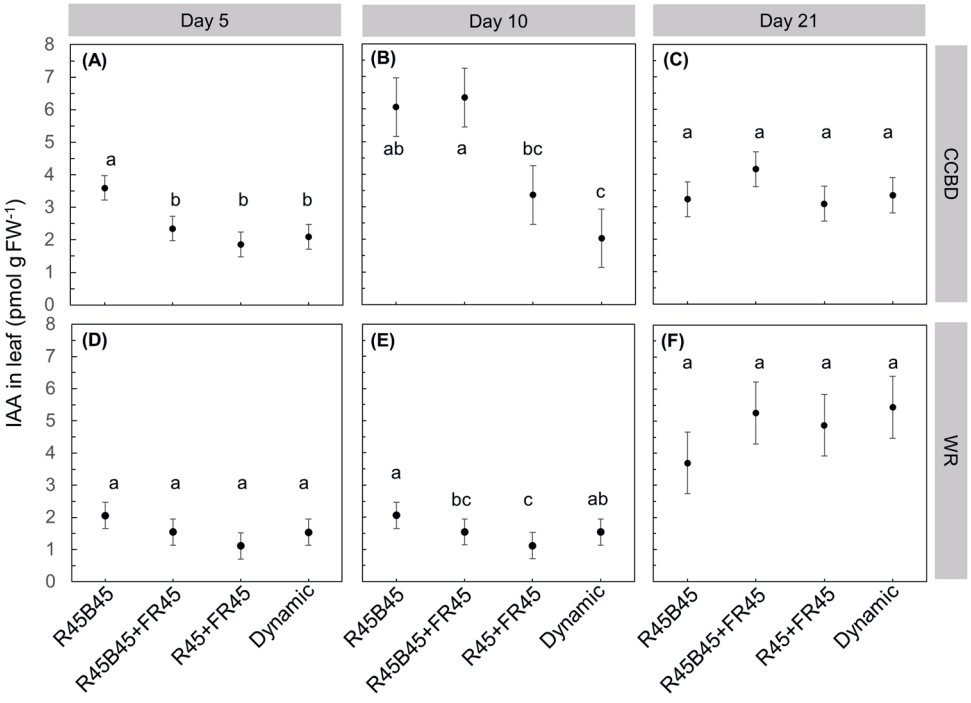

Figure 3. Effect of light spectrum on IAA content in young leaf of 'CCBD' (A-C) and 'WR' (D-F) cuttings after start of treatments for 5, 10 and 21 days in Experiment 2. Far-red (FR45) was added to red:blue (R45B45) and sole red (R45). The dynamic treatment means the cuttings were exposed to R45FR45 until day 7, followed by R45B45 on day 8-21. In none of the treatments external auxin was applied. All data are the means of 6 pooled samples per treatment from the same trial. The error bars indicate ± standard error of means. The different letters indicate the significant differences between means for each cultivar and timepoint separately according to Fisher's protected LSD test (n=6, P=0.05). F-Probability values are shown in Table S5.

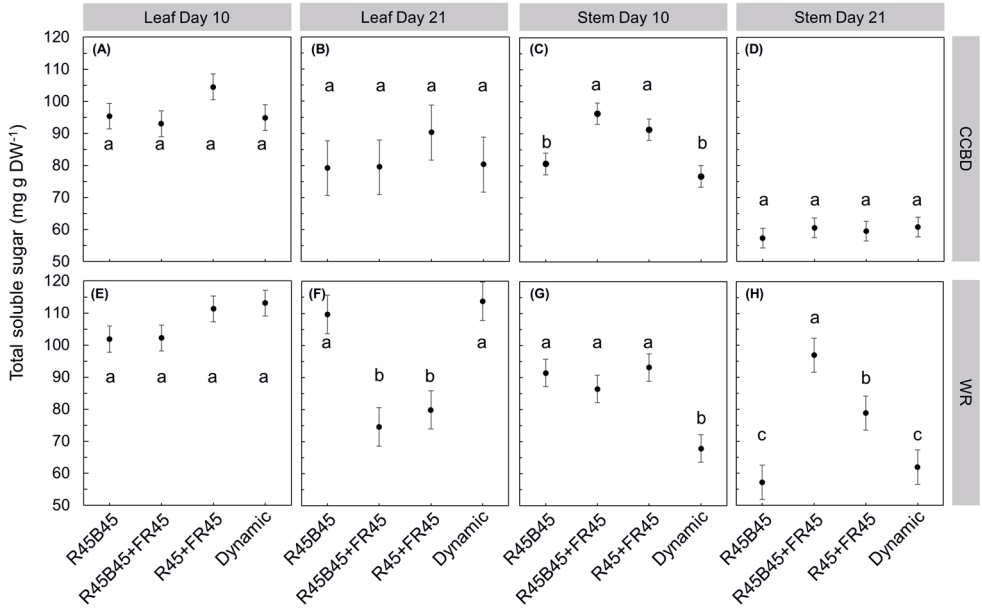

Figure 4. Effect of light spectrum on total soluble sugar content in young leaf and stem of 'CCBD' (A-D) and 'WR' (E-H) cuttings after start of treatments for 10 and 21 days in Experiment 2. Far-red (FR45) was added to red:blue (R45B45) and sole red (R45). The dynamic treatment means the cuttings were exposed to R45FR45 on day 1-7, followed by R45B45 on day 8-21. External auxins were not applied in any of the treatments. All data are the means of 6 pooled samples per treatment from the same trial. The error bars indicate ± standard error of means. The different letters indicate the significant differences between means for each cultivar, tissue and timepoint according to Fisher's protected LSD test (n=6, P=0.05). F-Probability values are shown in Table S5.

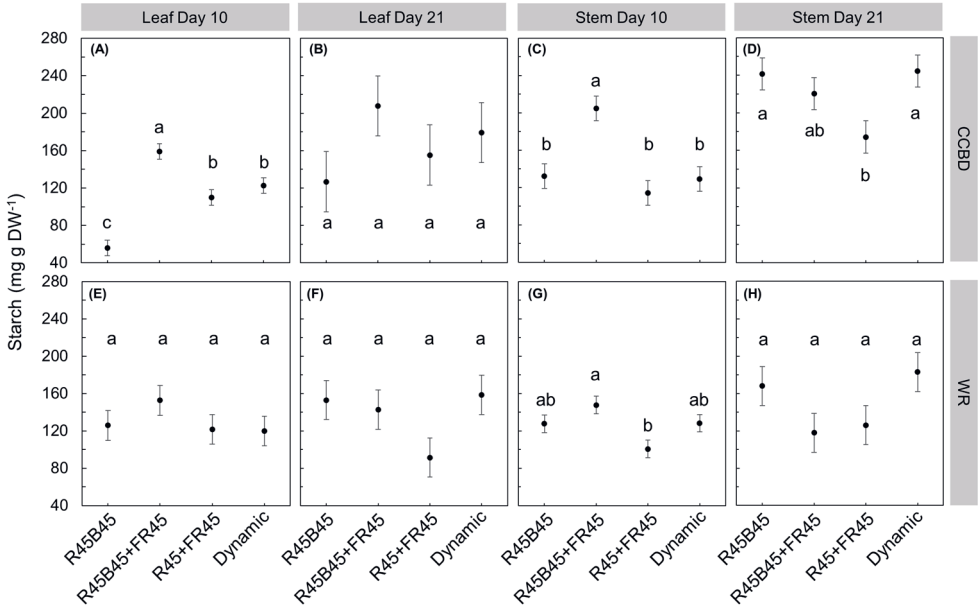

Figure 5. Effect of light spectrum (at 45 µmol $m^{-2}s^{-1}$) on starch content in leaf and stem of 'CCBD' (A-D) and 'WR' (E-H) cuttings after start of treatments for 10 and 21 days in Experiment 2. Far-red (FR45) was added to red:blue (R45B45) and sole red (R45). The dynamic treatment means the cuttings were exposed to R45FR45 on day 1-7, followed by R45B45 on day 8-21. All data are the means of 6 pooled samples per treatment from the same trial. The error bars indicate ± standard error of means. The different letters indicate the significant differences between means for each cultivar, tissue and timepoint according to Fisher's protected LSD test (n=6, P=0.05). F-Probability values are shown in Table S5.

4. Discussion

4.1. Adding far-red improved rooting in some conditions

In our study on cannabis stem cuttings, a positive effect of far-red on adventitious rooting was found in one of two experiments (Experiment 2). Adding far-red to either a red:blue (R45B45) or a sole red (R45) background promoted rooting. The presence of far-red during only the first 7 days had a similar positive effect on rooting as providing far-red for 21 days. A positive effect of far-red on rooting has also been reported for other species such as *Rhododendron*, Chinese *Thuja*, *Leucothoe* (Park et al., 2022), and *Chrysanthemum* in *vivo* (Christiaens et al., 2019) and in *vitro* (Kurilčik et al., 2008). It has been claimed that far-red upregulates the biosynthesis of auxin (Tao et al., 2008) which plays a crucial role in root formation (Muday and DeLong, 2001; Vanneste and

Friml, 2009); therefore, far-red may lead to rooting enhancement (Christiaens et al., 2016).

In commercial cannabis propagation, synthetic auxins such as indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) are used to enhance the success rate of rooting (Blythe et al., 2007; Caplan et al., 2018). This was confirmed by our results, where quick-dipping the cuttings in 0.25% IBA increased rooting by 20%-40% under all light treatments. Moreover, genotypes of cannabis may differ in rooting ability (Campbell et al., 2021), which was also shown in Experiment 1 (Fig 1, where 'WR' rooted better than 'CCBD' with about 12% higher rooted cuttings on average). However, in Experiment 2, there was no difference in rooting between cultivars; the positive effect of far-red on rooting was similarly found in both cultivars. The presence of blue (12% or 50%, while red was 88% or 50% and in both cases with additional far-red) did not affect the rooting of cannabis cuttings. Similarly, Moher et al. (2023) reported that changing the fraction of blue between 15%-75% (with the fraction red changing concomitantly from 85% to 25%) did not the alter rooting of cannabis cuttings. However, in *Chrysanthemum* and rosemary cuttings, blue light increased rooting and increased the expression of IAA synthesis-related genes (Christiaens et al., 2019; Gil et al., 2021, 2020).

There were several differences between the experimental conditions of Experiment 1 and 2 that we should consider as possible reasons for a positive effect of far-red on rooting in Experiment 2 but not in Experiment 1. First of all, PFD was different. The better rooting found in Experiment 2 may have resulted from the lower PFD at 90-135 µmol $m^{-2}s^{-1}$, while the PFD in Experiment 1 was 100-200 µmol $m^{-2}s^{-1}$. Zheng et al. (2019) showed that a high light intensity (PPFD) caused a delay in the rooting of strawberry runners. Second, the ratio of red:blue background light differed between Experiment 1 (R88B12) and 2 (R45B45). However, we assume that this was not causing the different influence of far-red on rooting, since red:blue resulted in similar rooting as sole red light. The rooting substrate and temperature also differed between the two experiments. In Experiment 2, the cuttings were placed in stonewool at 28 °C, while they were placed in river sand at 25 °C in Experiment 1. As a substrate, river sand allows for the easy removal of substrate such that roots can be observed, but stonewool is more often used in commercial production. Campbell et al. (2021) found that the rooting success of cannabis cuttings was 10-fold higher in stonewool than in peat-based substrates. Lastly, the mother plants did not grow under the same light spectrum in both experiments, potentially influencing the quality of the produced cuttings. In Experiment 1, the mother plants were grown under solar light with supplemental light from HPS lamps, whereas in

Experiment 2 the supplemental light was from LEDs with a spectrum consisting of 86% red, 6% green and 8% blue. Thus, we suspect that these factors might interact with far-red, causing positive effects of far-red on rooting to be observed in some conditions while not in other conditions.

4.2. Far- red stimulated stem elongation

The addition of far-red light tended to increase plant height, which is in line with the general shade avoidance response (Demotes-Mainard et al., 2016). Likewise, stem elongation by far-red occurred in our cannabis experiments. Far-red modulates stem elongation by inactivating phytochrome, which leads to auxin biosynthesis in the top of the plant and transport downward through the stem (Keuskamp et al., 2010), causing stem elongation. The stem elongation caused by additional far-red occurred regardless of the presence of blue (Fig 2C, Fig S1). The application of far-red to improve the rooting of cannabis cuttings, might not be adopted by growers if it would lead to too much stem elongation. Interestingly, applying far-red only during the first 7 days improved rooting without the stimulation of excessive stem elongation.

4.3. Far-red lowered endogenous IAA content in young leaf

Our study demonstrates that exogenous auxin application of IBA was sufficient to the improve rooting of cannabis cuttings (Fig 1), confirming previous reports (Campbell et al., 2021; Caplan et al., 2018). It is well established that far-red stimulates auxin biosynthesis in the young leaves of *Arabidopsis* (Ljung et al., 2001; Tao et al., 2008). This suggests that far-red may induce auxin biosynthesis in cannabis young leaves, which could result in the improved rooting of the cuttings as well. Indeed, in one of the two experiments (Experiment 2), we found that additional far-red light promoted rooting. We also observed a positive effect of far-red light on stem elongation, an effect often linked to auxin signaling (Gommers et al., 2018). However, in our experiment, far-red did not increase the endogenous IAA content in young leaves. On the contrary, exposure to far-red even decreased the level of IAA in these young leaves at 5 and 10 days after start of treatments. It is possible that, in contrast to what we found in leaves, the concentration of auxin in the lower part of stem, where root initiation takes place, would be affected by exposure to far-red light. For example, far-red may induce basipetal transport of IAA auxin away from its biosynthesis location in *Arabidopsis* leaves (Küpers et al., 2023). Such downward transport in cannabis could potentially drain the auxin from the young leaves and thus explain the lower IAA content measured in the leaves while still inducing rooting at the base of stem. However, the IAA content in the lower part of stem was not measured

in our study. Therefore, the selected leaf samples for these measurements might not have been the most optimal to see the effect of far-red on IAA. On the other hand, several studies found IAA in plant tissues to be rapidly changed (2 to 5 h) after far-red exposure (Ahkami et al., 2013; Druege et al., 2016; Küpers et al., 2023; Tao et al., 2008). It could be that rapid changes in IAA levels might lead to a variation that is difficult to analyse when IAA is measured at a few selected moments. Alternatively, heightened responsiveness to a stable level of auxin in the stem where elongation or rooting occurs could lead to increased auxin signaling without changing its absolute concentration.

4.4. Light spectrum did not influence carbohydrate status

Carbohydrates contribute to rooting as it is a substrate for root formation and supply energy (Corrêa et al., 2005). For example, in eucalyptus, high carbohydrate accumulation in the stem base of cuttings positively affected adventitious rooting (da Costa et al., 2013; Ruedell et al., 2015, 2013). It was shown in petunia that in well-rooted shoot cuttings, the sugar level at the stem base increased continuously from 1-8 days after excision (Ahkami et al., 2013).The carbohydrate status of cuttings is positively influenced by leaf photosynthesis during rooting, as observed in hazelnut (Tombesi et al., 2015). Far-red benefits photosynthesis (Yang et al., 2020; Zhen and van Iersel, 2017) and thus carbohydrate accumulation. Hence, we expected that the positive effect of far-red on rooting in cannabis cuttings was partly the consequence of improved carbohydrate accumulation. Although the total soluble sugar and starch measured in cannabis cuttings did not show a substantial increase when far-red was added, their rooting was significantly improved, we cannot exclude a role for carbohydrates. The timing of the measurements may play a role, and we measured in stem and leaf, but the carbohydrates at the base of the stem are probably most relevant.

5. Conclusion

Adding far-red to either red:blue or sole red background promoted the rooting of stem cuttings, compared to applying only red:blue light in one of two experiments, which experiments differed in growth conditions such as substrate, light, and air temperature. The presence or absence of blue did not significantly affect rooting. Auxin and carbohydrate concentrations did not correlate with rooting. Adding far-red only during the initial stage of rooting was sufficient to improve rooting while it did not result in excessive stem elongation.

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Supplementary material

Table S1. Realized light intensity in Experiment 1 and 2. The light was measured at top of plant canopy which was about 15 cm above the table. R is red light (600-700 nm), B is blue light (400-500 nm), and FR is far-red light (700-800 nm).

	Light treatments	Light intensity (µmol $m^{-2}s^{-1}$)		
		R	B	FR
Exp 1	R88B12	87.9±1.6	12.4±0.9	-
	R88B12+FR50	88.8±1.6	12.1±0.2	53.2±1.0
	R88B12+FR100	88.3±1.9	13.2±0.3	98.1±2.7
	R100+FR50	100.6±1.7	-	54.1±0.9
Exp 2	R45B45	45.1±0.6	45.6±0.5	-
	R45B45+FR45	45.4±0.3	44.9±0.4	45.5±0.4
	R45+FR45	45.9±0.4	-	46.1±0.4
	Dynamic: Day 1-7	45.9±0.4	-	46.1±0.4
	Day 8-21	45.1±0.6	45.6±0.5	-

Table S2. Number of cuttings per treatment used in each replication (trial)

Exp	Number of replications	Treatments		Number of cuttings (per replication)	
		Light	IBA	Critical CBD	White Russian
1	5	R88B12	-IBA	17, 18, 12, 17, 20	24, 18, 12, 24, 19
			+IBA	17, 18, 12, 16, 19	23, 18, 12, 23, 19
		R88B12+FR50	-IBA	17, 18, 11, 14, 20	35, 18, 12, 24, 20
			+IBA	16, 18, 12, 14, 19	20, 18, 12, 23, 19
		R88B12+FR100	-IBA	18, 18, 12, 14, 19	24, 20, 12, 24, 20
			+IBA	19, 18, 12, 15, 19	22, 18, 12, 23, 18
		R100+FR50	-IBA	18, 18, 13, 16, 19	25, 19, 12, 24, 19
			+IBA	16, 20, 12, 14, 19	23, 17, 12, 20, 18
2	2	R45B45	-	39, 72	39, 71
		R45B45+FR45	-	39, 70	39, 72
		R45+FR45	-	39, 72	39, 72
		Dynamic	-	39, 72	39, 71

Table S3. F-Probability values of each effect and interaction resulting from split-split-plot analysis of Experiment 1

Effect	% Rooted cutting	Root dry weight	Rooted cutting height
Light	0.22	0.50	0.14
IBA	<0.001**	<0.001**	0.20
Cultivar	0.01*	0.004**	0.002**
Light x IBA	0.90	0.76	0.84
Light x Cultivar	0.86	0.72	0.83
IBA x Cultivar	0.70	0.64	0.13
Light x IBA x Cultivar	0.95	0.88	0.41

Table S4. F-Probability values of each effect and interaction resulting from split-plot analysis of Experiment 2

Effect	% Rooted cutting	Rooting score	Cutting height
Light	0.04*	0.12	0.03*
Cultivar	0.90	0.29	0.06
Light x Cultivar	0.43	0.91	0.53

Table S5. F-Probability values for each effect and interaction resulting from split-plot analysis of IAA and carbohydrate contents in Experiment 2

Effect	IAA content in leaf			Total soluble sugar in leaf		Total soluble sugar in stem		Starch in leaf		Starch in stem	
	Day 5	Day 10	Day 21	Day 10	Day 21	Day 10	Day 21	Day 10	Day 21	Day 10	Day 21
Light	0.18	0.05	0.63	0.46	0.28	0.02	0.06	0.09	0.21	0.13	0.09
Cultivar	0.03	0.007	0.03	0.04	0.07	0.64	0.04	0.07	0.04	0.13	0.02
Light x Cultivar	0.65	0.17	0.56	0.61	0.07	0.15	0.14	0.07	0.08	0.32	0.77

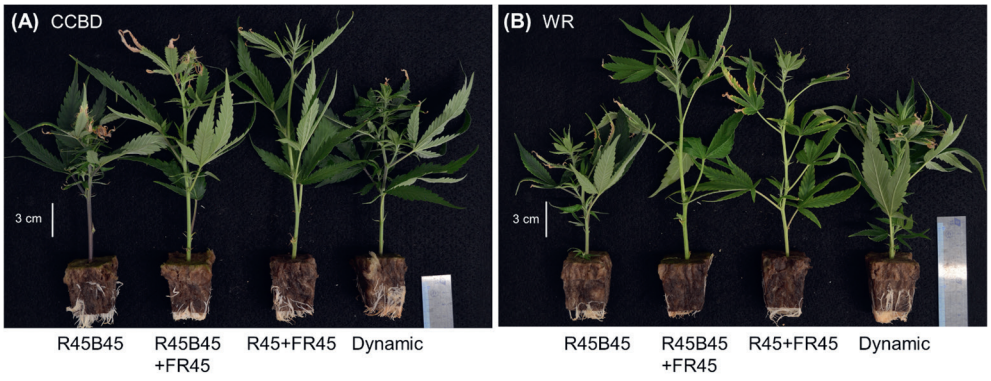

Figure S1. Cannabis cuttings of Critical CBD (CCBD) (**A**) and White Russian (WR) (**B**) at 21 days after start of treatments in Experiment 2. From left to right, the cuttings were rooted under red:blue (R45B45, 45 µmol $m^{-2}s^{-1}$ red + 45 µmol $m^{-2}s^{-1}$ blue); far-red at 45 µmol $m^{-2}s^{-1}$ added to R45B45 (R45B45+FR45) and far-red added to sole red (R45+FR45) from day 1-21. The dynamic treatment means the cuttings were exposed to R45FR45 on day 1-7, followed by R45B45 on day 8-21.

Chapter 3

Plant growth and specialized metabolites of medicinal cannabis are hardly influenced by fraction of blue light or additional far-red light

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Abstract

Medicinal cannabis (*Cannabis sativa* L.) is often cultivated indoors using artificial lights, where adjustments in the light spectrum can optimize plant morphology and yield, and potentially enhance the accumulation of specialized metabolites. This study aims to explore how blue and far-red light affect plant growth and concentrations of terpenoids and cannabinoids of medicinal cannabis. Two experiments were conducted in a climate chamber using LEDs providing a mixture of red, blue, white, and far-red light. In Experiment 1, fractions of blue light ranging from 8% to 21% substituting red light in a white background spectrum (at a total photosynthetic photon flux density (PPFD) of 400 µmol $m^{-2}s^{-1}$ during the vegetative phase and 690 µmol $m^{-2}s^{-1}$ during the generative phase) were studied for cultivars 'Critical CBD' and 'White Russian'. In Experiment 2, 200 µmol $m^{-2}s^{-1}$ of far-red was added to 800 µmol $m^{-2}s^{-1}$ PPFD of red/blue/white light during the last 4 weeks of the generative phase for 'Critical CBD'. The fraction of blue light did not significantly affect plant morphology and dry matter production. Adding far-red during the last 4 weeks of the generative phase did not result in significant changes in plant morphology, dry matter production, or inflorescence size. Moreover, both fraction blue and additional far-red light did not significantly influence the concentrations of terpenoids and cannabinoids. These results suggest that, for cannabis cultivation, using light with a relatively low fraction of blue may be preferable to reduce lighting costs. Adding far-red during the late stage of the generative phase is not needed. The limited effect of light spectrum on plant growth and specialized metabolites found in this study is inconsistent with some previous studies. This indicates the need for further research to understand these differences.

Keywords: light spectrum, LEDs, *Cannabis sativa* L., cannabinoids, terpenoids

1. Introduction

Indoor cultivation has been introduced to improve quantity and quality of plant production, due to finely controlled growing conditions including temperature, carbon dioxide, air humidity, irrigation, and light (SharathKumar et al., 2020). In indoor cultivation, artificial light – including light intensity, photoperiod, and light spectrum – can be customized. Light in the range of photosynthetically active radiation (PAR; 400-700 nm) is captured by light harvesting pigments and converted into chemical energy via photosynthesis, which is essential for plant growth (McCree, 1971). Photosynthetic efficiency can vary depending on the spectrum of light (Inada, 1976; McCree, 1971). Plants perceive the light spectrum through specific photoreceptors, which transduce light into signals that mediate photomorphogenesis (Higuchi and Hisamatsu, 2016).

Medicinal cannabis (*Cannabis sativa* L.), being a high value crop, is widely cultivated indoors to ensure both high and consistent yield and quality (Summers et al., 2021). This involves maximizing the mass of inflorescences and elevated concentrations of specialized metabolites, primarily cannabinoids and terpenoids, which primarily accumulate in the inflorescences. Manipulating the light spectrum in indoor cultivation has the potential to optimize plant growth through enhanced photosynthesis and desired plant morphology, leading to improved cannabis yield.

Blue light (400-500 nm) is essential for normal plant growth and development (Yorio et al., 1998). Blue activates the plant photoreceptor, phototropin, leading to increased stomatal conductance and subsequently higher photosynthetic rates (Boccalandro et al., 2012), thereby potentially promoting plant growth. Blue also triggers cryptochromes resulting in a reduced internode length and compact plant (Cope et al., 2014; Hernández and Kubota, 2016; Wollaeger and Runkle, 2015). Cannabis plants grown under blue-rich LEDs exhibit shorter and more horizontally oriented branches, compared to High Pressure Sodium (HPS) lamps (Magagnini et al., 2018; Namdar et al., 2019). Conversely, exposure to very high blue (>90%) or monochromatic blue light induced stem elongation in cucumber and basil (Hernández and Kubota, 2016; Larsen et al., 2020). Supplementary lighting with red:blue light in greenhouse tomato showed that a fraction of 6-12% blue was optimal for fruit yield (Kaiser et al., 2019). A high fraction blue (tested within a range of 10-80%) in red:blue light caused a reduction in leaf area and resulted in lower shoot dry mass in cucumber seedlings grown in a climate room (Hernández and Kubota, 2016). A high fraction of blue reduced yield in tomato due to a decrease in whole plant light interception (Kalaitzoglou et al., 2021). In cannabis, a decrease in

inflorescence yield was found when the fraction of blue increased, from low blue (4-8 %) of HPS lamps to high blue (20-24%) of LEDs (Magagnini et al., 2018; Westmoreland et al., 2021), while yields did not differ when fraction of blue of white LEDs was varied from 3 to 18% by applying spectral filters (Kotiranta et al., 2024).

Blue has been shown to enhance biosynthesis of specialized metabolites in several crops. Phenolics and flavonoids in leaves of roses, chrysanthemums, and campanulas increased from 0 to 40% blue added to red light (Ouzounis et al., 2014). Anthocyanins and carotenoids in leaves of pepper increased from 15 to 75% blue added to red light (Hoffmann et al., 2016). However, previous studies have shown inconsistent effects of blue light on cannabinoids in cannabis inflorescences. Magagnini et al. (2018) reported that blue-rich LEDs light increased concentrations of some cannabinoids including delta-9-tetrahydrocannabinol (THC), tetrahydrocannabivarin (THCV), cannabidiol (CBD), and cannabigerol (CBG), compared to HPS lamps. The increased in blue fraction was found to increase only THCVA but not other cannabinoids and terpernoids (Kotiranta et al., 2024). Conversly, Westmoreland et al. (2021) found blue fractions had no effect on either THC or CBD.

Besides PAR, plants can also sense a broader range of the light spectrum from UV-B (280-315 nm) to far-red (700-800 nm). A low red to far-red ratio results in shade avoidance responses such as stem elongation and leaf expansion (Demotes-Mainard et al., 2016; Franklin, 2008). Recently, it has also been suggested that far-red photons are equally efficient in driving canopy photosynthesis as PAR when provided together with PAR (Zhen and Bugbee, 2020). Additional far-red to red:blue light is often found to increase plant biomass, for example, in lettuce (Jin et al., 2021). Far-red also increased biomass in young tomato plants by enhancing total light absorption, accelerated flowering, and increased dry matter partitioning to fruits (Ji et al., 2020; Kalaitzoglou et al., 2019). In addition, far-red may have the potential to stimulate elongation of cannabis inflorescences which could help to minimize the risk of fungi infection. This infection is often caused by high humidity around the inflorescence (Williamson et al., 2007) which may worsen when the inflorescence is compact. However, effects of far-red on the accumulation of specialized metabolites in plants remains unclear. A study on *Artemisia annua* showed that adding far-red from LEDs to a backgroung of solar light with supplemental HPS lamps (having red:far-red =0.3) had no effect on artemisinin in leaves (Zhang et al., 2023). Another study on ginseng found that far-red enriched conditions, including 15 µmol $m^{-2}s^{-1}$ of far-red from LEDs and shaded white LEDs light, promoted ginsenoside biosynthesis in leaves (Mohanan et al., 2023). Interestingly, in cannabis,

exposure to low red: far-red (=1) light by replacing photosynthetic photon flux density (PPFD) with far-red throughout the generative phase resulted in a plant elongation and a large reduction in inflorescence yield compared to high red:far-red (=11) (Kotiranta et al., 2024). The reduction in yield might have been caused by the lower PPFD in the treatment of low red:far-red. This study also observed a negative impact of far-red on the concentration of some cannabinoids and terpenoids.

The medicinal cannabis industry is relatively new, and many cultivation strategies are embraced by commercial growers, which may lack scientific validation (Eaves et al., 2020). There is a noticeable shift from traditional lighting for indoor cultivation with fluorescent lamps and HPS lamps to LEDs technology due to various advantages, including high photosynthetic efficacy, long lifetime, less thermal radiation and customizable spectral composition (Jin et al., 2019). While the effect of light spectrum on growth and development is well-known in several crops, this can vary depending on light intensity in terms of absolute or relative photon amount (Cope and Bugbee, 2013; Utasi et al., 2023). Thus, the known effects of light spectrum might be different for medicinal cannabis, which is often grown under relatively high light intensity, i.e., 500-1500 µmol $m^{-2}s^{-1}$ PPFD (Chandra et al., 2017).

This study aimed to explore how blue and far-red light affect plant morphology, dry mass production, and concentration of cannabinoids and terpenoids in medicinal cannabis. We hypothesized that higher fractions of blue leads to higher concentrations of specialized metabolites, more compact plants, but less plant growth. Furthermore, we hypothesized that applying far-red only during the latter part of the generative phase increases concentration of specialized metabolites, inflorescence yield and inflorescence elongation without strong stem elongation. Two experiments were conducted in a climate chamber using LEDs providing a mixture of red, blue, white, and far-red. In Experiment 1, different fractions of blue light from 8% to 21% were studied throughout the vegetative phase at 250-400 µmol $m^{-2}s^{-1}$ PPFD and the generative phases at 800 µmol $m^{-2}s^{-1}$ PPFD. In Experiment 2, 200 µmol $m^{-2}s^{-1}$ of far-red was added during the second half of the generative phase at 800 µmol $m^{-2}s^{-1}$ PPFD.

2. Materials and methods

2.1. Plant cultivation

Medicinal *Cannabis sativa* L. plants were grown in a climate-controlled walk-in chamber. Plants were propagated by stem cuttings (details on the rooting phase are described in Supplemental material 1). Well-rooted cuttings were transplanted into 15 x 15 x 15 cm stonewool blocks (Grodan, Roermond, the Netherlands). The planting density was kept constant at 9 plants m^{-2} throughout the whole experiment. Plants were pruned three times during the cultivation. At 8 days after transplanting (DAT) the apex was removed. At 11 DAT the lowest side shoots were removed such that four upper side shoots were retained. Finally, at 16 DAT, the second-order side shoots were removed except for the three upper second-order side shoots on each side shoot.

During the vegetative phase, i.e. a phase of longdays to promote vegetative growth, the average temperature was 27/24 °C (light/dark), relative humidity (RH) was 80%/85 %, and CO_2 during the light period was 400 µmol mol^{-1}. During the generative phase, i.e. a phase of shortdays for flower induction and flower development, temperature was 28/26 °C, RH was 66/68%, while CO_2 during the light period was gradually increased from 600 to 1200 µmol mol^{-1}, adding 200 µmol mol^{-1} every 2 weeks. The realized weekly air temperature and RH for each of the light treatments is shown in Table S1. Before transplanting, the stonewool blocks were pre-soaked in a nutrient solution with an EC of 1.6 dS $m^{-1}$and a pH of 5.8. The solution consisted of the following macro- and micronutrients; 1.25 mM NH_4^+; 6.2 mM K^+; 1.9 mM Ca^{2+}; 0.9 mM Mg^{2+}; 10.5 mM NO_3^-; 0.85 mM SO_4^{2-}; 0.85 mM PO_4^{3-}; 60 µM Fe^{2+}; 20 µM Mn^{2+}; 3 µM Zn^{2+}; 20 µM B^{2+}; 0.5 µM Cu^{2+}; 0.5 µM Mo^{2+}. Irrigation from transplanting onward was supplied with the same nutrient solution by discharge-regulated drippers in the stonewool blocks, each discharge was 100 mL. Frequency of dripping ranged from four to six cycles per day, and it was adjusted to ensure that all plants received sufficient water and nutrients. The EC value was first raised to 2 dS m^{-1} during the vegetative phase, then further raised to 2.5 dS m^{-1} during the generative phase, by increasing the concentration of macronutrients, while keeping the ratios between individual macronutrients constant.

2.2. Light spectrum treatments

The research consisted of two experiments in which the light treatments were arranged differently. Exp 1 studied the effect of blue fraction in two cultivars: ‘Critical CBD’ and ‘White Russian’. Exp 2 studied the effect of supplemental far-red light in ‘Critical CBD’.

PPFD was measured using a LI-250A light meter (LI-COR Inc., Lincoln, NE, USA); and the light spectrum was measured using a specbos 1210 spectrophotometer (JETI Technische Instrumente GmbH, Jene, Germany). The light was measured at 45, and 75 cm above the table, which was at final canopy height at the vegetative phase and the generative phase, respectively.

Blue fraction (Exp 1)

After transplanting, the plants were randomly assigned to three compartments within the climate room, partitioned by white plastic screens. Each compartment measured 3.3 m^2, placed with 15 'Critical CBD' plants and 15 'White Russian' plants. Three different fractions of blue light were applied throughout the cultivation cycle: 8%, 13%, and 21% blue (400-500 nm), achieved by substituting the % red (600-700 nm), while green (500-600 nm) remained constant at 6% (Table 1), provided by a mixture of red-blue-white light LEDs (Green Power DRW LB; MB; HB 1.2, Philips, the Netherlands). For the first five days of the vegetative phase (0-5 DAT), the light intensity was at 245±7 µmol $m^{-2}s^{-1}$ and later this increased to 388±8 µmol $m^{-2}s^{-1}$ (6-20 DAT). The photoperiod was 18/6 h (light/dark). The generative phase lasted for 8 weeks (21-76 DAT); the light intensity was increased to 692±10 µmol $m^{-2}s^{-1}$. The photoperiod was 12/12 h (light/dark).

Additional far-red (Exp 2)

After transplanting, the plants were randomly assigned to two compartments within the climate room, partitioned by white plastic screens. Each compartment measured 2 m^2, placed with 18 Critical CBD plants. The vegetative phase lasted from 0 to 11 DAT, all plants were grown under 404 ± 11 µmol $m^{-2}s^{-1}$ PPFD, with 13% blue, 6% green, 81% red, provided by a mixture of red-blue-white LEDs (Green Power DRW MB 1.2, Philips, Eindhoven, the Netherlands). The photoperiod was 18/6 h (light/dark). During the 8 weeks of the generative phase (12-69 DAT), control plants (800) were grown under 800±15 µmol $m^{-2}s^{-1}$ PPFD where the light was provided by the same fixtures as used in the vegetative phase with the same light spectrum. Far-red exposed plants (800+FR) were grown under the same light condition as the control plants for the first 4 weeks (week 1-4), while during the last 4 weeks (week 5-8), 197±7 µmol $m^{-2}s^{-1}$ of far-red, provided by LEDs (Green Power Dynamic 2.0 LED research modules and Green Power PM FR 1.2, Philips, Eindhoven, the Netherlands), was added during the light period (Table 2). The photoperiod was 12/12 h (light/dark).

Table 1. Overview of light treatments in the experiment on blue fractions (Exp 1) with their spectral distribution over 400-700 nm which were remained the same throughout the vegetative and generative phase. B is blue light (400-500 nm), G is green light (500-600 nm), and R is red light (600-700 nm). Phytochrome stationary state (PSS) is calculated according to Sager et al. (1988). PPFD means photosynthetic photon flux density (400-700 nm; µmol $m^{-2}s^{-1}$).

Parameters		**Light treatment**		
		8% blue	**13% blue**	**21% blue**
%B		8	13	21
%G		6	6	6
%R		86	81	73
B:R		0.1	0.2	0.3
PSS		0.88	0.88	0.87
PPFD (µmol $m^{-2}s^{-1}$)	**- Vegetative phase** (0-20 DAT)	250-400	250-400	250-400
	- Generative phase (21-76 DAT)	690	690	690

Table 2. Overview of light treatments in the experiment on additional far-red (Exp 2) with their spectral distribution over 400-700 nm. B is blue light (400-500 nm), G is green light (500-600 nm), R is red light (600-700 nm), and FR is far-red light (700-800 nm). Phytochrome stationary state (PSS) is calculated according to Sager et al. (1988). PPFD means photosynthetic photon flux density (400-700 nm). PFD means photon flux density (400-800 nm).

Parameters		**Light treatment**		
		800 (control)	**800+FR**	
Vegetative phase (0-11 DAT)	**%B**	13	13	
	%G	6	6	
	%R	81	81	
	PPFD (µmol $m^{-2}s^{-1}$)	400	400	
Generative phase (12-69 DAT)		**Week 1-8**	**Week 1-4**	**Week 5-8**
	%B	13	13	13
	%G	6	6	6
	%R	81	81	81
	FR (µmol $m^{-2}s^{-1}$)	0	0	200
	R:FR	123	123	2.9
	PSS	0.88	0.88	0.81
	PPFD (µmol $m^{-2}s^{-1}$)	800	800	800
	PFD (µmol $m^{-2}s^{-1}$)	800	800	1000

2.3. Growth and morphology measurements

Final harvest was conducted after 8 weeks of the generative phase. Plant height was measured as the longest distance from the base of the stem towards the top of the canopy. The diameter of the main stem was determined at 1 cm above the top of the stonewool block. Each plant was dissected into leaves, stems, and inflorescences. The inflorescence leaves and stems were trimmed by an electronic trimmer (Bowl Trimmer 40 cm, Dutchmasters Fertraso, the Netherlands), and their trimmed mass was added to that of the stems. Leaf area was determined using a LI-3100C area meter (LI-COR Inc., Lincoln, NE, USA). In Exp 1, leaf area based on average measured area of 20 random leaves per plant and leaf dry weight, while in Exp 2 leaf area of all leaves was measured. Inflorescence mass was determined after trimming, measured as fresh mass, approximately 10% moisture dry mass (air-dried, as is the commercial standard), and dry mass. Drying to 10% moisture of inflorescences took place for five days in a dark climate-controlled room with ample ventilation, at 45% RH, and 25 °C. Dry mass was determined after drying the material at 70 °C for 24 h, followed by 105 °C for 72 h in a ventilated oven. Dry matter content was calculated as the ratio between dry mass and fresh mass. Light use efficiency (LUE) was calculated as dry mass accumulated over the whole cultivation cycle divided by cumulative incident mol of PPFD (400-700 nm) during the generative phase, while radiation use efficiency (RUE) in Exp 2 considered cumulative incident mol of PFD (400-800 nm) during the generative phase. Plant compactness was calculated as the ratio of total plant dry mass to plant height. Specific leaf area (SLA; $cm^2\ g^{-1}$) was calculated as the ratio between leaf area and leaf dry mass. In Exp 2, two apical inflorescences were collected from each plant to assess their size and density. The size measurements included length and diameter, while density was determined as a ratio of fresh weight to volume (Supplemental material 2).

2.4. Specialized metabolites determination

Fresh inflorescences were collected from the top 5 cm of inflorescences during the 6, 7, and 8 weeks of the generative phase and stored at -80°C. A total of five biological samples per treatment was prepared in which each sample was pooled from 4-6 plants. Per sample, 200 mg fresh inflorescences were homogenized before extraction in 2 mL of n-Hexane with 0.2 g L^{-1} of squalene as an internal standard (IS). Sample extracts were sonicated for 10 min in an ultrasonic bath (Branson 2800), subsequently filtered through a column containing siloxilated glass-wool and sodium sulphate in a Pasteur pipet and collected in a 2 mL glass vial. Two µL of each filtered extract was analysed in splitless

mode on a Gas Chromatography-Mass Spectrometry (GC-MS) Agilent (7890) equipped with a 30-m length × 0.25-mm inner diameter, 0.25-µm film thickness column (Zebron, 5MS) and a mass-selective detector (model 5972A, Hewlett–Packard). The GC was programmed at an initial temperature of 60 °C for 2 min, with a ramp of 5 °C min^{-1} to 250 °C, and then with 10 °C min^{-1} to 280 °C and final time of 5 min. The injection port, interface, and MS source temperatures were 250 °C, 290 °C, and 180 °C, respectively, and the Helium inlet pressure was controlled with an electronic pressure control to achieve a constant column flow of 1 mL min^{-1}. The ionization potential was set at 70 eV, and scanning was performed from 45 to 400 amu. Metabolites were identified by comparing mass spectra with those of the National Institute of Standards and Technology mass spectral library (NIST) MS search 2.0 and their relative retention time. Detected compounds were classified as either terpenoids or cannabinoids. Relative abundances (IS ratio response) for individual compounds were determined by the area under the curve, and normalized by IS and sample weight. Total terpenoids were calculated as the sum of metabolites at retention time (RT) 6.2-26.2 min, from which the proportion of total monoterpenoids at RT 6.2-11.3 min and total sesquiterpenoids at RT 19.5-26.2 min was determined by dividing to the total terpenoid abundance. Total cannabinoids were a sum of metabolites at RT 35.0-43.2 min.

2.5. Statistical set-up and analysis

In each experiment, there was only one experimental plot per light treatment, therefore no independent statistical replicates. In Exp 1, per plot, there were 15 plants per cultivar and each cultivar was analyzed separately. In Exp 2, there were 18 plants per plot. In both experiments, the individual plants within a treatment were considered as replicates (n=15 or 18) in a one-way Analysis of Variance (ANOVA). This might have led to an underestimation of variance. Therefore, statistical significance was tested at P= 0.01. In Exp 1, the effect of blue fractions was partitioned into a linear and a quadratic component (orthogonal polynomial contrasts). In Exp 2, Fisher's protected LSD test at P=0.01 was used for mean separation. The normality of the residuals was examined with Shapiro-Wilk test at P=0.05. The homogeneity of residuals was tested with Bartlett's test at P=0.05. Both assumptions were met in all cases. All tests were performed using Genstat 21st edition (VSN International, Hemel Hempstead, UK).

3. Results

3.1. Blue Fraction (Exp 1)

Blue fractions ranging from 8% to 21% substituting red light in a white light background throughout the vegetative and generative phases had no significant effect on plant dry mass of medicinal cannabis in both 'Critical CBD' and 'White Russian' (Fig 1A-B). An increase in blue fraction tended to increase the dry mass partitioned to the leaves at the expense of the stems, while the dry mass partitioned to inflorescences remained unchanged (Fig 1C-D). Dry matter content as well as light use efficiency (LUE) calculated for inflorescences as well as the total plant were not influenced by fraction of blue light (Fig 1E- H).

Plant height decreased by approximately 6% when blue fraction increased from 8% to 21%, although a statistically significant decrease was found only in 'White Russian' (Fig 2A-B). Internode length tended to also decrease when blue fraction increased (Fig 2C-D). Main stem diameter of 'White Russian' decreased with increasing blue fraction (Fig 2B). When the blue fraction increased, specific leaf area tended to decrease, while leaf area remained unchanged (Fig 2E-F). Plant compactness was not affected by blue fraction (Fig 2G-H). The developmental stage of flowers was not affected by fractions of blue. After ten days into the generative phase, a group of multiple stigmas became visible at the apex, which occurred similarly among the different blue fractions (Fig S1). Blue fraction had no significant effect on concentrations of terpenoids and cannabinoids in the inflorescences at 6, 7, or 8 weeks into the generative phase of either cultivar (Fig 3-4).

3.2. Additional far-red (Exp 2)

Adding 200 µmol $m^{-2}s^{-1}$ of far-red to 800 µmol $m^{-2}s^{-1}$ PPFD of red/blue/white light during the last 4 weeks of the generative phase did not have any statistically significant effect on dry mass (Fig 5A). Dry mass partitioning among plant organs and dry matter content were not different between the control and far-red exposed plants (Fig 5B-C). LUE (biomass per unit cumulative incident mol of PPFD, 400-700 nm) of both inflorescence and total plant were not statistically affected by adding far-red. Radiation use efficiency (RUE, biomass per unit cumulative incident mol of PFD, 400-800 nm) of both inflorescence and total plant seemed to decrease by 18% by adding far-red, although this was not statistically significant (Fig 5D-E).

Adding far-red did not statistically influence plant height, main stem diameter, internode length, and plant compactness (Fig 6A-B,D). The additional far-red resulted in a decreased leaf area (Fig 6C). The size (both length and diameter) and density of inflorescences were not affected by the presence of far-red (Fig 6E-F). Concentrations of terpenoids and cannabinoids in the inflorescences were not altered by additional far-red at 6, 7, or 8 weeks into the generative phase (Fig 7).

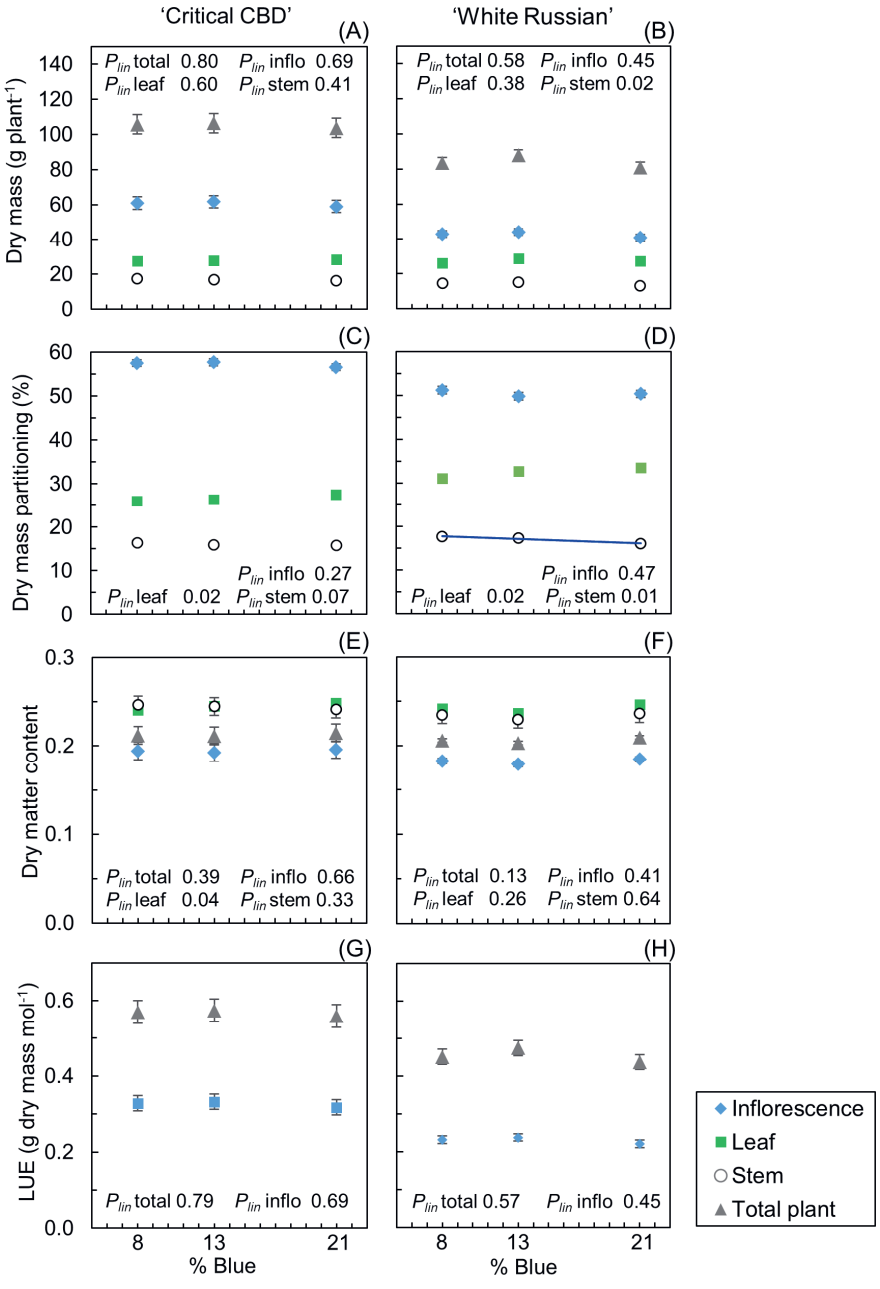

Figure 1. Dry mass (A-B), dry mass partitioning (C-D), dry matter content (ratio dry to fresh mass) (E-F), and light use efficiency (G-H) of medicinal cannabis 'Critical CBD' and 'White Russian' grown under 8, 13, and 21% blue light after 8 weeks into the generative phase. Data based on 15 replicated plants per treatment. Error bars represent SEM based on the common variance, when larger than the symbols. When linear effects of blue fraction were significant at P=0.01, trendlines are depicted.

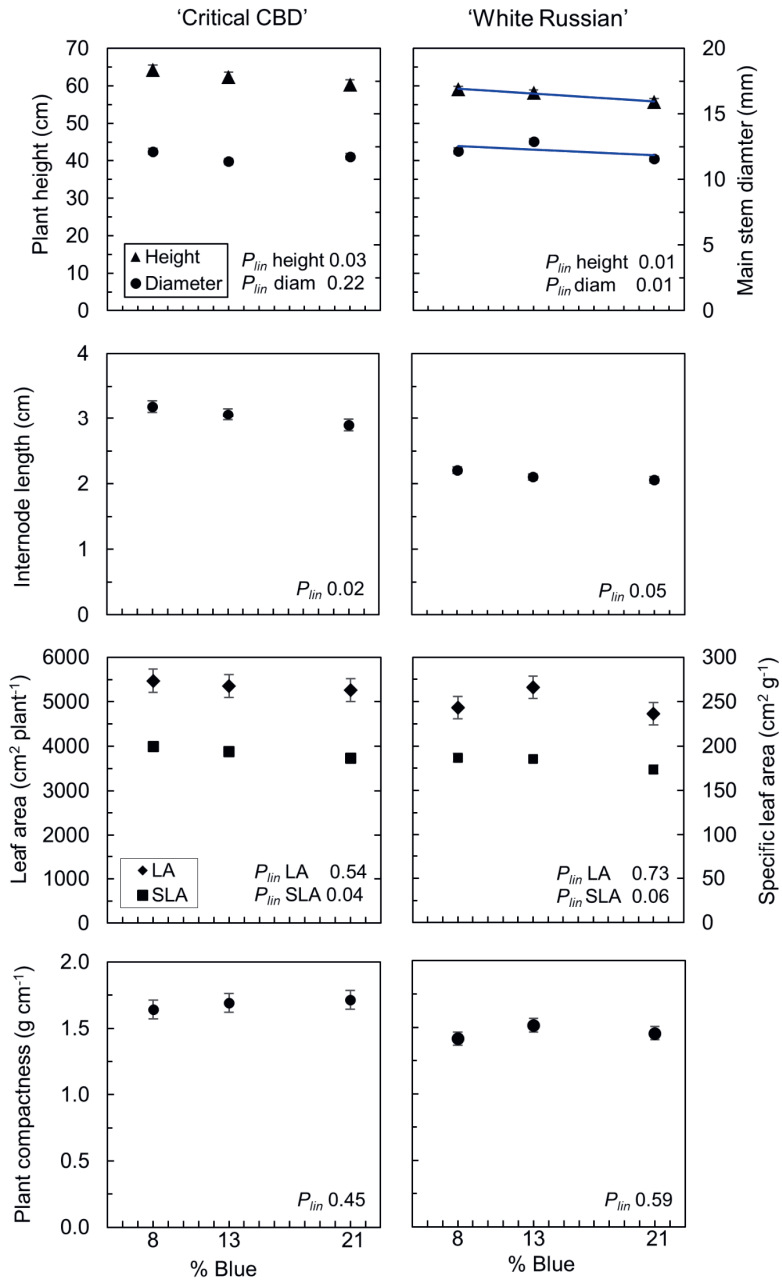

Figure 2. Plant height and main stem diameter (A-B), internode length (C-D); leaf area (E-F) and specific leaf area (SLA) (G-H), and plant compactness (D) of medicinal cannabis 'Critical CBD' and 'White Russian' grown under 8, 13, and 21% blue light after 8 weeks into the generative phase. Data based on 14-15 replicated plants per treatment. Error bars represent SEM based on the common variance, when larger than the symbols. When linear effects of blue fraction were significant at P=0.01, trendlines are depicted.

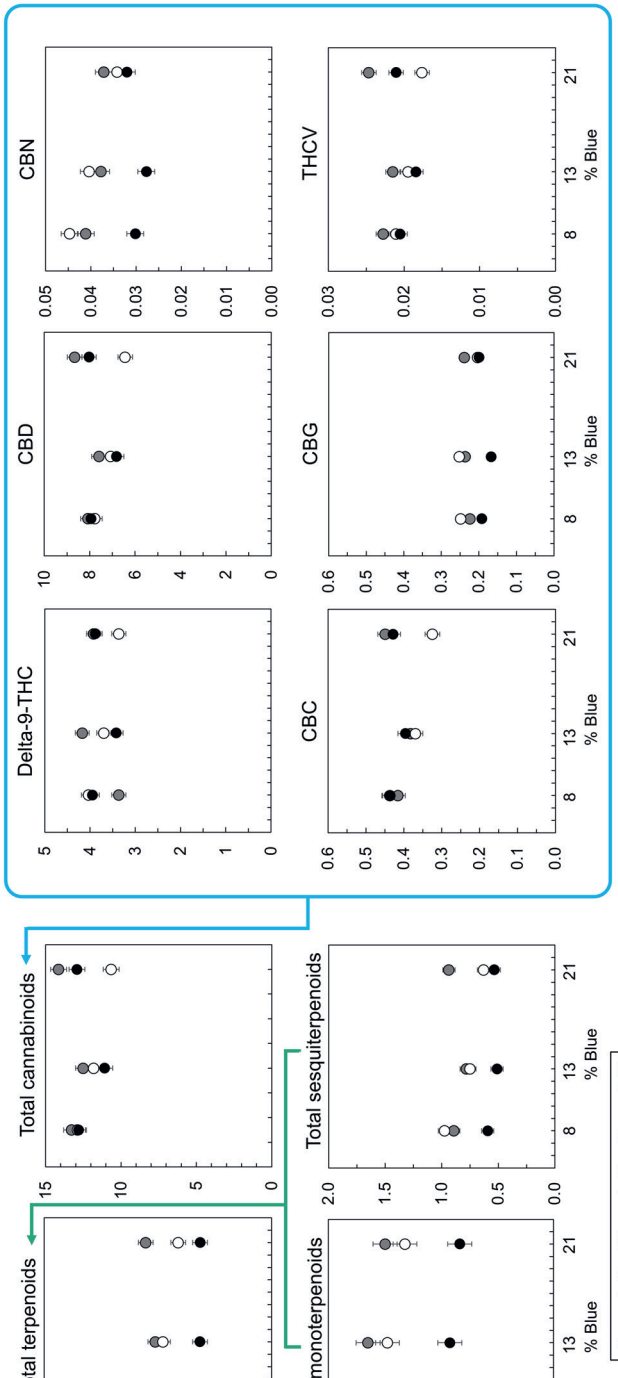

Figure 3. Concentration of terpenoids and cannabinoids in fresh inflorescences of medicinal cannabis 'Critical CBD' grown under 8, 13, and 21% blue light after 6, 7, and 8 weeks into the generative phase. Data are the averages of the internal standard (IS) response ratio based on 5 replicated samples per treatment. Error bars represent SEM based on the common variance. There was no significant effect of blue fraction (all P-values larger than 0.05).

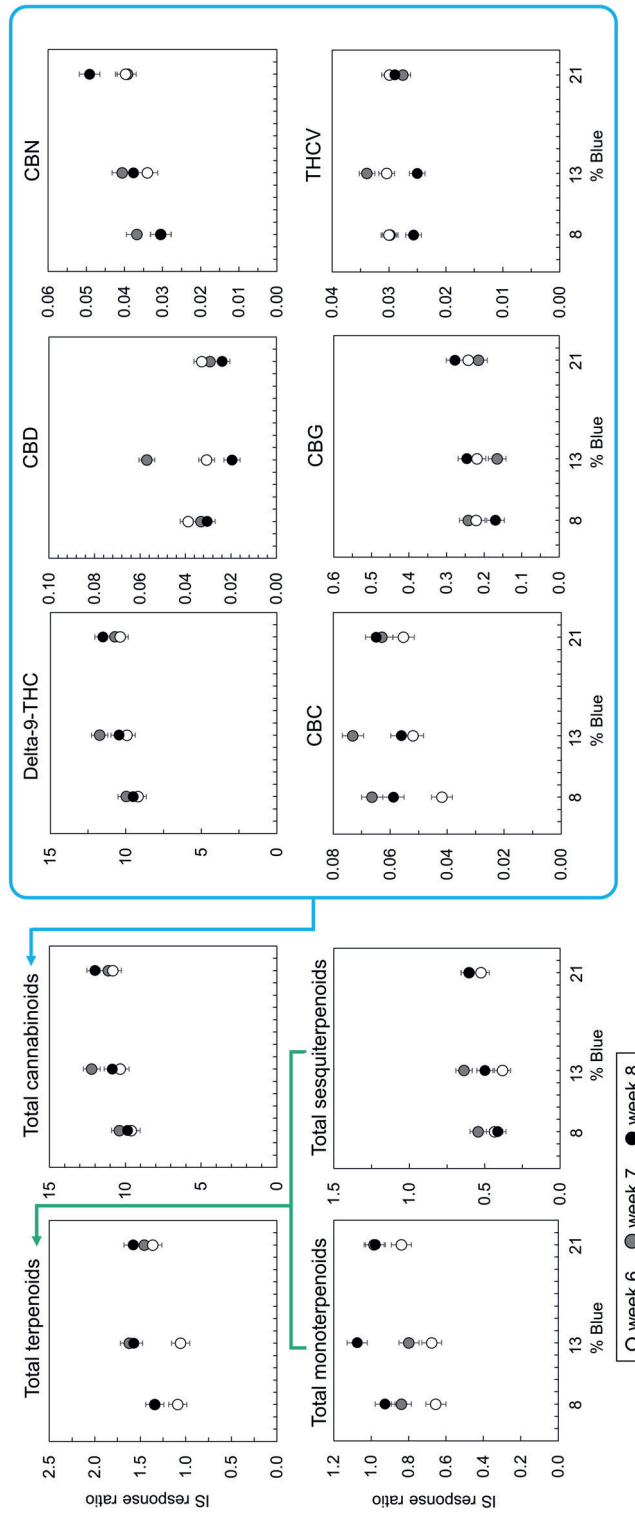

Figure 4. Concentration of terpenoids and cannabinoids in fresh inflorescences of medicinal cannabis 'White Russian' grown under 8, 13, and 21% blue light after 6, 7, and 8 weeks into the generative phase. Data are the averages of the internal standard (IS) response based on 5 replicated samples per treatment. Error bars represent SEM based on the common variance. There was no significant effect of blue fraction (all P-values larger than 0.05).

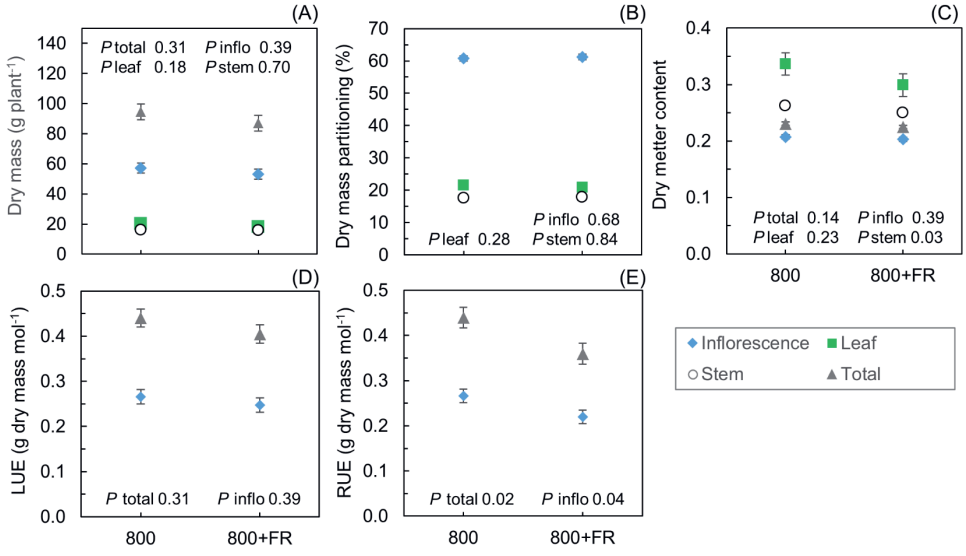

Figure 5. Plant dry mass (A), dry mass partitioning (B), dry matter content (C), light use efficiency (D), and radiation use efficiency (E) of medicinal cannabis cultivar 'Critical CBD' after 8 weeks into the generative phase. The plants were grown at 800 µmol $m^{-2}s^{-1}$ PPFD or 800 µmol $m^{-2}s^{-1}$ PPFD with an addition of 200 µmol $m^{-2}s^{-1}$ far-red during the last 4 weeks of the generative phase. Data based on 18 replicated plants per treatment. Error bars represent SEM based on the common variance, when larger than the symbols. There is no significant effect of light treatments on any parameters at P=0.01.

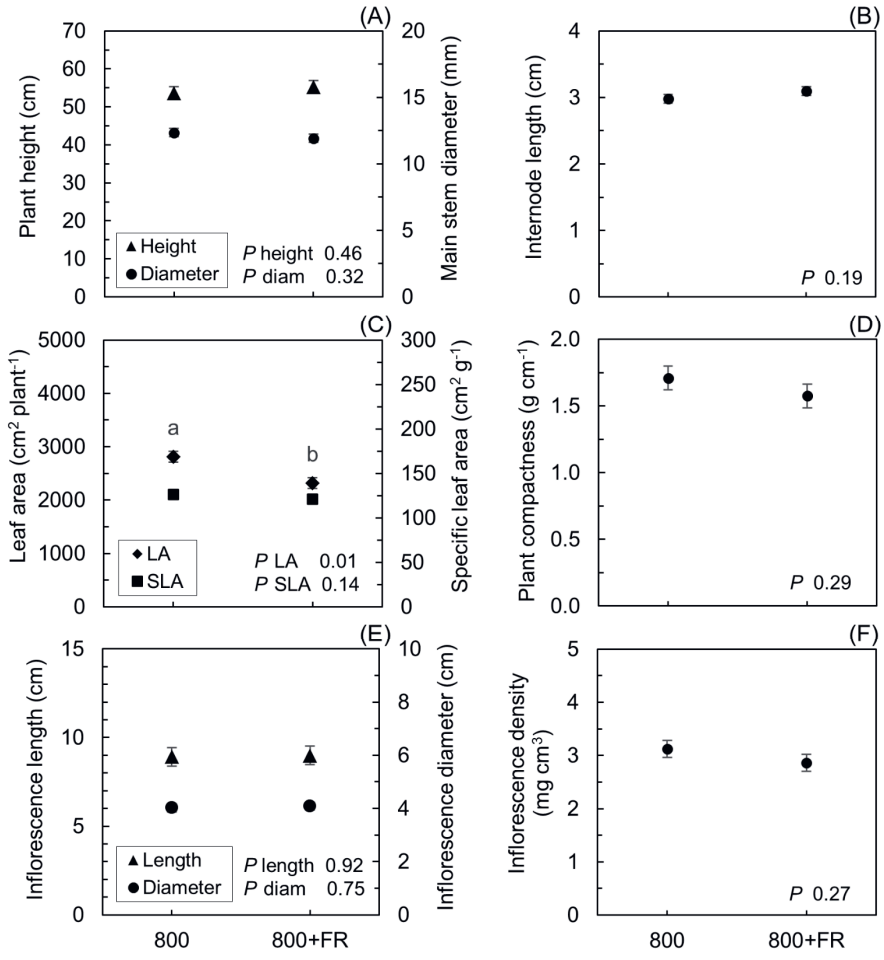

Figure 6. Plant height and main stem diameter (A); internode length (B); leaf area (LA) and specific leaf area (SLA) (C); plant compactness (D); Length and diameter of inflorescence (E); and inflorescence density (F) of medicinal cannabis cultivar 'Critical CBD' after 8 weeks into the generative phase. The plants were grown at 800 µmol $m^{-2}s^{-1}$ PPFD or 800 µmol $m^{-2}s^{-1}$ PPFD with an addition of 200 µmol $m^{-2}s^{-1}$ far-red during the last 4 weeks of the generative phase. Data based on 18 replicated plants per treatment. Error bars represent SEM based on the common variance, when larger than the symbols. Different letters indicate significant difference between treatments at P=0.01, according to Fisher's protected LSD test.

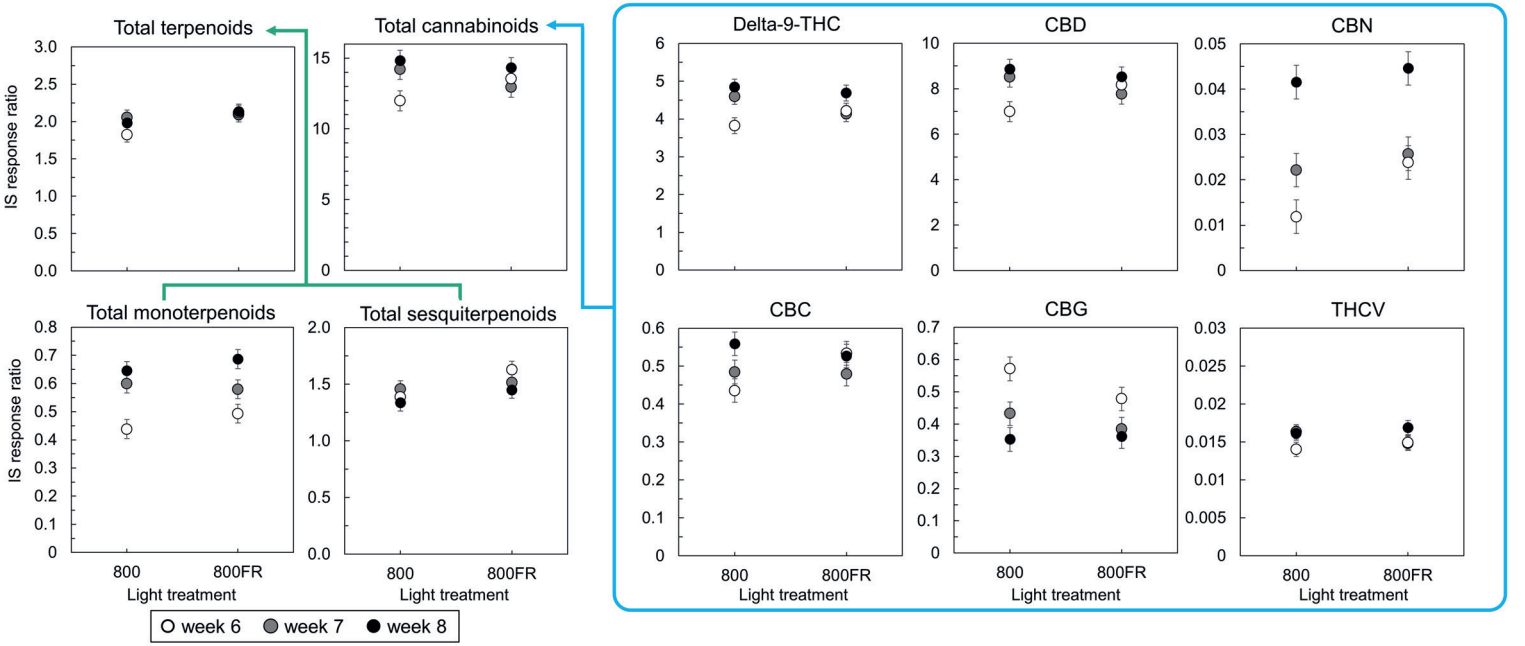

Figure 7. Concentration of terpenoids and cannabinoids in fresh inflorescences of medicinal cannabis cultivar 'Critical CBD' after 6, 7, and 8 weeks into the generative phase. The plants were grown at 800 µmol $m^{-2}s^{-1}$ PPFD or 800 µmol $m^{-2}s^{-1}$ PPFD with an addition of 200 µmol m^{-2} s^{-1} far-red during the last 4 weeks of the generative phase. Data are the averages of the internal standard (IS) response based on 5 replicated samples per treatment. Error bars represent SEM based on the common variance. There was no significant effect of light treatments (all P-values larger than 0.05).

4. Discussion

4.1. Limited effect of blue fraction on plant morphology and dry mass

Blue light serves as a crucial signal for plant growth and development. However, the optimal range for benefiting plant production can vary depending on species and other environmental factors (Huché-Thélier et al., 2016). Our study shows that blue fractions ranging from 8% to 21% substituting red fraction in white light applied throughout the vegetative and generative phases hardly affected dry mass and morphology in two cannabis cultivars. In contrast to our study, previous studies in cannabis have shown that an increase in blue fraction reduced inflorescence yield; Westmoreland et al. (2021) reported a 1% increase in blue light resulted in a 1% decrease in yield, while Magagnini et al. (2018) found a 0.6% decrease. The previous studies also suggested that plants grown under blue-rich LEDs lights tend to be shorter and have more horizontally oriented branches compared to those grown under lower blue from HPS lighting (Magagnini et al., 2018; Namdar et al., 2019). We also found a tendency of decreases in plant height, internode length, and specific leaf area (P-values = 0.02-0.06, Fig 2). Blue triggers cryptochrome resulting in a reduced internode length and compact plants (Cope et al., 2014; Wollaeger and Runkle, 2015). As a consequence, these plants have lower light absorption, possibly leading to reduced biomass production (Kalaitzoglou et al., 2021). These morphological responses to blue could explain reduction of biomass but were not observed in our study. Blue has been found to enhance flowering in some crops such as chrysanthemum (Jeong et al., 2012) and petunia (Gautam et al., 2015). This effect is potentially mediated through cryptochromes and phytochromes (Shibuya and Kanayama, 2014). However, the blue fractions (8%-21%) in our study did not influence flower induction and development (Fig S1-2).

4.2. Adding far-red during the late generative phase does not influence on plant morphology and yield

Adding 200 µmol $m^{-2}s^{-1}$ of far-red to 800 µmol $m^{-2}s^{-1}$ PPFD of red/blue/white light during the last 4 weeks of the generative phase did not result in an increase in dry mass, compared to the condition without far-red at the same PPFD. This is unexpected, as far-red light has a positive effect on dry mass production in various crops (Demotes-Mainard et al., 2016; Ji et al., 2020; Jin et al., 2021; Park and Runkle, 2017). In general, far-red enhances a shade avoidance response, characterized by stem elongation and leaf expansion, to maximize light interception, and consequently an increase in yield can be

expected (Franklin, 2008). In addition, although far-red is outside PAR range, it potentially improves photosynthetic efficiency (Zhen and Bugbee, 2020). In our study, the morphology of far-red exposed plants was not significantly different from those grown without far-red (Fig 6; Fig S3) in terms of plant height, stem diameter, internode length, and leaf area. Since the plant architecture was not modified, this may explain why we did not observe a clear effect on dry mass. The limited effect of far-red in our study could be attributed to the timing of application. Far-red was provided only during the last 4 weeks of the generative phase when plants had already finished vegetative growth, as shown by no increase in plant height by far-red (Fig S4) and plants prioritized reproductive growth instead (Potter, 2014 and Chapter 5). Furthermore, the period of far-red application in our study is also a crop stage where a decline in photosynthesis has been reported due to leaf senescence (Chapter 4). Therefore, the plants may not effectively utilize additional far-red for photosynthesis benefits.

The inflorescences of cannabis are formed when intense branching occurs at the apex while their internode length is reduced (Spitzer-Rimon et al., 2019). Low red:far-red ratio by replacing PPFD with far-red throughout the generative phase resulted in elongation of not only inflorescence but also whole plant (Kotiranta et al., 2024). We initially hypothesized that applying far-red during the latter part of the generative phase, at the moment of flower development, results in more elongated inflorescences without affecting the whole plant. This adjustment might help to minimize the risk of fungal infection and potentially create additional space for flowers to develop, thus increasing the final yield of inflorescences. However, the size and density of inflorescences were not altered by added far-red (Fig 6E-F), nor the yield. This could be due to the late timing of far-red when the inflorescences might have already stopped elongating. as the plant height did not change during that time (Fig S4).

4.3. Specialized metabolites are unaffected by fractions of blue and additional far-red

Light spectrum has the potential to affect the biosynthesis of plant specialized metabolites (Ouzounis et al., 2015; Zhang et al., 2021). Blue as a short wavelength radiation, has shown positive effects on specialized metabolites, for instance, high fraction of blue increased anthocyanins and carotenoids in leaves (Hoffmann et al., 2016; Huché-Thélier et al., 2016; Van Brenk et al., 2024), fruits (Liu et al., 2022), and flowers (An et al., 2020; Meng et al., 2004). Blue may increase terpenoid production via cryptochrome, which can upregulate biosynthetic genes (reviewed by Contreras-Avilés

et al., 2024). Previous research in cannabis found that high fraction of blue (8%-24%) increased THC, THCV, CBD, and CBG in inflorescences, compared to low blue (Magagnini et al., 2018). Additionally, 18% blue light resulted in higher THCVA than 3% blue light (Kotiranta et al., 2024). Westmoreland et al. (2021) found no effect of blue light (4%-20%) on either THC or CBD concentrations. In our study, blue fractions ranging from 8% to 21% did not change concentrations of both terpenoids and cannabinoids (Fig 3-4). It should be noted that in those previous studies, the blue fractions were compared across various light sources, such as LEDs and HPS lamps, and these tested spectra differed not only in blue but also in other ranges of the light spectrum. In contrast, the blue fraction in our study was increased by substituting red light, while green light remained constant. Moreover, the light spectra in the previous studies had a higher green fraction (about 40%), compared to the constant 6% used in our study. These variations could result in different effects of blue light, making it challenging to eliminate their influence.

Adding far-red to red/blue/white light during the last 4 weeks of the generative phase did not significantly influence concentrations of terpenoids and cannabinoids in inflorescences, compared to plants grown without far-red light. Kotiranta et al. (2024) found that replacing PPFD by far-red (i.e., low red:far-red) throughout the whole generative phase decreased concentrations of monoterpenoids and some cannabinoids. It should be noted that the decrease in metabolites might have been caused by lower PPFD in far-red treatment, which effect of PPFD is shown in Chapter 5. The reduction in cannabinoid due to low red:far-red was also observed by Magagnini et al. (2018) and Reichel et al. (2022). However, in those studies the red:far-red ratios were compared across different light sources, which affected not only the far-red spectrum but all other spectra. These authors proposed that low red:far-red could reduce the biosynthesis of geranyl pyrophosphate (GPP), a precursor of cannabinoids and monoterpenoids. Moreover, far-red reduces accumulation of metabolites related to plant defense mechanisms (Courbier et al., 2020; Demotes-Mainard et al., 2016). The absence of effects of far-red in our study may be attributed to the shorter and later timing of its application. In our study, far-red was only applied for the last 4 weeks of the generative phase, whereas most studies applied far-red throughout the entire generative phase (longer than 8 weeks). The reason for applying far-red only during the last period was to avoid elongated plants, as reported by Kotiranta et al. (2024) and Reichel et al. (2021) which would make crop management more difficult.

Considering that there is no significant benefit for biomass or concentrations of specialized metabolites from a high fraction of blue or additional far-red, it may be best

to produce medicinal cannabis at a relatively low fraction of blue, since efficacy of blue LEDs is lower than for red LEDs (Kusuma et al., 2020; Westmoreland et al., 2021). In addition, adding far-red during the last 4 weeks of the generative phase is not needed.

Understanding the effects of light spectrum on growth and specialized metabolites in medicinal cannabis remains challenging. Several studies have been conducted but comparing them is difficult due to variations in background light spectrum and other environmental factors that could alter plant growth and specialized metabolite production. We suggest further studies to vary only the colors of interest while carefully controlling other light colors to the best extent possible. Additionally, the limited effects of light spectrum observed here for cannabis could be due to the interaction between effects of light spectrum and light intensity. Such interaction has been reported for the effect of blue light on soybean, radish (Cope and Bugbee, 2013), and tomato (Utasi et al., 2023). Since cannabis experiments are often conducted at high light intensity, above 500 µmol $m^{-2}s^{-1}$ PPFD, which is higher than light levels applied in studies in other crops, it would be interesting for further studies on the light spectrum to include also light intensity as factor. Investigating the effects of the light spectrum at various light intensities could elucidate the effect of the interaction.

5. Conclusion

Within the range of 8% to 21%, blue light fraction substituting red light in a white light background did not significantly affect plant morphology and dry matter production. Adding 200 µmol $m^{-2}s^{-1}$ of far-red to 800 µmol $m^{-2}s^{-1}$ PPFD of red/blue/white light during the last 4 weeks of the generative phase did not lead to significant changes in plant morphology and dry matter production. Furthermore, the fractions of blue and the additional far-red light did not significantly influence the concentrations of terpenoids and cannabinoids.

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Supplementary material

Supplemental method 1 Rooting phase

In Exp 1, stem cuttings of medicinal cannabis (cultivar 'Critical CBD' and 'White Russian') were cut from 3-6 months old mother plants grown in a glasshouse (Wageningen University and Research, Greenhouse Horticulture, Bleiswijk, the Netherlands). The lower end of the stem cuttings was dipped in 0.1% IBA powder (Rhizopon, the Netherlands) and rooted in a propagating box with a transparent cover filled with a mixture of fine sand and cutting soil (Lensli Potgrond, Horticoop, the Netherlands) (1:1). The propagation boxes were place at 100 µmol $m^{-2}s^{-1}$ PPFD, consisting of 11% blue (400-500nm), 16% green (500-600nm), and 73% red light (600-700nm) provided by a mixture of red-blue-white LEDs (Philips, GPL production DRW LB 1.2) with 40 µmol $m^{-2}s^{-1}$ of far-red (Philips, GPL production module far red 120). The photoperiod was 18/6 h (light/dark). The rooting took 21 days. Climate setpoints were 23/21°C air temperature, 85% relative humidity (RH) and ambient CO_2. The irrigation of the rooting phase was done by water can with a nutrient solution with an EC of 1.6 dS m^{-1} and a pH of 5.8. The solution consisted of the following macro- and micronutrients; 1.25 mM NH_4^+; 6.2 mM K^+; 1.9 mM Ca^{2+}; 0.9 mM Mg^{2+}; 10.5 mM NO_3^-; 0.85 mM SO_4^{2-}; 0.85 mM PO_4^{3-}; 60 µM Fe^{2+}; 20 µM Mn^{2+}; 3 µM Zn^{2+}; 20 µM B^{2+}; 0.5 µM Cu^{2+}; 0.5 µM Mo^{2+}.

In Exp 2, stem cuttings of medicinal cannabis (cultivar 'Critical CBD') were cut from 3-6 months old mother plants grown in a glasshouse (Wageningen University and Research, Greenhouse Horticulture, Bleiswijk, the Netherlands). The lower end of the stem cuttings was dipped in 0.25% IBA powder (Rhizopon, the Netherlands) and rooted in 3.6 x 3.6 x 4 cm stonewool plugs (Grodan, the Netherlands). Cuttings were placed in an enclosed transparent plastic tent at 100 µmol $m^{-2}s^{-1}$ PPFD, consisting of 8% blue (400-500nm), 6% green (500-600nm), and 86% red light (600-700nm), provided by a mixture of red-blue-white LEDs (Green Power DRW LB 1.2, Philips, the Netherlands) with 50 µmol $m^{-2}s^{-1}$ of supplemental far-red (Green Power far-red 1.2, Philips, the Netherlands). The photoperiod was 18/6 h (light/dark). The rooting took 21 days. Climate setpoints were 28/28°C air temperature, 80% relative humidity (RH) and ambient CO_2. The irrigation of the rooting phase was done by soaking the plugs in a nutrient solution with an EC of 1.6 dS m^{-1} and a pH of 5.8. The solution consisted of the following macro- and micronutrients; 1.25 mM NH_4^+; 6.2 mM K^+; 1.9 mM Ca^{2+}; 0.9 mM Mg^{2+}; 10.5 mM NO_3^-; 0.85 mM SO_4^{2-}; 0.85 mM PO_4^{3-}; 60 µM Fe^{2+}; 20 µM Mn^{2+}; 3 µM Zn^{2+}; 20 µM B^{2+}; 0.5 µM Cu^{2+}; 0.5 µM Mo^{2+}.

Table S1. Realized air temperature and relative humidity, represent overall average of 3 blue light treatments in Exp 1. The measurement was done at plant level, recorded every 10 minutes by dataloggers (ML4160, Hanwell Solutions, UK).

Week of the generative phase		Air temperature (°C)	%RH
Vegetative	Light	26.7	78.7
	Dark	23.2	82.9
Week 1	Light	28.3	59.9
	Dark	25.6	67.4
Week 2	Light	25.6	74.4
	Dark	26.0	69.2
Week 3	Light	26.3	68.6
	Dark	25.0	73.3
Week 4	Light	26.9	66.7
	Dark	25.4	68.0
Week 5	Light	25.8	72.1
	Dark	26.0	66.3
Week 6	Light	26.6	66.1
	Dark	26.5	54.3
Week 7	Light	26.5	62.8
	Dark	26.3	53.1
Week 8	Light	26.7	62.2
	Dark	26.5	53.0

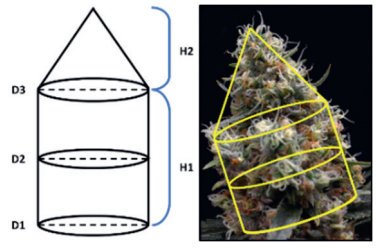
Table S2. Realized air temperature and relative humidity for each light treatment in Exp 2. The measurement was done at plant level, recorded every 10 minutes by dataloggers (Easylog USB-1-LCD, Lascar electronics, Wiltshire, UK).

Week of generative phase		Air temperature (°C)		%RH	
		800	800+FR	800	800+FR
Week 1	Light	28.0	28.4	81.2	79.4
	Dark	26.2	26.5	88.1	87.3
Week 2	Light	28.4	30.2	81.0	73.0
	Dark	26.0	26.1	89.4	88.0
Week 3	Light	27.5	29.4	83.5	76.1
	Dark	26.9	27.0	89.8	88.5
Week 4	Light	27.5	28.3	76.4	71.1
	Dark	26.1	26.2	83.0	80.4
Week 5	Light	28.1	28.5	67.0	64.6
	Dark	26.1	25.8	74.7	74.2
Week 6	Light	27.9	29.7	62.7	56.1
	Dark	26.5	27.0	71.3	67.9
Week 7	Light	27.5	29.6	61.4	54.5
	Dark	26.4	26.9	66.6	63.3
Week 8	Light	27.5	29.8	61.2	53.0
	Dark	26.2	26.8	63.7	59.5

Supplemental method 2 Inflorescence measurement

The volume of the inflorescence was modelled as a cylinder with a cone on top. The denominator of the formula consists of the volume of the cylinder, where D_{avg} (cm) is the average diameter taken at 3 positions along the inflorescence (position D1, D2, and D3). H_1 (cm) is the height of the cylindrical part of the inflorescence. The volume of the cone is determined by D_3 (cm) and H_2 (cm). If an inflorescence's shape lacked either a cylinder or cone, that volume calculation was removed. Schematic representation of an apical inflorescence and the schematic overlaid over an actual inflorescence. Diameters are determined at three points (dotted lines), D1, D2, and D3. D3 is defined as the part of the inflorescence where cylindrical part tapers towards a conical shape. H1 and H2 are determined accordingly.

$$Inflorescence\ density\ (g \cdot cm^3) = \frac{Apical\ inflorescence\ weight\ (g\ FW)}{\left(\pi * \left(\frac{Davg}{2}\right)^2 * H_1\right) + \left(\frac{1}{3} * \pi * \left(\frac{D_3}{2}\right)^2 * H_2\right)}$$



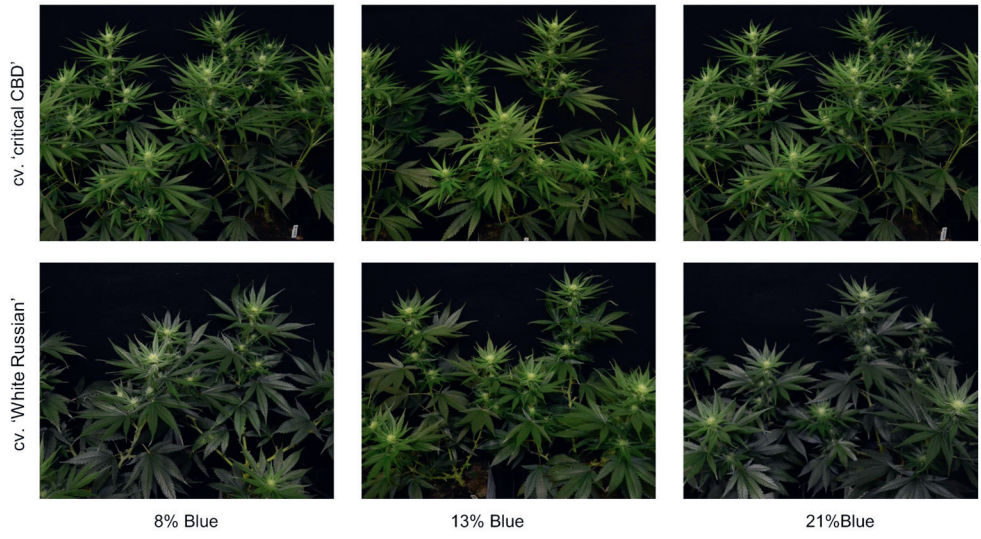


Figure S1. Pictures of plants at 13 days of medicinal cannabis cultivar 'Critical CBD' and 'White Russian' grown under 8, 13, and 21% blue after the generative phase started (33 DAT), showing no differences in developmental stage among blue light treatments. (Exp 1)

Figure S2. Pictures of plants of medicinal cannabis 'Critical CBD' and 'White Russian' grown under 8, 13, and 21% blue at the end of the vegetative phase (20 DAT) and the generative phase (76 DAT), showing no differences in developmental stage among blue light treatments. (Exp 1)

Figure S3. Pictures of plants of medicinal cannabis cultivar 'Critical CBD' at the end of the generative phase (69 DAT). The plants were grown under 800 µmol $m^{-2}s^{-1}$ PPFD or 800 µmol $m^{-2}s^{-1}$ PPFD with an addition of 200 µmol $m^{-2}s^{-1}$ far-red during the last 4 weeks of the generative phase. (Exp 2)

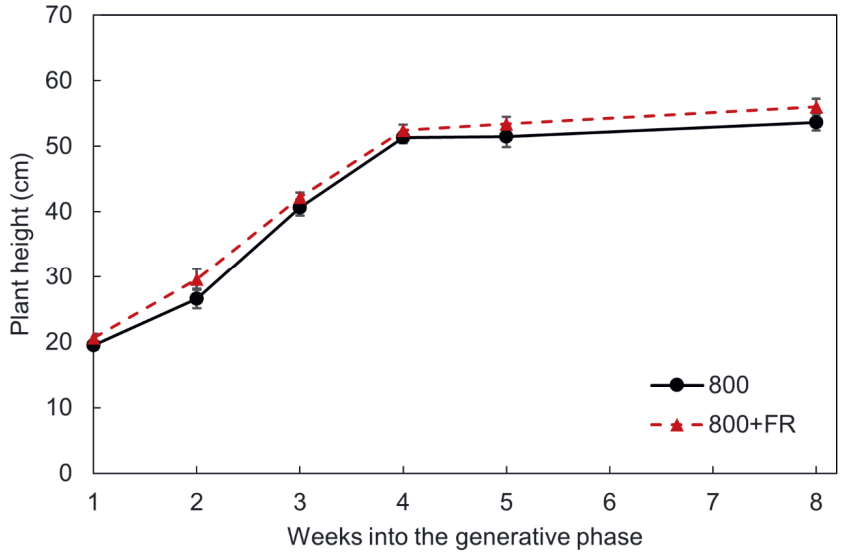


Figure S4. Plant height of medicinal cannabis cultivar 'Critical CBD' during 8 weeks of the generative phase. The plants were grown at 800 µmol $m^{-2}s^{-1}$ PPFD or 800 µmol $m^{-2}s^{-1}$ PPFD with an addition of 200 µmol $m^{-2}s^{-1}$ far-red during the last 4 weeks of the generative phase. Data based on 18 replicated plants per treatment. Error bars represent SEM based on the common variance, when larger than the symbols. (Exp 2).

Chapter 4

High light intensity improves yield of specialized metabolites in medicinal cannabis, resulting from both higher inflorescence mass and concentrations of metabolites

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(submitted)

Abstract

Medicinal cannabis (*Cannabis sativa* L.) contains various plant specialized metabolites, such as cannabinoids and terpenoids. These metabolites are mainly accumulated in inflorescences and are the primary focus of cultivation. Medicinal cannabis is often cultivated in indoor farming with artificial light, which allows for light intensities to optimize quantity and quality of production. Although it is known that an increase in light intensity results in increased inflorescence yield in cannabis, its impact on specialized metabolites remains unclear. We aim to quantify the effects of light intensity on the yield of specialized metabolites, and to elucidate which plant traits explain these effects, using a yield component analysis. Hereto, we conducted an experiment in a climate-controlled chamber using broad-band white LEDs at three light intensities: 600, 800 and 1000 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD), applied during the generative phase (8 weeks with a 12-h photoperiod) in cannabis cv. 'Critical CBD'. Yield of specialized metabolites, including cannabinoids and terpenoids, strongly increased with increasing PPFD due to increases in both their concentrations and inflorescence yield. The inflorescence yield showed a proportional increase with PPFD, resulting in a constant light use efficiency. The higher inflorescence dry mass was mainly caused by an increase in total plant dry matter production, and to a lesser extent a larger fraction of dry mass partitioned to the inflorescences. Leaf photosynthesis was higher for plants grown at higher PPFD. This study shows that cannabis can use very high light intensity, resulting in high yield of specialized metabolites due to high inflorescence mass and metabolite concentrations.

Keywords: LEDs, *Cannabis sativa* L., cannabinoids, terpenoids

1. Introduction

Indoor cultivation has been introduced to several horticultural crops, as it allows for control over plant quantity and quality through finely controlled growing conditions such as light, temperature, carbon dioxide, and air humidity (SharathKumar et al., 2020). Light in indoor cultivation can be designed according to many desired regimes, including light intensity, light spectrum, and photoperiod. Among others, medicinal cannabis (*Cannabis sativa* L.) as a high value cop, has benefited from indoor cultivation, as this allows for high quality and quantity control (Summers et al., 2021).

The primary focus of medicinal cannabis cultivation is to produce specialized metabolites, also named secondary metabolites, including cannabinoids, terpenoids, and flavonoids. Groups of cannabinoids and terpenoids accumulate in the glandular trichomes, which are mainly present on mature female inflorescences (Andre et al., 2016; Livingston et al., 2020). The major bioactive forms of cannabinoids consist of delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabigerol (CBG), cannabichromene (CBC) and cannabinol (CBN) (Richins et al., 2018), and there are more than 120 other cannabinoids which have been isolated from cannabis plants (ElSohly et al., 2017). Additionally, an abundance of terpenoids results in the unique aroma of cannabis (Hanuš and Hod, 2020). As these compounds are found together, they might have synergistic and/or entourage effects (Russo, 2011). From an ecological point of view, cannabis is hypothesised to produce cannabinoids and terpenoids to protect itself from the damage of UV light, pathogens and pest insects (Gülck and Møller, 2020).

The production of specialized metabolites can be manipulated by influencing their concentrations and the yield of inflorescence. Biosynthesis of specialized metabolites is known to be light-dependent, with both light intensity and spectrum influencing their concentrations (Contreras-Avilés et al., 2024; Ntagkas et al., 2020; Ouzounis et al., 2015; Ramakrishna and Ravishankar, 2011). In cannabis, however, studies on specialized metabolites have primarily focused on the effects of light spectrum rather than light intensity (Danziger and Bernstein, 2021b; Magagnini et al., 2018; Morello et al., 2022; Rodriguez-Morrison et al., 2021b; Westmoreland et al., 2021). Light intensity can increase carbon gain via enhanced photosynthesis, and subsequently increases carbon-based specialized metabolites (Darko et al., 2014b). It was reported that light intensity ranging from 120 to 1800 µmol $m^{-2}s^{-1}$ had no significant effect on concentrations of cannabinoids, but slightly increased those of total terpenes, in cannabis inflorescences (Rodriguez-Morrison et al., 2021a).

To promote the formation of inflorescences, cannabis requires a 'short-day' phase of maximum 12 h of light per day during the generative phase (Spitzer-Rimon et al., 2019). It has been recommended to use very high light intensities, with a photosynthetic photon flux density (PPFD) between 500 and 1500 µmol $m^{-2}s^{-1}$ PPFD (Chandra et al., 2017a) to obtain a high daily light integral (DLI) which drives plant biomass production**.** These light intensities are much higher than those used for other horticultural crops: e.g., a PPFD of 250 µmol $m^{-2}s^{-1}$ is recommended for lettuce and basil for indoor cultivation (Pennisi et al., 2020) and 300 µmol $m^{-2}s^{-1}$ for dwarf tomato (Ke et al., 2023). A recent study using a light intensity gradient in a cannabis cultivation room found that increasing light intensity, ranging from 120 to 1800 µmol $m^{-2}s^{-1}$ PPFD using LED, resulted in higher inflorescence yield of cannabis (Rodriguez-Morrison et al., 2021a).

Photosynthesis uses most of the visible light absorbed by green plant tissue to power the assimilation of carbon dioxide into triose phosphates, which are then converted into carbohydrates (Calvin and Benson, 1948; Simkin et al., 2022). In many horticultural crops, there is a 0.7-1% increase in harvestable biomass for every 1% increase in light intensity (Marcelis et al., 2006). To maximize plant productivity, a light intensity above the photosynthetic light compensation point (i.e., the light intensity at which net photosynthesis rate is zero) and below the light saturation point of the photosynthetic response is often sought (Eichhorn Bilodeau et al., 2019; Simkin et al., 2019). The net photosynthesis rate of cannabis leaves increases with higher light intensities, and it remains unsaturated (i.e. it keeps increasing) even at levels up to 1500-2000 µmol $m^{-2}s^{-1}$ (Chandra et al., 2015, 2008). However, light intensity cannot be increased indefinitely; photosynthesis will eventually become light-saturated, caused by limitation of the photosynthetic machinery, leading to photodamage from excessive light (Raven, 2011; Zivcak et al., 2014).

The allocation of photo assimilates among plant organs determines harvestable yield and can be influenced by light intensity. In fruit crops, light intensity indirectly affects biomass partitioning by increasing the number of fruits or reducing rates of fruit abortion, thereby enhancing the overall sink strength of all fruits (Marcelis, 1996). In chrysanthemum, higher light intensity leads to a larger number of flowers, resulting in greater allocation of dry mass towards flowers (Heuvelink et al., 2002; Hosseinzadeh et al., 2021). In cannabis**,** higher light intensities have been shown to increase the harvest index, calculated as inflorescence yield over total aboveground biomass (Rodriguez-Morrison et al., 2021a), as well as the flower-to-leaf ratio (Potter and Duncombe, 2012)**.**

Although it is known that an increase in light intensity results in increased inflorescence yield in cannabis, its impact on specialized metabolites remains unclear. Moreover, it is uncertain which components are responsible for these increases. This can be further investigated using a yield component analysis (Higashide and Heuvelink, 2009) to quantify the contributions of underlying components to yield. Therefore, this study aims to quantify the effects of light intensity on the yield of specialized metabolites and to elucidate which plant traits explain these effects. We hypothesized that high light intensity increases specialized metabolites yield due to higher concentrations and higher inflorescence yield, where the increase of inflorescence yield is caused by higher total biomass production and higher dry mass partitioning towards inflorescences. We conducted an experiment in a climate-controlled chamber using light emitting diodes (LEDs) at three light intensities: 600, 800, and 1000 µmol $m^{-2}s^{-1}$ PPFD, applied during 8 weeks of the generative phase (i.e. short-day phase for flower induction and flower development) of medicinal cannabis.

2. Materials and methods

2.1. Plant cultivation

Medicinal *Cannabis sativa* L. cultivar 'Critical CBD' (chemotype II, with an intermediate THC/CBD ratio of approximately 0.5) plants were grown in a climate-controlled walk-in chamber. Plants were propagated by stem cuttings, which were rooted in stonewool plugs for 21 days (details on the rooting phase are described in Supplemental methods 1). Well-rooted cuttings were transplanted into 15 x 15 x 15 cm stonewool blocks (Grodan, Roermond, the Netherlands). The vegetative phase (i.e. long-day phase to promote vegetative growth), lasted from 0 to 11 days after transplanting (DAT) at 423 ± 24 µmol $m^{-2}s^{-1}$ PPFD, with 13% blue (400-500nm), 6% green (500-600nm), 81% red (600-700nm), and <0.6% far-red (700-800 nm), provided by a mixture of red-blue-white light-emitting diodes (LEDs, Green Power DRW MB 1.2, Philips, the Netherlands). The photoperiod was 18/6 h (light/dark). The light (PPFD and spectrum) was measured at 45 cm above the table, which was at final canopy height during the vegetative phase. The PPFD was measured using a LI-250A light meter (LI-COR Inc., Lincoln, NE, USA) and the light spectrum was measured using a specbos 1210 spectrophotometer (JETI Technische Instrumente, Jena, Germany).

The planting density was kept constant at 9 plants m^{-2} throughout the whole experiment. Plants were pruned three times during cultivation. At 8 DAT, the apex was removed. At

11 DAT, the lowest side shoots were removed, such that the four upper side shoots were retained. Finally at 16 DAT, the lower second-order side shoots were removed to retain three second-order side shoots per side shoot.

During the vegetative phase (0-11 DAT), the climate was 28.7/26.3 °C (light/dark), 79/86 %RH, and CO_2 was 400 µmol mol^{-1}. During the generative phase (12-69 DAT), the climate was 28.0/26.7 °C, and 71/75% RH, while CO_2 was gradually increased from 600 to 1200 µmol mol^{-1}, adding 200 µmol mol^{-1} every 2 weeks. Realised air temperature and RH per light treatment and week are shown in Table S1. The maximum differences of average realised air temperature and RH between treatments were 1.5/0.3 °C and 5.3/1.2 %RH (light/dark values). Before transplanting, stonewool blocks were pre-soaked in a nutrient solution with an EC of 1.6 dS m^{-1} and a pH of 5.8. The solution consisted of the following macro- and micronutrients: 1.25 mM NH_4^+; 6.2 mM K^+; 1.9 mM Ca^{2+}; 0.9 mM Mg^{2+}; 10.5 mM NO_3^-; 0.85 mM SO_4^{2-}; 0.85 mM PO_4^{3-}; 60 µM Fe^{2+}; 20 µM Mn^{2+}; 3 µM Zn^{2+}; 20 µM B^{2+}; 0.5 µM Cu^{2+}; 0.5 µM Mo^{2+}. Irrigation from transplanting onward was supplied with the same nutrient solution by discharge-regulated drippers into the stonewool blocks, each discharge was 100 mL. Frequency of dripping ranged from four to six cycles per day, and it was adjusted to ensure that all plants received sufficient water and nutrients. The EC value was first raised to 2 dS m^{-1} (0-11 DAT), then further raised to 2.5 dS m^{-1} during the generative phase (12-69 DAT), by increasing the concentration of macronutrients, while keeping the ratios between individual macronutrients constants.

2.2. Light intensity treatments

During the 8 weeks of the generative phase (12-69 DAT), three light intensity treatments were applied: 600, 800, or 1000 µmol $m^{-2}s^{-1}$ PPFD. Realized light intensities were 595±11, 791±10, and 991±15 µmol $m^{-2}s^{-1}$ PPFD respectively, measured at 75 cm above the table, which was at final canopy height. The light was provided by the same fixtures that were used in the vegetative phase; the light spectrum was therefore similar (described above). The photoperiod was 12/12 h (light/dark). These three light treatments were randomly assigned to each compartment within the climate room and were partitioned by white plastic screens. Each compartment measured 3.3 m^2 (replicate 1) and 2 m^2 (replicate 2).

2.3. Growth and morphology measurements

Final harvest was conducted after 8 weeks into the generative phase (67-69 DAT). Plant height was measured as the longest distance from the base of the stem towards the top of the canopy. The diameter of the main stem was determined at 1 cm above the top of the stonewool block. Each plant was dissected into leaves, stems and inflorescences. Inflorescence leaves and stems were trimmed by an electronic trimmer (Bowl Trimmer 40 cm, Dutchmasters Fertraso, the Netherlands), and their trimmed mass was added to that of the stems. Leaf area per plant was determined using a LI-3100C area meter (LI-COR Inc., Lincoln, NE, USA). Inflorescence mass was determined after trimming, measured as fresh mass, approximately 10% moisture dry mass (air-dried, as is the commercial standard), and dry mass. Drying to 10% moisture of inflorescences took place for five days in a dark climate-controlled room with ample ventilation, at 45% RH, and 25 °C. Dry mass was determined after drying the material at 70 °C for 24 h, followed by 105 °C for 72 h in a ventilated oven. Dry matter content was calculated as the ratio between dry and fresh mass. Light use efficiency (LUE) was calculated as dry mass accumulated over the whole cultivation cycle divided by cumulative incident mol of PPFD during the generative phase (treatment period). Plant compactness was calculated as the ratio of total plant dry mass to plant height. Specific leaf area (SLA; $cm^2\ g^{-1}$) was calculated as the ratio between leaf area and leaf dry mass.

2.4. Photosynthesis light response curves

Photosynthesis light response curves were determined at 3, 5 and 7 weeks into the generative phase in replicate 2, using a LI-6800 Portable Photosynthesis System (LI-COR) equipped with a Multiphase Flash Fluorometer and Chamber (LI-COR part No. 6800-01A, area 2 cm^2). Measurements were taken on one leaf per plant using the youngest, fully developed, unshaded leaves, with 3-5 plants chosen at random per light treatment (n=3-5). At 3 weeks into the generative phase, net photosynthesis rate (*A*) (µmol CO_2 $m^{-2}s^{-1}$) was measured at 2000, 1500, 1000, 800, 600, 400, 200, 100, and 0 µmol $m^{-2}s^{-1}$, with each step taking 3-5 min. At 5 and 7 weeks into the generative phase, a light intensity step at 3000 µmol $m^{-2}s^{-1}$ was added. Light was provided by a mixture of red (87%) and blue (13%) LEDs in the fluorometer (at the light step of 3000 µmol $m^{-2}s^{-1}$, the ratio changed to 90% red and 10% blue, as the capacity of blue LEDs was exceeded at that intensity). Leaves were first adapted to the highest light intensity (either 2000 or 3000 µmol $m^{-2}s^{-1}$) for 15 min, or until *A* and stomatal conductance (g_s) were stable. Data were logged after stability was reached, i.e. when the slope of ΔCO_2 was <0.1 over a

period of 20 s. A rolling average of 10-15 s was used. CO_2 concentration was 800 µmol mol^{-1}, air flow rate was 500 µmol s^{-1}, RH was 60-70%, and air temperature was 27 °C, yielding a leaf temperature of 27-30 °C across all light steps. Light response curves were fitted to a non-rectangular hyperbola (Ogren and Evans, 1993), yielding the parameters maximum net photosynthesis rate (A_{max}; µmol CO_2 $m^{-2}s^{-1}$), (α; µmol CO_2 µmol photon s^{-1}), a curvature parameter (θ), and dark respiration (R_d; µmol CO_2 $m^{-2}s^{-1}$) (Eq. 1). The light compensation point (*LCP*; µmol $m^{-2}s^{-1}$ PPFD) was determined from R_d and α.

$$A = -R_d + \frac{\alpha \cdot \text{PPFD} + A_{max} - \sqrt{(\alpha \cdot \text{PPFD} + A_{max})^2 - 4\theta \ \text{PPFD} \cdot A_{max} \cdot \alpha}}{2\theta} \quad (1)$$

2.5. Specialized metabolites

Fresh inflorescences were collected from the top 5 cm of inflorescences in 6, 7, and 8 weeks into the generative phase and stored at -80 °C. A total of five biological samples per treatment was prepared, in which each sample was pooled from 4-6 plants. Per sample, 200 mg fresh inflorescences were homogenized before extraction in 2 mL of *n*-Hexane with 0.2 g L^{-1} of squalene as an internal standard (IS). Sample extracts were sonicated for 10 min in an ultrasonic bath (Branson 2800; Sigma-Aldrich, St. Louis, MI, USA), subsequently filtered through a column containing siloxilated glass-wool and anhydrous sodium sulphate in a Pasteur pipet and collected in a 2 mL glass vial. Two µL of each filtered extract was analysed in splitless mode on a Gas Chromatography-Mass Spectrometry (GC-MS) Agilent (7890) equipped with a 30-m length × 0.25-mm inner diameter, 0.25-µm film thickness column (Zebron, 5MS) and a mass-selective detector (model 5972A, Hewlett–Packard). The GC was programmed at an initial temperature of 60 °C for 2 min, with a ramp of 5 °C min^{-1} to 250 °C, and then with 10 °C min^{-1} to 280 °C and final time of 5 min. The injection port, interface, and MS source temperatures were 250 °C, 290 °C, and 180 °C, respectively, and the Helium inlet pressure was controlled with an electronic pressure control to achieve a constant column flow of 1 mL min^{-1}. The ionization potential was set at 70 eV, and scanning was performed from 45 to 400 atomic mass unit (amu). Metabolites were identified by comparing their mass spectra with those of the National Institute of Standards and Technology mass spectral library (NIST) MS search 2.0 and an in-house spectral library generated with authentic standards. In addition, the relative retention times of individual metabolites were compared to those of Adams (2017). Detected compounds were classified as either terpenoids or cannabinoids. Relative abundances (IS ratio response) of individual

compounds were determined by the area under the curve, and normalized by IS and fresh mass multiplied by the ratio between dry to fresh mass (as described in section 2.3). Total terpenoids were calculated as the sum of metabolites at retention time (RT) 6.2-26.2 min, from which the proportion of total monoterpenoids at RT 6.2-11.3 min and total sesquiterpenoids at RT 19.5-26.2 min was determined by dividing to the total terpenoid abundance. Total cannabinoids were a sum of metabolites at RT 35.0-43.2 min.

2.6. Yield component analysis

The effect of light intensity on specialized metabolites yield was analyzed by dissecting the effects on the underlying components, as illustrated in Fig 1. The yield of specialized metabolites per plant is defined by the product of inflorescence dry mass and the concentrations of specialized metabolites. The inflorescence dry mass is the product of total plant dry mass and fraction of dry mass partitioned to the inflorescences. The total plant dry mass is the result of fraction intercepted light (depends on leaf area index) and net photosynthesis rate, measured at its growth light intensity treatments (e.g., 600, 800, 1000 µmol $m^{-2}s^{-1}$) at 5 weeks into the generative phase. These values were extracted from the light response curves of each treatment. The leaf area index is the product of leaf dry mass per m^2 ground area and specific leaf area. A linear relationship was fitted to yield and each of the yield components with PPFD as regressor, and this regression was utilized to calculate the percentage change of each component from 600 to 1000 µmol $m^{-2}s^{-1}$ PPFD.

2.7. Statistical set-up and analysis

The experiment was performed twice in the same climate chamber, with a shift in time. The replicate experiments represented two blocks, each having a new randomization. There were 30 and 18 replicate plants per experiment (i.e., per treatment per experiment; hence 60 and 36 plants per treatment for the two experiments together) in replicate experiment 1 and 2, respectively. For morphological traits and dry mass, a one-way Analysis of Variance (ANOVA) in two blocks was conducted using the average values for each experimental unit. The effect of light intensity was partitioned into a linear and a quadratic component (orthogonal polynomial contrasts). For the concentrations of specialized metabolites, average values of 5 biological samples per experimental unit were used in a two-way ANOVA in blocks, with sampling week and light intensity (partitioned into a linear and a quadratic component) as factors (no interaction). For the photosynthesis parameters, which were determined only in replicate 2, the

measurement on each plant (n=3-5) was considered as an independent replicate in a one-way ANOVA. Statistical tests were conducted at P=0.05. The normality of the residuals was examined with Shapiro-Wilk test at P=0.05. For the photosynthesis parameters, homogeneity of residuals was tested with Bartlett's test at P=0.05, while homogeneity for the other traits was assumed as it could not be tested because of the small number of replicates (n=2). These assumptions were met in all cases. All tests were performed using Genstat 21st edition (VSN International, Hemel Hempstead, UK). For the multivariate analysis, the relative abundance of specialized metabolites (as described in section 2.3) was uploaded to MetaboAnalyst 5.0 online platform (Chong et al., 2019). The dataset included individual biological replicates from both repetitions (n=10), underwent log-10 transformation and mean centering before being subjected to analysis.

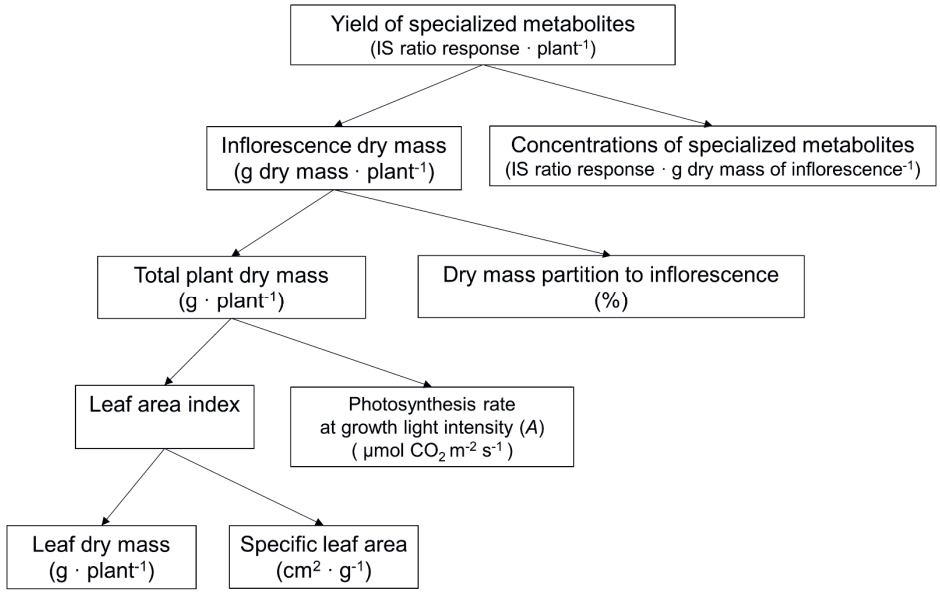

Figure 1. General scheme of a top-down analysis of specialized metabolites yield, separated into underlying components. *A* represents net photosynthesis rate measured at growth light intensity (i.e. 600, 800, and 1000 µmol $m^{-2}s^{-1}$), 5 weeks into the generative phase. Units are provided between brackets. Metabolites were calculated as internal standard (IS) ratio response.

3. Results

3.1. Concentrations of specialized metabolites in inflorescences

In total, 56 specialized metabolites that were either terpenoids or cannabinoids were detected in inflorescences. Unsupervised principal component analysis (PCA) did not show a separation of specialized metabolite profiles between the three PPFD treatments (Fig S1). However, using a sparse partial least squares-discriminant analysis (sPLS-DA) as a supervised model showed that the groups of 600 and 1000 µmol $m^{-2}s^{-1}$ PPFD could be distinguished from each other, while results from the intermediate PPFD (800 µmol $m^{-2}s^{-1}$ PFD) were to a relatively large extent overlapping with those of the other two PPFD levels (Fig 2A-C). This separation between PPFD treatments was more apparent in the early phase, after 6 weeks into the generative phase (Fig 2A). Variability in projection scores of individual metabolites, for example of Component 1, indicated that multiple metabolites were responsive to increasing PPFD, and this was most obvious for sesquiterpenoids (Fig 2D-F). However, the error rate of the classification from these sPLS-DA models were considerably high, over 30% (Fig 2H-J). Analyzing metabolite abundance with ANOVA, considering sampling weeks and experimental blocks, showed a linear increase in total terpenoids, total cannabinoids, and also multiple individual compounds with increasing PPFD (Fig 3). Considering the individual weeks, a significant increase of CBN, CBG, and some terpenoids, including β-myrcene, β-caryophyllene, α-humulene, β-farnesene, and γ-eudesmol was found after 6 weeks into the generative phase, compared to their respective abundances after 7 or 8 weeks (Fig S2).

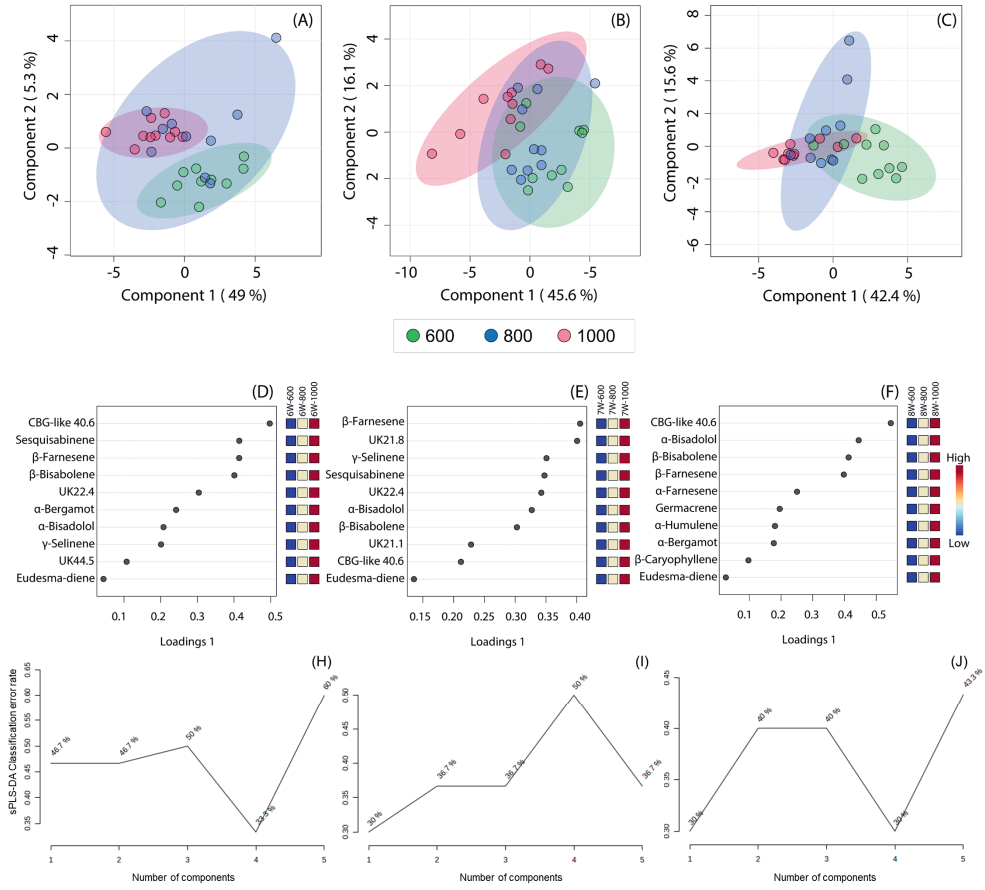

Figure 2. Sparse partial least-squares discriminant analysis (sPLS-DA) plots (A-C) and corresponding variable in projection plots indicating the most discriminating compounds of component 1 (D-F) of specialized metabolites in inflorescences of medicinal cannabis grown under 600 (green points), 800 (blue points), and 1000 (red points) µmol $m^{-2}s^{-1}$ PPFD at 6 (A, D, H), 7 (B, E, I) and 8 weeks (C, F, J) into the generative phase. When the compounds cannot be matched with those of the NIST library, they are represented as unknown (UK) with the retention time. The data are based on variation in the relative abundance of 56 specialized metabolites detected by GC-MS from 5 biological samples per light treatment per replicate experiment, 2 replicates were analyzed together (n=10). The color coding in (D-F) indicates the relative abundance from lowest (blue) to highest (red). Mean classification error rate (5-fold cross-validation) for each sPLS-DA component (H-J).

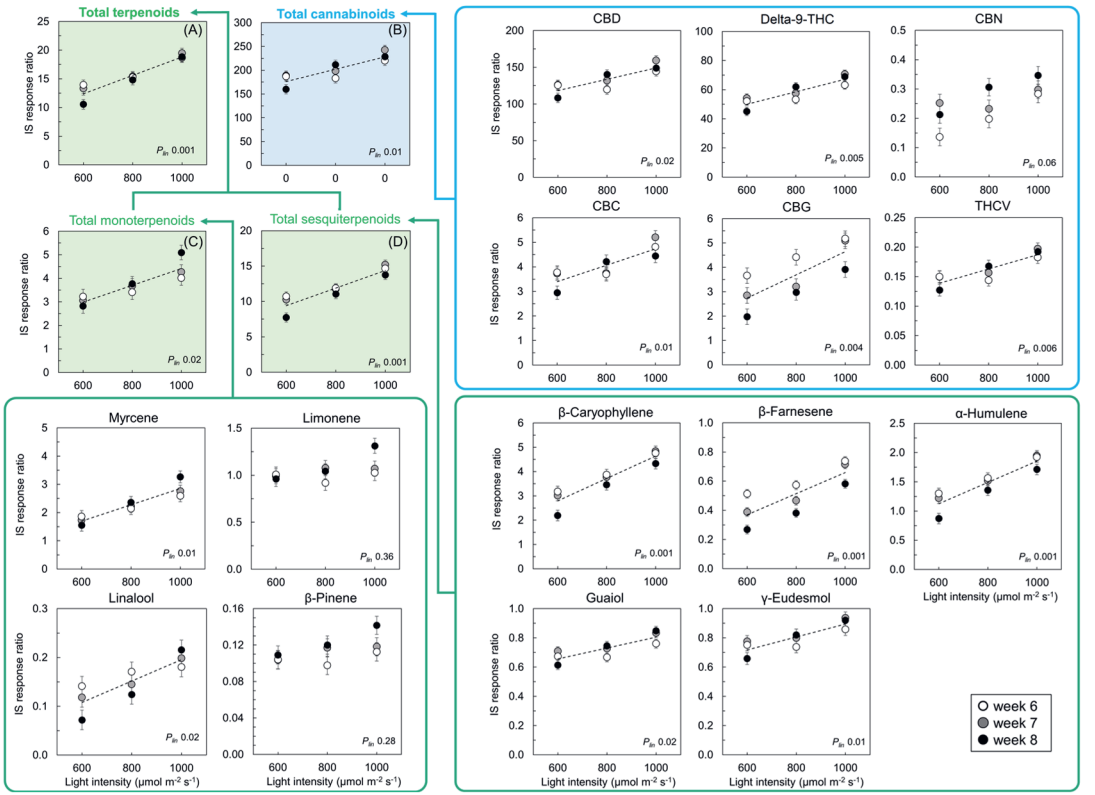

Figure 3. Relative abundance of specialized metabolites in inflorescences of medicinal cannabis grown under 600, 800, and 1000 μmol $m^{-2}s^{-1}$ PPFD after 6 (white points), 7 (grey points), and 8 (black points) weeks into the generative phase. The metabolites are calculated to dry mass basis. Two major groups are total terpenoids (A) and total cannabinoids (B). Total terpenoids consist of two subgroups: total monoterpenoids (C), including myrcene, limonene, linalool, and β-pinene, and total sesquiterpenoids (D), including β-caryophyllene, β-farnesene, α-humulene, guaiol, and γ-eudesmol. Total cannabinoids consist of CBD, THC, CBN, CBC, CBG, and THCV. Data are the averages of the internal standard (IS) response ratio based on two replicated experiments (n=2), each consisting of 5 pooled biological samples per treatment. Error bars represent SEM based on the pooled variance. When linear effects of light intensity for the overall 3 weeks were significant at P=0.05, trendlines are depicted. The abundance of these metabolites on fresh mass basis shown in Fig S3.

3.2. Plant dry mass, partitioning, and morphology

The total dry mass of the aboveground plant, as well as the individual dry masses of the inflorescences, leaves, and stems, all showed linear increases with increasing PPFD (Fig 4A). The fraction of dry mass partitioned to the inflorescences also increased linearly with PPFD, though this effect was small (only 3.4% increase in partitioning when PPFD increased from 600 to 1000 µmol $m^{-2}s^{-1}$). No significant change was found in the partitioning to leaves nor to stems (Fig 4B). Dry matter content of neither whole plant nor individual organs was affected by light intensity (Fig 4C). Also, remarkably the light use efficiency (LUE) of both inflorescences and whole plants remained constant over the PPFD range from 600 to 1000 µmol $m^{-2}s^{-1}$ (Fig 4D, Fig S4). Plant height was unaffected (Fig 5A, Fig S5), while the diameter of the main stem increased linearly with PPFD (Fig 5B). High PPFD increased plant compactness (Fig 5C). Specific leaf area showed a linear decrease with rising PPFD, while leaf area per plant remained stable (Fig 5D).

3.3. Photosynthesis light response curves

Increasing PPFD increased photosynthetic capacity, but this effect depended on plant developmental stage: At 5 weeks into the generative phase, net photosynthesis rate (*A*) in the range of 1000-3000 µmol $m^{-2}s^{-1}$ was significantly higher in the 800-1000 µmol $m^{-2}s^{-1}$ growth PPFD treatments compared to the 600 µmol $m^{-2}s^{-1}$ (Fig 6B). Maximum photosynthesis rate (A_{max}) increased linearly with growth light PPFD at 3 and 5 weeks, but not at 7 weeks (Fig 6D). Dark respiration (R_d) and the light compensation point (*LCP*) increased linearly with PPFD of growth light at 3 weeks (Fig 6F). The apparent quantum yield (α) was not affected by PPFD of growth light (Fig 6E). After 7 weeks, there was no effect of PPFD on any of the parameters of the light response curve (Fig 6C-G). Remarkably, *A* was not saturated (i.e. kept increasing with increasing PPFD even at the highest measurement PPFD (2000-3000 µmol $m^{-2}s^{-1}$), regardless of treatment and measurement week (Fig 6A-C).

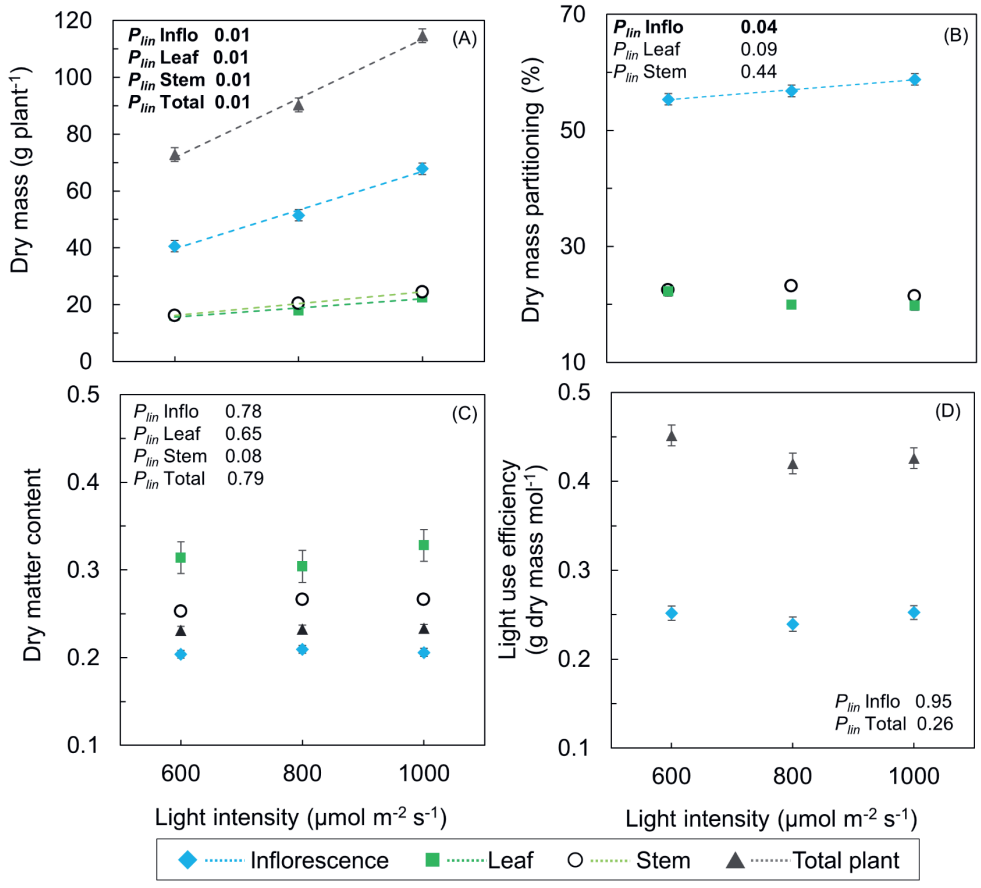

Figure 4. Plant dry mass (A), dry mass partitioning (B), dry matter content (C), and light use efficiency (D) of medicinal cannabis grown at 600, 800, and 1000 µmol $m^{-2}s^{-1}$ PPFD after 8 weeks into the generative phase. Data based on two replicated experiments (n=2), each consisting of 30 (replicate 1) and 18 (replicate 2) plants per treatment. Error bars represent standard error of means (SEM) based on the common variance and are visible when larger than the symbols. When linear effects of light intensity were significant at P=0.05, trendlines are depicted.

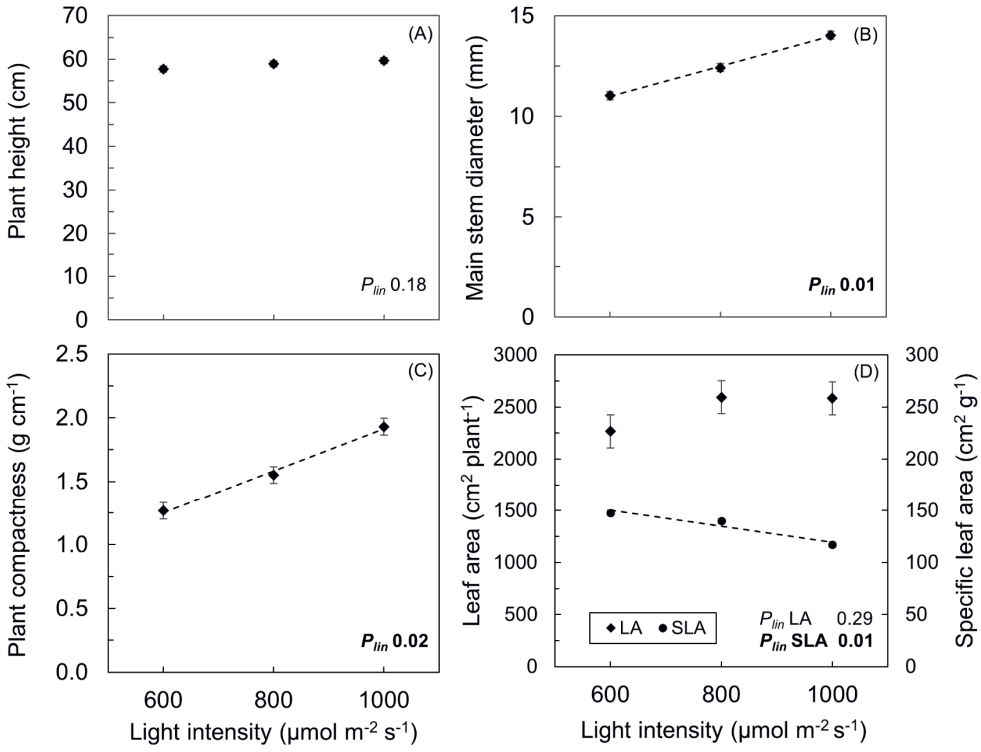

Figure 5. Plant height (A), diameter of the main stem (B), plant compactness (C), leaf area (LA) and specific leaf area (SLA) (D) of medicinal cannabis grown at 600, 800, and 1000 µmol $m^{-2}s^{-1}$ PPFD after 8 weeks into the generative phase. Data are based on 2 replicated experiments (n=2), each consisting of 30 (replicate 1) and 18 (replicate 2) plants per treatment. Error bars represent SEM based on the common variance and are visible when larger than the symbols. When linear effects of light intensity were significant at P=0.05, trendlines are depicted.

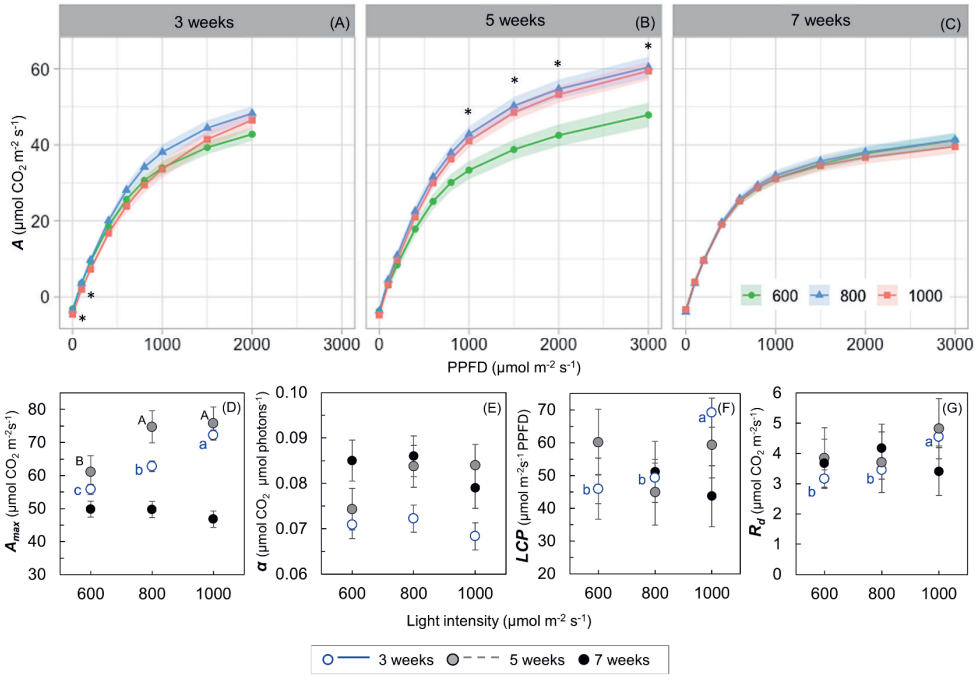

Figure 6. Light response curves of net photosynthesis rate (*A*) for medicinal cannabis grown under 600, 800, and 1000 µmol $m^{-2}s^{-1}$ PPFD at 3 (A), 5 (B), and 7 (C) weeks into the generative phase. Parameters maximum estimated photosynthesis rate (A_{max}) (D), apparent quantum yield (α) (E), light compensation point (*LCP*) (F), and dark respiration (R_d) (G) were derived from light response curves. Symbols represent means of 3-5 plants measured in replicate experiment 2. Ribbons in A-C and error bars in D-G represent SEM (n=3-5). When effects of light intensity were significant at P=0.05, asterisks (A-C) or different letters (D-G) are depicted.

3.4. Yield component analysis

When PPFD increased from 600 to 1000 µmol $m^{-2}s^{-1}$, metabolites yield per plant went up by 140% for cannabinoids and 214% for terpenoids, due to increases in both inflorescence yield and concentrations of metabolites in the inflorescences. Remarkably, increasing the light intensity during the generative phase by 67% led to a similar increase (69%) in dry mass of the inflorescences (Fig 7). This increased inflorescence dry mass was caused by an increase in total plant dry mass rather than a higher fraction of dry matter partitioned to the inflorescences. This increase in total plant dry mass was primarily attributed to an increase in photosynthesis rate, measured at growth light intensities, and was also partially attributed to a rise in leaf area. The higher leaf area was related to an increase in leaf dry mass, while specific leaf area (SLA) decreased.

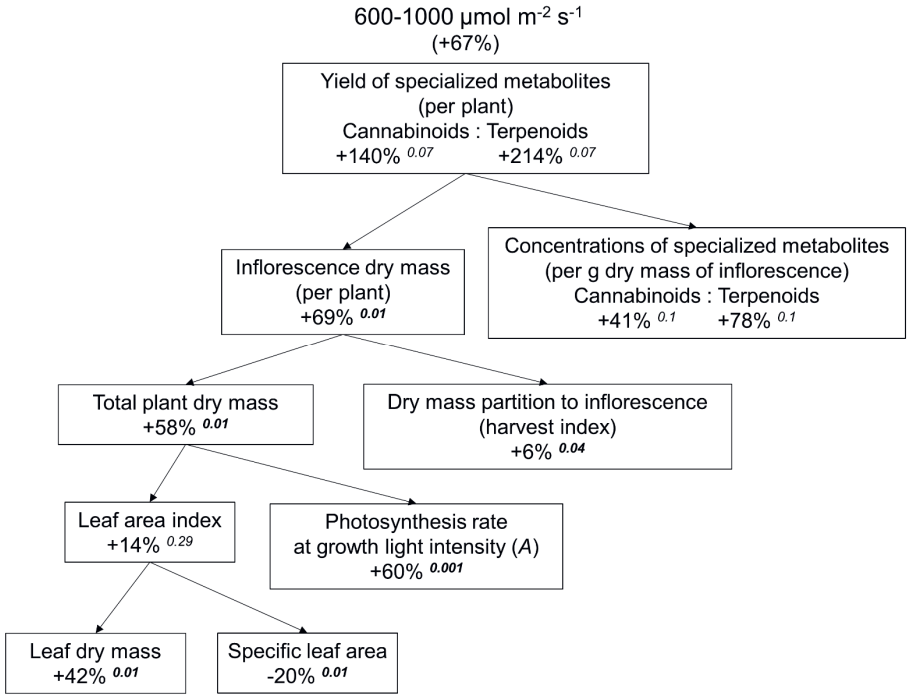

Figure 7. Yield component analysis of the effects of light intensity during 8 weeks into the generative phase on specialized metabolites (IS response ratio on fresh mass basis) in medicinal cannabis. Percentages indicate the relative change in the components when PPFD increased from 600 to 1000 µmol $m^{-2}s^{-1}$, based on a linear regression (P-values indicated as superscripts) making use of the data at 600, 800 and 1000 µmol $m^{-2}s^{-1}$ (Table S2). The specialized metabolites, plant mass, and leaf area were measured after 8 weeks of generative phase. *A* represents net photosynthesis rate measured at growth light intensity (i.e. 600, 800, and 1000 µmol $m^{-2}s^{-1}$), 5 weeks into the generative phase.

4. Discussion

4.1. Concentrations of specialized metabolites increased strongly with increasing PPFD

The yield of specialized metabolites - both terpenoids and cannabinoids - in fresh inflorescences increased strongly with increasing PPFD, due to higher inflorescence yield as well as higher concentrations of these metabolites. The increased concentrations of specialized metabolites in response to a higher light intensity was found in several other crops (Bian et al., 2015). An increase in light intensity increases available photoassimilates, which are not only essential for plant growth, but also serve as

precursors for specialized metabolites biosynthesis (Darko et al., 2014b) as well as energy source for their biosynthesis (Zavala and Ravetta, 2001). Moreover, elevated light intensity enhances the necessity for photoprotection (Bassi and Dall'Osto, 2021) to prevent oxidative stress by promoting specialized metabolite biosynthesis (Ramakrishna and Ravishankar, 2011). Concentrations of both sesquiterpenoids and monoterpenoids increased linearly with PPFD in plants during the 6-8 weeks into the generative phase (Fig 3). Sesquiterpene biosynthesis was found to be positively correlated with photosynthetic efficiency and the production of stress-related phytohormones in *Atractylodes lancea* (X. Guo et al., 2022). In our study, total terpenoids concentration in fresh inflorescences increased by 76% with a 67% increase in light intensity. Rodriguez-Morrison et al. (2021b) observed a smaller effect of light intensity on dry inflorescences, with only a 43% increase when light intensity was increased by 1400%. This increase was mainly observed for individual monoterpenoids like limonene and myrcene. However, during drying of inflorescences, a considerable loss of (mono-) terpenoids can occur, which may have reduced the effects reported by (Rodriguez-Morrison et al., 2021a). A higher light intensity led to increased concentration and yield of total essential oil in leaves of mint (*Mentha arvensis* L.) that were mainly contributed of monoterpenoids (de Souza et al., 2015). In addition, high light intensity increased concentration of a diterpenoid steviol glycoside in leaves of *Stevia rebaudiana*, which was linked to an up-regulation of related biosynthesis genes (Hernández et al., 2022). We found concentrations of detected cannabinoids increased with PPFD. In contrast, other studies found that light intensity had no effect on THC (Rodriguez-Morrison et al., 2021a; Vanhove et al., 2011). In some cases, cannabinoid concentration decreased due to a dilution effect caused by increased inflorescence yield (Bevan et al., 2021), but this was not observed in our study. Such contrasting effects of increased light intensity between studies may partly be explained by the cellular localization of different metabolites. Sesquiterpenoids are mostly synthesized in the cytosol from the precursor farnesyl pyrophosphate (FPP), which is generated in the mevalonate (MVA) pathway. On the other hand, monoterpenoids and cannabigerol (CBG) are synthesized in the plastids from geranyl pyrophosphate (GPP), which is generated in the methyl-D-erythritol phosphate (MEP) pathway (Romero et al., 2020), and the latter is suggested to be light-induced (Tholl, 2015). The transformation of CBG to other cannabinoids occurs in the secretory vesicles (Romero et al., 2020). Furthermore, the variation in the increase of different metabolites in response to light intensity might be related to a distinct effect of light on an induction of different trichome types. For instance, sessile glandular trichomes tend

to accumulate more sesquiterpenoids, while stalked trichome types accumulate more monoterpenoids (Livingston et al., 2020). As genes involved in both terpenoid and cannabinoid biosynthesis were identified in a co-expression network (Zager et al., 2019), a light effect on genes related to the MEP and/or the MVA pathway might affect the mutual balance between terpenoids and cannabinoids. To what extent these factors determine the responses in specialized metabolite abundances as found in our study needs further exploration of the regulation of biosynthetic genes in response to light intensity.

4.2. Inflorescence dry mass increased proportionally with PPFD, while LUE remained unchanged

Besides the higher concentrations of metabolites, a higher inflorescence yield contributed significantly to the increase in the yield of metabolites. This higher inflorescence yield at higher PPFD aligns with other studies on cannabis (Eaves et al., 2020; Potter and Duncombe, 2012; Rodriguez-Morrison et al., 2021a; Vanhove et al., 2011). In our experiment, a 1% increase in PPFD resulted in a ~1% increase in inflorescence yield (Fig 7), which matches with the 1%-rule of thumb in harvestable yield used and observed for several greenhouse crops (Marcelis et al., 2006). This also implies that the light use efficiency (LUE) remained constant at about 0.25 g inflorescence dry mass per mol of light (g mol^{-1}) within a PPFD ranging from 600 to 1000 µmol $m^{-2}s^{-1}$. This constant LUE at PPFD levels up to at least 1000 µmol $m^{-2}s^{-1}$ is remarkable, as it is rare at such high light intensities. This stability in LUE may partially be attributed to optimal growth conditions that were likely achieved in our setup, including factors such as CO_2, water, temperature, and nutrients (Jin et al., 2023; Medlyn, 1996), but additionally testifies to the remarkable ability of cannabis to use high light intensities productively (see below). In many other species, LUE usually decreases with increasing PPFD even at lower PPFD ranges (i.e. for 200 up to 750 µmol $m^{-2}s^{-1}$ in lettuce; 250 up to 300 µmol $m^{-2}s^{-1}$ in basil (Carotti et al., 2021; Heuvelink et al., 2002; Jeong et al., 2003; Pennisi et al., 2020). This response is likely due to a saturation of leaf photosynthesis response to light intensity (Fu et al., 2012).

4.3. Higher inflorescence dry mass was mainly due to an increase in total plant dry matter production, with a small contribution of increased harvest index

The increase in inflorescence dry mass with increasing PPFD was mainly due to increased total plant dry matter production, while there was a small contribution from a higher dry mass partitioning to inflorescences (i.e., higher harvest index) (Fig 7). The

increase in total plant dry mass correlated with an increase in leaf photosynthesis. Specific leaf area (SLA) decreased with increasing PPFD, indicating thicker leaves, a common crop response to high light intensity (Evans and Poorter, 2001; Poorter et al., 2019). This leaf thickening, caused by larger palisade cells and more spongy parenchyma layers, may improve photosynthetic capacity via an increase in the number of chloroplasts and photosynthetic proteins per chloroplast (Murchie et al., 2022; Zheng and Van Labeke, 2018). PPFD did not influence plant height, although total plant dry mass increased, hence higher PPFD promoted plant compactness. Simultaneously, the diameter of the main stem increased with PPFD, which may be a result of supporting the higher inflorescence weight. Flowering triggers secondary bast fiber formation and an intense lignification of the fiber to prepare the plant to hold heavier inflorescences (Westerhuis et al., 2019). Although the measured air temperature and relative humidity varied slightly among PPFD treatments (Table S1; 24-h average temperature differed less than 1 °C), we anticipated little interference with the positive effect of increasing PPFD on plant growth and yield.

4.4. Cannabis is a plant with exceptionally high photosynthetic capacity

We found that cannabis is a high photosynthesis capacity species, as *A* did not saturate even at 3000 µmol $m^{-2}s^{-1}$ PPFD and showed values of 40-55 µmol CO_2 $m^{-2}s^{-1}$ (Fig 6A-C). Even though light response curves were conducted at high CO_2 (800 µmol CO_2 mol^{-1}), these rates are higher than reported for many other C_3 plants (<40 µmol CO_2 $m^{-2}s^{-1}$ for tomato (Pan et al., 2020; Qian et al., 2012); <30 µmol CO_2 $m^{-2}s^{-1}$ for strawberry (X. Li et al., 2020), measured at similar light and CO_2 condition (2000 µmol $m^{-2}s^{-1}$ PPFD and 800 µmol CO_2 mol^{-1})). An example of a C_3 crop with high photosynthetic capacity crop is sunflower (English et al., 1979), which was found to exhibit *A* of ~45 µmol CO_2 $m^{-2}s^{-1}$ (Atkins and Boldt, 2022). Garassino et al. (2022) and Taylor et al. (2023) proposed *Hirschfeldia incana* as a model plant of C_3 species with high photosynthetic LUE at high irradiance, with an *A* of 50-52 µmol CO_2 $m^{-2}s^{-1}$, measured at 2000 µmol $m^{-2}s^{-1}$ PPFD and 400 µmol CO_2 mol^{-1}. To our knowledge, these rates are only surpassed by *Amaranthus palmeri*, a C4 desert annual, whose *A* was shown to reach ~80 µmol CO_2 $m^{-2}s^{-1}$ at 2000 µmol $m^{-2}s^{-1}$ PPFD and 325 µmol CO_2 mol^{-1} (Ehleringer, 1983). Altogether, we posit that cannabis is likely a photosynthesis extremophile, i.e., a species that can productively use very high light intensities for growth.

In addition, cannabis plants grown at higher PPFD showed a higher leaf photosynthetic capacity, in line with what is often found in other crops (Poorter et al., 2019). However,

unlike many other crops that tend to show saturation of *A* well below full sunlight (2000 µmol $m^{-2}s^{-1}$; (Taylor et al., 2023), *A* in cannabis leaves did not saturate at even 3000 µmol $m^{-2}s^{-1}$ (Fig 6B). The fact that biomass accumulation of both inflorescences and total plant dry mass was unsaturated at the highest PPFD of 1000 µmol $m^{-2}s^{-1}$, indicates that cannabis is a light-demanding species that can grow under extremely high light intensity without yield reduction. Rodriguez-Morrison et al. (2021b) found that *A* of cannabis leaves started to saturate at 1000 µmol $m^{-2}s^{-1}$ PPFD of measurement light. However, their measurement was done at 400 µmol CO_2 mol^{-1}, which is only half of that in our experiment (i.e. at 800 µmol mol^{-1}, commonly used in commercial practice (Zheng and Llewellyn, 2022). This suggests that increasing light intensity in commercial cannabis production should be combined with sufficient supplemental CO_2 in order to increase inflorescence dry mass. Dark respiration was about 3-5 µmol CO_2 $m^{-2}s^{-1}$, which was relatively high compared to other crops (at similar temperature), for example, 1-2 µmol CO_2 $m^{-2}s^{-1}$ has been reported for tomato (Kaiser et al., 2019; Zhang et al., 2022) and 1-3 µmol CO_2 $m^{-2}s^{-1}$ for chrysanthemum (Zhou et al., 2013). This high respiration rate in cannabis can be expected due to its high photosynthetic capacity.

5. Conclusion

Yield of specialized metabolites, including cannabinoids and terpenoids, strongly increased with increasing light intensity (600-1000 µmol $m^{-2}s^{-1}$) resulting from increases in both their concentrations and inflorescence yield. Within this range of light intensities, the inflorescence yield showed a proportional increase with light intensity, meaning that light use efficiency remained constant. Higher inflorescence yield was mainly due to higher total plant dry matter production, and to a lesser extent due to an increase in the fraction of dry mass partitioned to the inflorescences. Cannabis is a species with exceptionally high photosynthetic capacity, in which leaf photosynthesis rate kept on increasing up to the highest measuring light level (3000 µmol $m^{-2}s^{-1}$).

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Supplementary material

Supplemental method 1 Rooting phase

Rooting of stem cutting was conducted following a protocol in Sae-Tang et al. (2024). Stem cuttings of medicinal cannabis (cultivar 'Critical CBD') were cut from 3-6 months old mother plants grown in a glasshouse (Wageningen University and Research, Greenhouse Horticulture, Bleiswijk, the Netherlands). The lower end of the stem cuttings was dipped in 0.25% IBA powder (Rhizopon, the Netherlands) and rooted in 3.6 x 3.6 x 4 cm stonewool plugs (Grodan, the Netherlands). Cuttings were placed in an enclosed transparent plastic tent at 100 µmol $m^{-2}s^{-1}$ PPFD, consisting of 8% blue (400-500nm), 6% green (500-600nm), and 86% red light (600-700nm), provided by a mixture of red-blue-white LEDs (Green Power DRW LB 1.2, Philips, the Netherlands) with 50 µmol $m^{-2}s^{-1}$ of supplemental far-red (Green Power far-red 1.2, Philips, the Netherlands). The photoperiod was 18/6 h (light/dark). The rooting took 21 days. Climate setpoints were 28/28 °C air temperature, 80% relative humidity (RH) and ambient CO_2. The irrigation of the rooting phase was done by soaking the plugs in a nutrient solution with an EC of 1.6 dS m^{-1} and a pH of 5.8. The solution consisted of the following macro- and micronutrients; 1.25 mM NH_4^+; 6.2 mM K^+; 1.9 mM Ca^{2+}; 0.9 mM Mg^{2+}; 10.5 mM NO_3^-; 0.85 mM SO_4^{2-}; 0.85 mM PO_4^{3-}; 60 µM Fe^{2+}; 20 µM Mn^{2+}; 3 µM Zn^{2+}; 20 µM B^{2+}; 0.5 µM Cu^{2+}; 0.5 µM Mo^{2+}.

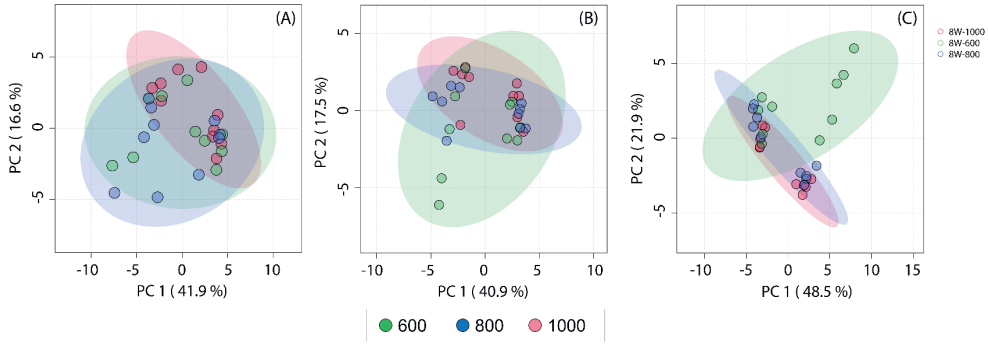

Figure S1. Principal component analysis (PCA) plots of specialized metabolites in fresh inflorescences of medicinal cannabis grown under 600 (green points), 800 (blue points), and 1000 (red points) µmol $m^{-2}s^{-1}$ PPFD after 6 (A), 7 (B) and 8 weeks (C) of the generative phase. The data are based on their variations in relative peak area of 56 specialized metabolites as detected by GC-MS.

Table S1. Averages of realized air temperature and relative humidity ± SEM from 2 replicated experiments. The measurement was done at plant level, recorded every 10 minutes by dataloggers (Easylog USB-1-LCD, Lascar electronics, Wiltshire, UK).

Week of generative phase		Air temperature (°C)			%RH		
		600	800	1000	600	800	1000
		µmol $m^{-2}s^{-1}$	µmol $m^{-2}s^{-1}$	µmol $m^{-2}s^{-1}$	µmol $m^{-2}s^{-1}$	µmol $m^{-2}s^{-1}$	µmol $m^{-2}s^{-1}$
Week 1	Light	29.2±1.6	28.6*	30.6±0.4	78.6±4.7	80.5*	72.1±1.2
	Dark	26.0±0.2	26.1*	26.0±0.1	87.0±1.5	89.1*	84.0±3.8
Week 2	Light	27.4±0.3	27.7*	28.9±0.7	85.2±0.2	83.3*	77.5±2.2
	Dark	26.5±0.3	26.7*	26.4±0.3	87.5±2.0	89.9*	84.3±4.1
Week 3	Light	27.1±0.3	27.4*	28.0±0.6	83.5±5.4	77.9*	78.5±6.5
	Dark	26.8±0.1	26.3*	26.6±0.3	86.9±5.0	84.8*	84.3±1.9
Week 4	Light	27.2±0.8	28.2*	27.7±0.9	72.2±0.0	67.9*	75.0±9.7
	Dark	26.4±0.2	26.0*	25.9±0.0	77.4±2.1	76.7*	82.0±6.5
Week 5	Light	26.2±0.3	26.8±1.1	28.0±1.6	74.1±7.1	71.0±8.3	64.2±7.2
	Dark	26.2±0.2	25.9±0.5	26.2±0.5	75.1±3.0	76.5±4.7	73.4±4.2
Week 6	Light	27.5±0.4	28.2±0.7	29.6±0.1	66.0±4.0	61.6±0.4	63.2±8.9
	Dark	27.9±1.3	27.5±0.9	27.7±0.3	69.3±1.9	69.8±2.2	69.6±5.8
Week 7	Light	27.1±0.5	27.8±0.3	29.1±0.7	67.0±2.1	61.8±0.6	60.5±7.2
	Dark	27.6±1.4	27.7±1.5	27.8±1.1	62.5±1.1	59.0±4.5	59.6±0.1
Week 8	Light	27.2±0.8	27.5±0.1	28.9±1.0	65.5±0.9	63.0±1.9	58.5±5.6
	Dark	27.8±1.6	26.5±0.3	27.0±0.2	60.8±2.7	64.8±1.3	63.6±4.2

**Data only available for replicate 2*

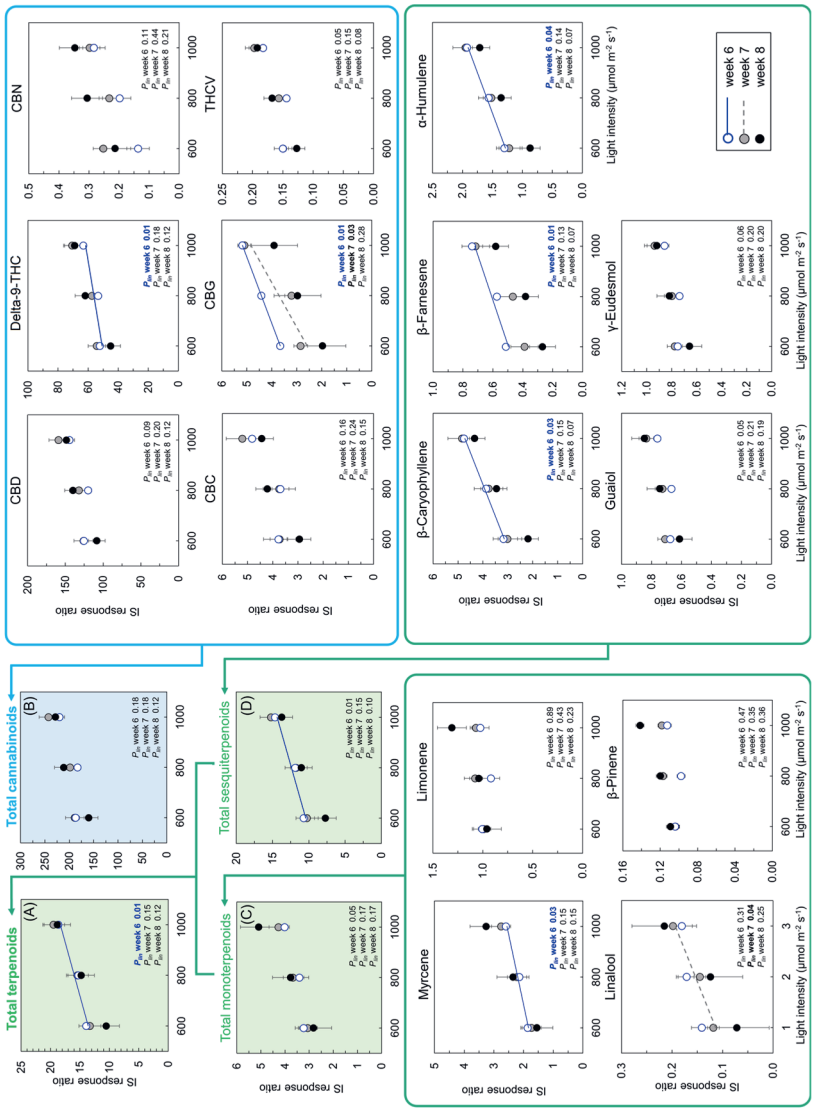

Figure S2. Relative abundance of specialized metabolites in inflorescences of medicinal cannabis grown under 600, 800, and 1000 µmol $m^{-2}s^{-1}$ PPFD after 6 (white points), 7 (grey points), and 8 (black points) weeks into the generative phase. The metabolites are calculated to dry mass basis. Two major groups are total terpenoids (A) and total cannabinoids (B). Total terpenoids consist of two subgroups: total monoterpenoids (C), including myrcene, limonene, linalool, and β-pinene, and total sesquiterpenoids (D), including β-caryophyllene, β-farnesene, α-humulene, guaiol, and γ-eudesmol. Total cannabinoids consist of CBD, THC, CBN, CBC, CBG, and THCV. Data are the averages of the internal standard (IS) response ratio based on two replicated experiments (n=2), each consisting of 5 pooled replicated samples per treatment. Error bars represent SEM based on the common variance. When linear effects of light intensity for individual weeks were significant, trendlines are depicted.

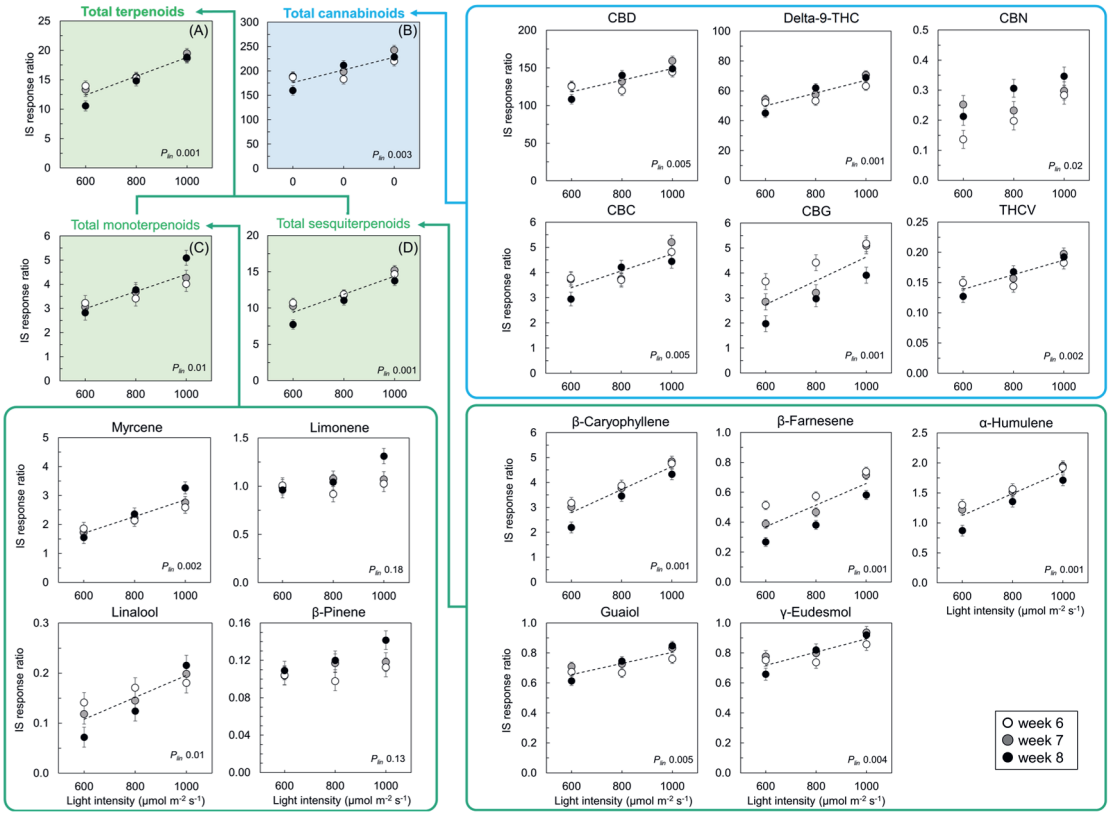

Figure S3. Relative abundance of specialized metabolites in inflorescences of medicinal cannabis grown under 600, 800, and 1000 μmol $m^{-2}s^{-1}$ PPFD after 6 (white points), 7 (grey points), and 8 (black points) weeks into the generative phase. The metabolites are on fresh mass basis. Two major groups are total terpenoids (A) and total cannabinoids (B). Total terpenoids consist of two subgroups: total monoterpenoids (C), including myrcene, limonene, linalool, and β-pinene, and total sesquiterpenoids (D), including β-caryophyllene, β-farnesene, α-humulene, guaiol, and γ-eudesmol. Total cannabinoids consist of CBD, THC, CBN, CBC, CBG, and THCV. Data are the averages of the internal standard (IS) response ratio based on two replicated experiments (n=2), each consisting of 5 pooled replicated samples per treatment. Error bars represent SEM based on the common variance. When linear effects of light intensity for the overall 3 weeks were significant, trendlines are depicted.

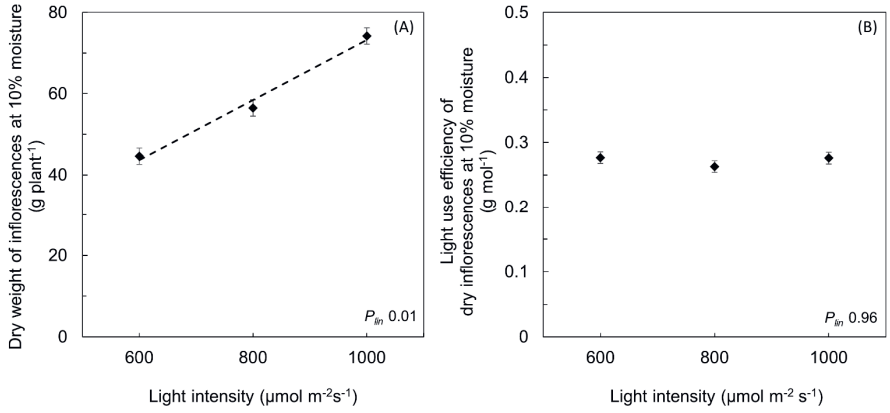

Figure S4. Weight (A) and light use efficiency (B) of dry inflorescences at 10% moisture of medicinal cannabis grown under 600, 800, and 1000 µmol $m^{-2}s^{-1}$ PPFD after 8 weeks of generative phase. Data based on 2 replicated trials (n=2), each consisting of 30 (Experiment 1) or 18 (Experiment 2) replicated plants per treatment. Error bars represent SEM based on the common variance, when larger than the symbols. When linear effects of light intensity are significant, trendlines are depicted.

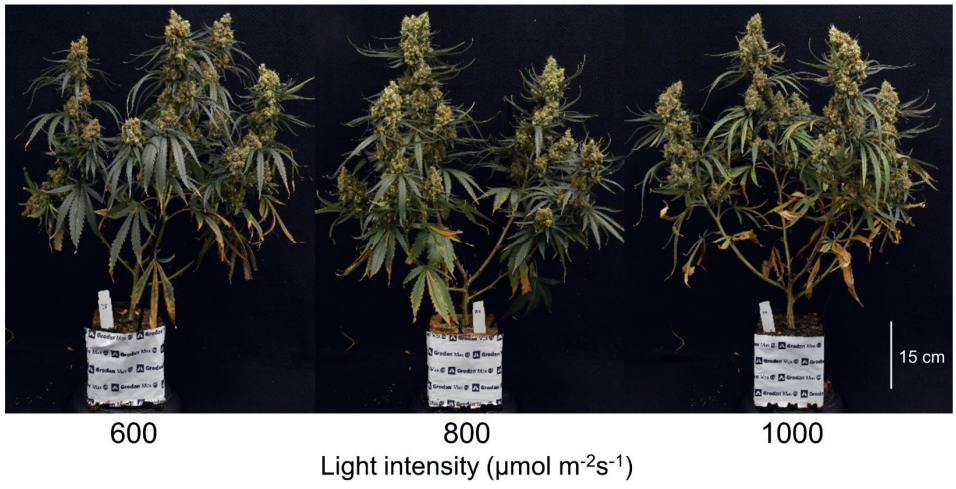

Figure S5. Pictures of representative plants of medicinal cannabis grown under 600, 800, and 1000 µmol $m^{-2}s^{-1}$ PPFD after 8 weeks into the generative phase

Table S2. Table of linear regression of each parameter from 600 to 1000 µmol $m^{-2}s^{-1}$ PPFD as used in the yield component analysis (Fig 7)

Parameter	Linear regression equation	R^2
Total cannabinoids (per plant)	y=22.5x-7076.29	0.92
Total cannabinoids (per g inflorescence)	y=0.17x+62.91	1.00
Total terpenoids (per plant)	y=2.12x-876.95	1.00
Total terpenoids (per g inflorescence)	y=0.02x-1.83	0.98
Inflorescence dry mass	y=0.07x-1.27	0.99
Total plant dry mass	y=0.10x+9.08	0.99
Dry mass partitioning to inflorescence	y=0.01x-0.50	0.99
LAI	y=0.01x+1.66	0.73
A at growing PPFD	y=0.04x+2.81	0.89
Leaf dry mass	y=0.02x-5.87	0.95
SLA	y=(-0.08)x+196.30	0.93

Chapter 5

Longday in the last two weeks before harvest to shortday medicinal cannabis can improve inflorescence yield without affecting concentrations of cannabinoids

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(submitted)

Abstract

Most medicinal cannabis cultivars are shortday plant requiring a photoperiod of approximately 12 h or less to induce flowering. Short photoperiods limit the daily light integral and consequently daily photosynthesis, growth, and dry mass production. In shortday-chrysanthemum, extending a shortday with blue light for a whole generative phase did not inhibit flowering, and it also increased number of flowers. However, this strategy was found to impair flowering of cannabis. The aim of this study is to explore if it is possible to increase inflorescence yield of medicinal cannabis by extended photoperiod when flowers have been initiated. We conducted a series of experiments in a climate-controlled chamber to test several scenarios of extending the photoperiod from 12 (shortday) to 18 h (longday). When the photoperiod was extended by 250 µmol $m^{-2}s^{-1}$ of blue light for 6 and 4 weeks, from 2 or 4 weeks after start of shortday, the plants reversed back to vegetative growth by producing leaves on top of inflorescences while maturity of flowers was delayed. Interestingly, the plants exposed to the extended photoperiod by blue for the last 2 weeks, from 6 weeks after they had first been grown under shortday, showed comparable development and inflorescence yield to the plants under continuous shortday. Subsequently, the photoperiod was extended for the last 2 weeks by 250 µmol $m^{-2}s^{-1}$ of red, blue or white light. There was no difference among light spectra. The increase in light from the extended photoperiod treatment may have been limited by low light intensity at 250 µmol $m^{-2}s^{-1}$. Therefore, in the last experiments, the photoperiod was extended for the last 2 weeks with a constant light intensity of either 600 or 800 µmol $m^{-2}s^{-1}$ of white light. Inflorescence yield increased with the extended photoperiod for the last 2 weeks, although a significant increase was found only at the light intensity of 600 µmol $m^{-2}s^{-1}$. Concentrations of cannabinoids in inflorescence declined by extending the photoperiod but not when the extended photoperiod was only the last two weeks. These findings suggest the potential to increase inflorescence yield without negative effect on cannabinoids by extending the photoperiod, but it appears effective only during the last 2 weeks before harvest and with a substantial increase in amount of light.

Keywords: *Cannabis sativa* L., LEDs, Spectrum, flower induction, flowering, photoperiod

1. Introduction

Most cannabis genotypes are sensitive to photoperiod. Cannabis is identified as a shortday (SD) plant, naturally flowering in early autumn when the photoperiod becomes shorter and the nights longer than the critical photoperiod threshold (Dowling et al., 2021; Salentijn et al., 2019). Some studies argued that cannabis flower initiation is autonomous since first solitary flowers can form under both long and short photoperiods (Spitzer-Rimon et al., 2022). However, short photoperiods are strongly required post-induction for flower bud maturation, and also for branching at the apex to obtain dense inflorescences (Duchin et al., 2020; Spitzer-Rimon et al., 2019). In commercial production, medicinal cannabis plants are typically grown under approximately 2-3 weeks of longday (LD) (≥ 16 h photoperiod, i.e., vegetative phase) to promote vegetative plant development. This is followed by 7-10 weeks of SD (12 h photoperiod, i.e., generative phase) for flower induction and development, hence until harvest (Chandra et al., 2017a; Moher et al., 2022).

Short photoperiods limit daily light integral (DLI), consequently limiting crop photosynthesis and plant growth (Eichhorn Bilodeau et al., 2019; Kelly et al., 2020). Therefore, extending photoperiods at fixed light intensity is expected to improve biomass production, however this could negatively affect flowering. A delay in cannabis flowering was found at photoperiods longer than 12 h (Lisson et al., 2000; Zhang et al., 2021). Flowering percentage of *in vitro* cannabis explants was highest when exposed to a photoperiod less than 13.2 h (Moher et al., 2021). Many cannabis cultivars initiate flowers under photoperiods up to 14-15 h, with only minor delays in flower initiation times. However, a decrease in apical inflorescence size and early stage floral yield was observed under photoperiods longer than 13 h (Ahrens et al., 2023). In some cultivars inflorescence yield increased by a lengthened photoperiod from 12 to 13 h (Ahrens et al., 2024) and 14 h (Peterswald et al., 2023).

Besides photoperiod, light spectrum also determines photoperiodic flowering response in short-day plants. In several photoperiodic plants especially floricultural plants, daylight extension with specific light spectrum has been applied to control flowering (Runkle and Heins, 2006). For instance, flowering of SD chrysanthemum was not inhibited when a SD of 11 h of red:blue light was extended with 4 h of sole blue light (Jeong et al., 2014; SharathKumar et al., 2021). This extension of the photoperiod with blue light resulted in a larger number of flowers, which might be a consequence of high expression of *CRY1* and *PHYA* during early flowering measured at an hour after photoperiod began (Park and

Jeong, 2020). However, the photoperiod extension with blue light inhibited flowering of some SD-species, for example kalanchoe, perilla, and stevia (SharathKumar et al., 2024a). A previous study on medicinal cannabis showed that photoperiod extension of 11 h of either white or red:blue with 4 h sole blue throughout the whole generative phase significantly delayed flowering (SharathKumar et al., 2024b *unpublished manuscript*).

An alternative strategy for extending the photoperiod could focus on the period once the flowers have been initiated but are still developing. This might improve biomass production, without flowering inhibition in SD plants. In some SD plants a single SD is enough to initiate flowering, for example cocklebur (*Xanthium strumarium* L.) and Japanese morning glory (pharbitis, *Ipomea* nil.) (Thomas and Vince-Prue, 1996). In contrast, strawberry (*Fragaria ananassa* Duch.) requires certain number of SD (i.e. 7-23 SDs) for floral induction, depending on genotype, temperature and plant age (Warner, 2009). Nonetheless, the question of how flowering in cannabis responds to extended photoperiods once flowers have been initiated has not been answered yet.

Not only the inflorescence yield, but also cannabinoids such as delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), are important aspects of medicinal cannabis cultivation. These metabolites might be manipulated by light, either photoperiod, light spectrum, or light intensity. In two high-THC lines, a 14-h photoperiod decreased THC concentration compared to a 12-h photoperiod (Peterswald et al., 2023). Conversely, in a CBD line, a 14-h photoperiod led to a significant increase in the CBD concentration, compared to a 12-h photoperiod (Peterswald et al., 2023). A study comparing spectra properties among various lamp modules discussed that a higher fraction of blue light increased cannabinoids content, while green light had a negative effect (Magagnini et al., 2018). Danziger and Bernstein (2021) observed that CBG accumulation is stimulated by blue-rich light, while the response on CBD, and THC was cultivar specific. Therefore, influence of photoperiod associated with light spectrum on accumulation of cannabinoids has to be taken into account.

The aim of this study is to explore possibilities for an extended photoperiod (from 12 to 18 h) in medicinal cannabis to increase inflorescence yield and concentrations of cannabinoids. We hypothesized that extended photoperiod by blue light once flowers have been initiated does not impair flower development. Hence, extending the photoperiod by blue light increases inflorescence yield due to higher cumulative light interception and increases concentration of cannabinoids due to high amount of blue light. We conducted a series of experiments in a climate-controlled chamber to

investigate several ways of extending photoperiod, including various durations, light spectra and light intensities.

2. Materials and methods

2.1. Plant cultivation

Medicinal cannabis cultivar 'White Russian', a high THC (10-16%w/w) and low CBD (<0.05%w/w) genotype, was grown in a climate-controlled walk-in chamber. Plants were propagated by stem cuttings which were rooted in stonewool plugs for 21 days (details on the rooting phase are described in Supplement 1). Well-rooted cuttings were transplanted into 15 x 15 x 15 cm stonewool blocks (Grodan, the Netherlands). The vegetative phase, meaning a phase of longdays (LD) (i.e. a 18-h photoperiod) to promote vegetatively grow, was applied from 0 to 11 days after transplanting (DAT) at 423±24 µmol $m^{-2}s^{-1}$ PPFD, 13% blue (400-500 nm), 6% green (500-600 nm), 81% red (600-700 nm), and <0.6% far-red (700-800 nm), provided by a mixture of red-blue-white LEDs (Green Power DRW MB 1.2, Philips, the Netherlands). The light (PPFD and spectrum) was measured at 45 cm above the table, which was at final canopy height. The PPFD was measured using a LI-250A light meter (LI-COR Inc., Lincoln, NE, USA) and the light spectrum was measured using a specbos 1210 spectrophotometer (JETI Technische Instrumente GmbH, Jene, Germany).

The planting density was kept constant at 9 plants m^{-2} throughout the whole experiment. Plants were pruned three times during the cultivation. At 8 DAT the apex was removed. At 11 DAT the lowest side shoots were removed such that four upper side shoots were retained. Finally at 16 DAT, the lower second-order side shoots were removed to remain the three upper second-order side shoots on each side shoot. The dry mass of removed leaves and stems was determined and included in plant dry mass calculation.

During the vegetative phase (0-11 DAT), the climate was 28.7/26.3°C (light/dark), 79/86 %RH, and CO2 was 700 mol mol^{-1}. During the generative phase (12-67 DAT), the climate was 28.0/26.7°C, and 71/75% RH while CO_2 was gradually increased from 600 to 1200 mol mol^{-1}, adding 200 mol mol^{-1} every 2 weeks. The realized air temperature and RH separated in each light treatment and week are shown in Table S1. Before transplanting, the stonewool blocks were pre-soaked in a nutrient solution with an EC of 1.6 dS m^{-1} and a pH of 5.8. The solution consisted of the following macro- and micronutrients; 1.25 mM NH_4^+; 6.2 mM K^+; 1.9 mM Ca^{2+}; 0.9 mM Mg^{2+}; 10.5 mM NO_3^-; 0.85 mM SO_4^{2-}; 0.85 mM PO_4^{3-}; 60 µM Fe^{2+}; 20 µM Mn^{2+}; 3 µM Zn^{2+}; 20 µM B^{2+}; 0.5 µM

Cu^{2+}; 0.5 µM Mo^{2+}. Irrigation from the vegetative phase onward supplied with the same nutrient solution by discharge-regulated drippers into the stonewool blocks, each discharge contained 100 mL. Frequency of dripping was adjusted to maintain proper water content in the stonewool blocks depending on the light treatment to ensure that all plants received sufficient water and nutrients. The EC value was raised to 2 dS m^{-1} then further raised to 2.5 dS m^{-1} during generative phase by increasing concentration of macronutrients, while keeping the ratios between individual ions constant.

2.2. Photoperiod treatments during generative phase

This research consisted of two topics each consisting of two experiments. In each individual experiment, during 8 weeks of generative phase, plants were randomly assigned to four photoperiod treatments (Fig 1), arranged in four compartments in the climate chamber divided by white plastic sheets (most experiments were replicated 2-3 times, see section on statistical set-up). The light (PPFD and spectrum) was measured at 70 cm above the tabletop, which was the expected canopy height at the end of cultivation.

Photoperiod extension with different spectra (Topic 1)

- ***Duration of blue extended photoperiod after flower initiation*** *(Exp 1.1)*

There were four combinations of shortdays (SD) and blue extended white photoperiod (LD); 8 weeks SD as control (SD; 0-week LD); 6 weeks SD followed by 2 weeks LD (2-week LD-Blue); 4 weeks SD followed by 4 weeks LD (4-week LD-Blue), and 2 weeks SD followed by 6 weeks LD (6-week LD-Blue). SD was a 12-h photoperiod of 600 µmol $m^{-2}s^{-1}$ white light consisting of 11% blue (400-500 nm), 6% green (5000-600 nm) and 83% red (600-700 nm). LD was a 18-h photoperiod consisting of 12 h of 600 µmol $m^{-2}s^{-1}$ white light (SD), extended with 6 h of 250 µmol $m^{-2}s^{-1}$ 100% blue light (400-500 nm, peak at 450 nm).

- ***Blue, white, or red extended photoperiod for last 2 weeks before harvest*** *(Exp 1.2)*

All photoperiod treatments started with 6 weeks SD followed by 2 weeks LD with three different light spectra used for photoperiod extension; blue (2-week LD-Blue), white (2-week LD-White) or red light (2-week LD-Red), compared with 8 weeks SD, as control (SD). SD was a 12-h photoperiod of 600 µmol $m^{-2}s^{-1}$ white light. LD was a 18-h photoperiod consisting of 12 h at 600 µmol $m^{-2}s^{-1}$ white light (SD) extended with 6 h of 250 µmol $m^{-2}s^{-1}$ either 100% blue (400-500 nm, peak at 450 nm), white or 100% red (600-700 nm, peak at 665 nm).

Photoperiod extension with constant intensity of white light (Topic 2)

- ***LD for last 2 weeks before harvest at 600 µmol $m^{-2}s^{-1}$*** *(Exp 2.1)*

Two treatments: 6 weeks SD followed by 2 weeks LD (LD600), compared with 8 weeks SD, as control (SD600). The photoperiod of SD was 12 h, while LD was 18 h. The light intensity was constantly at 600 µmol $m^{-2}s^{-1}$ of white light in both treatments.

- ***LD for last 2 weeks before harvest at 800 µmol $m^{-2}s^{-1}$*** *(Exp 2.2)*

The two light treatments from Exp 2.1 were repeated at 800 µmol $m^{-2}s^{-1}$, hence LD800 and SD800.

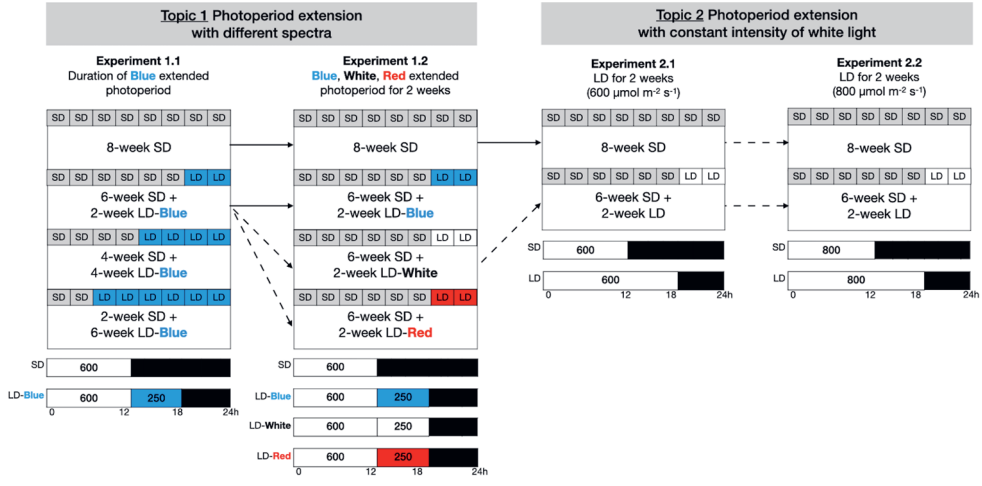

Figure 1. Schematic overview of the four experiments with *Cannabis Sativa* L. 'White Russian'. The large boxes show composition of different photoperiod treatments during 8 weeks of generative phase. The strips with 8 squares in each treatment show 8 weeks before harvest, with either SD (a 12-h photoperiod) and LD (a 18-h photoperiod). The bars below indicate changes in light spectrum and light intensity (µmol $m^{-2}s^{-1}$) throughout the day (0-24 h) during SD and LD. White means 11% blue, 6% green and 83% red; blue is 100% blue; red is 100% red; and black stands for dark period. The solid arrows indicate repetitions across the experiments, the dash arrows indicate adapted treatments by either light spectrum or intensity.

Table 1. Light intensity, daily light integral (DLI) averaged over 8 weeks of the generative phase. Realized light intensity is shown in Table S2.

Experiment	Treatment	Light intensity during 12 h of SD ($\mu mol\ m^{-2}s^{-1}$)	Light intensity during 6 h of LD ($\mu mol\ m^{-2}s^{-1}$)	Average DLI ($mol\ m^{-2}d^{-1}$)
1.1, 1.2, 2.1	8-week SD	600	-	25.9
1.1, 1.2	6-week SD + 2-week LD	600	250	27.3
1.1	4-week SD + 4-week LD	600	250	28.6
1.1	2-week SD + 6-week LD	600	250	30.0
2.1	6-week SD + 2-week LD	600	600	29.2
2.2	8-week SD	800	-	34.6
2.2	6-week SD + 2-week LD	800	800	38.9

2.3. Measurements of growth and morphology

The final harvest was done after 8 weeks of generative phase (i.e. at 65-67 DAT). Plant height was measured as the longest distance from the base of the stem towards the top of the canopy. Main stem diameter was determined at 1 cm above the top of the stonewool block using a digital caliper (model Mitutoyo- series 500). Each plant was dissected to leaves, stems and inflorescences. Leaf and stem dry mass was determined by exposing the material to 105 °C for 72 h in a ventilated oven. Air-dried inflorescence mass was determined after five days in a dark climate-controlled room with ample ventilation, at 45% RH, and 25 °C. This represents commercial practice and results in a moisture content of approximately 10%. After that, inflorescence material was divided into two halves based on weight. One half of the air-dry inflorescences were stored at -20 °C for cannabinoid analysis, whereas the other half was dried in a ventilated oven at 105 °C for 72 h to obtain oven-dry mass. The total inflorescence dry mass was calculated from the mass determined at air-dry multiplied by the ratio between oven-dry and air-dry mass. Light use efficiency (LUE) was calculated as either inflorescence dry mass or total plant dry mass divided by cumulative incident mol of PPFD during 8 weeks of generative phase.

2.4. Cannabinoids analysis

Air-dried Inflorescences at the final harvest (8 weeks of generative phase) were randomly sampled from the whole plant and used for extraction. A total of six biological samples for each light treatment per replicated experiment was prepared in which each sample was pooled from 2-3 plants. Per sample, 100 mg air-dried inflorescences were

homogenized and stems removed before extraction in 5 ml of n-Hexane with 0.2 g L^{-1} of squalene as an internal standard (IS). Sample extracts were sonicated for 10 min in an ultrasonic bath (Branson 2800), subsequently filtered through a column containing siloxilated glass-wool and sodium sulphate in a Pasteur pipet and collected in a 2 mL glass vial. Two µL of each filtered extract was analysed in splitless mode on a Gas Chromatography-Mass Spectrometry (GC-MS) Agilent (7890) equipped with a 30-m length × 0.25-mm inner diameter, 0.25-µm film thickness column (Zebron, 5MS) and a mass-selective detector (model 5972A, Hewlett–Packard). The GC was programmed at an initial temperature of 60 °C for 2 min, with a ramp of 5 °C min^{-1} to 250 °C, and then with 10 °C min^{-1} to 280 °C and final time of 5 min. The injection port, interface, and MS source temperatures were 250 °C, 290 °C, and 180 °C, respectively, and the Helium inlet pressure was controlled with an electronic pressure control to achieve a constant column flow of 1 mL min^{-1}. The ionization potential was set at 70 eV, and scanning was performed from 45 to 400 amu. Metabolites were identified by comparing mass spectra with those of the National Institute of Standards and Technology mass spectral library (NIST MS search 2.0) and their relative retention time. The concentration of delta-9-THC and CBD were calculated using the area under the curve, which was then normalized by IS and weight. These values were expressed as %(W/W), based on calibrations using authentic standard compounds.

2.5. Statistical set-up and analysis

The photoperiod treatments were randomly located over the four different compartments within a climate chamber in all experiments. Exp 1.1 was conducted once, Exp 1.2 was conducted twice over time. Two treatments were similar in both experiments (SD and 2-week LD-Blue treatments). Exp 1.1 and 1.2 were combined in one statistical analysis using an unbalanced design. Exp 2.1 was conducted three times, the first replicate was conducted in the first round separately and the last two replicates were conducted in parallel in the second round to obtain a total of three statistical replicates. Exp 2.2 was conducted twice in parallel. The numbers of independent statistical replicates and replicate plants are shown in Table S3-S4. Repetitions were taking into account as blocks. One-way Analysis of Variance (ANOVA) was conducted followed by mean separation according to Fisher's protected LSD test at P=0.05. The assumption of normality of the residuals was met for all variates, according to the Shapiro-Wilk test at P=0.05. Homogeneity of variances was assumed as it could not be tested because of the low number of real replicates ($n \leq 3$). The statistical tests were performed in Genstat 19th edition (VSN International, London, UK).

3. Results

3.1. Photoperiod extension with different light spectra (Exp 1.1, 1.2)

Cannabis plants reverted to vegetative growth when the plants were exposed to blue extended photoperiod (LD-Blue) during the last 4 or 6 weeks before harvest, despite flowers were already initiated before the blue extended LD started. During these periods of LD-Blue, newly formed leaves appeared on top of the inflorescence part (Fig 2). Inflorescences under 4 and 6 weeks of LD-Blue were undeveloped at final harvest (after 8 weeks of the generative phase), with groups of flowers appearing loose (Fig S3). This phenomenon was not observed in the plants exposed to LD-Blue during only the last 2 weeks before harvest and this treatment resulted in plants comparable to those under SD (Fig 2, 4, S3).

The plants exposed to 6 weeks of LD-Blue obtained the highest total plant dry mass and the lowest inflorescence dry mass: total plant dry mass consisted for 87% of leaves and stems (Fig 3A). In comparison to SD plants, 2 or 4 weeks of LD-Blue had no impact on total plant nor inflorescence dry mass, indicating no significant change in dry mass partitioning to the inflorescences (Fig 3A-B). Two weeks of extended photoperiod with white or red light, similar to blue light, did not have a statistically significant effect on dry mass or its partitioning to the inflorescence, compared to SD (Fig 5A-B). Inflorescence dry mass averaged over the three light spectra applied during 2 weeks of extended photoperiod was 7.5% higher than under SD (Fig 3A).

The light use efficiency (LUE; g dry mass per mol light) of inflorescence was notably lower when plants received 6 weeks of LD-Blue than the rest of treatments, whereas the LUE of total plant was substantially higher though not statistically significant (P=0.06) (Fig 3C). The LUE of either inflorescence or total plant was not statistically different between the plants exposed to 2 weeks of LD and those under SD (Fig 3C, 5C). Plant height and main stem diameter showed a significant increase with the application of 4 or 6 weeks of LD-Blue. In contrast, extending photoperiod for 2 weeks, regardless of whether using blue, red, or white light, had no significant impact on plant height and stem diameter compared to plants under SD (Table 2). The concentration of THC, a dominant cannabinoid in the studied genotype, decreased by LD-Blue, and this effect was more pronounced with longer periods of LD-Blue (Fig 3D). Two weeks of extended photoperiod with either blue, red, or white light, did not significantly affect THC or CBD concentration (Fig 5D).

In summary, two weeks of extended photoperiod with either blue, red, or white at 250 µmol $m^{-2}s^{-1}$ neither reversed cannabis plants back to vegetative growth nor clearly improved the inflorescence yield compared to SD condition. This may be attributed to the marginal increase in cumulative incident light from SD to 2 weeks LD (5.2%) as a relatively low light intensity was used (250 µmol $m^{-2}s^{-1}$). Therefore, in the next section extension was applied by the same light intensity as during the SD for the last 2 weeks before harvest.

3.2. Photoperiod extension by constant intensity of white light (Exp 2.1 and 2.2)

Two weeks of extended photoperiod (LD) with white light (600 or 800 µmol $m^{-2}s^{-1}$) at the same light intensity as during the SD increased inflorescence, leaves, and stem dry mass and therefore also total plant dry mass (Fig 6A). The 2 weeks of LD led to a 12.5% boost in inflorescence yield compared to SD plants at 600 µmol $m^{-2}s^{-1}$ and a 4.9% increase at 800 µmol $m^{-2}s^{-1}$, though the effects at 800 µmol $m^{-2}s^{-1}$ was not statistically significant (Fig 6A). On the other hand, the fraction dry matter partitioned to inflorescences slightly reduced by the 2 weeks of LD (Fig 6B). At 600 µmol $m^{-2}s^{-1}$, the LUE of inflorescence remained the same under both SD and 2-week LD. However, at 800 µmol $m^{-2}s^{-1}$, the LUE of inflorescences seemed marginally decreased under LD, although not statistically significant (Fig 6C). Plant height was not affected by LD (Table 2, Fig S1), and only at 600 µmol $m^{-2}s^{-1}$ the main stem diameter slightly increased in 2 weeks LD. Nevertheless, the concentration of THC and CBD in inflorescences was not influenced by 2 weeks of photoperiod extension (Fig 6D).

Figure 2. Plant architecture of *Cannabis sativa* L. 'White Russian' after 8 weeks of generative phase. During 8 weeks of generative phase, the plants were grown under 8, 6, 4, or 2 weeks of SD followed by 0 (SD; control), 2, 4, and 6 weeks of blue extended photoperiod (LD-Blue) respectively. LD-Blue means 12 h of 600 µmol $m^{-2}s^{-1}$ white light (SD), extended with 6 h of 250 µmol $m^{-2}s^{-1}$ blue light. For visualization purpose, the fan leaves at the lower part of the canopy were removed. (Exp 1.1)

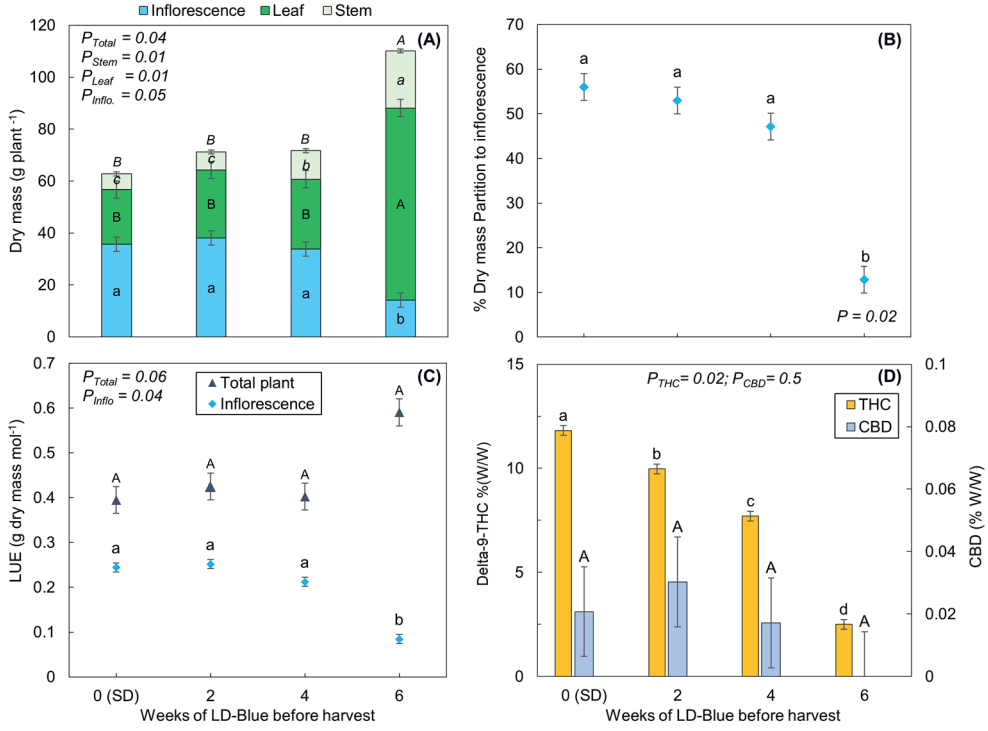

Figure 3. Effect of number of weeks of blue extended photoperiod (LD-Blue), applied before harvest, on plant dry mass (A), dry mass partition to inflorescences (B), light use efficiency of inflorescence and total plant dry mass per cumulative incident light (C), and concentrations of THC and CBD in inflorescences (D) of *Cannabis sativa* L. 'White Russian'. During 8 weeks of generative phase, the plants were grown during 8, 6, 4, or 2 weeks under SD followed by 0 (SD; control), 2, 4, and 6 weeks of LD-Blue respectively. LD-Blue means 12 h of 600 µmol $m^{-2}s^{-1}$ white light (SD), extended with 6 h of 250 µmol $m^{-2}s^{-1}$ blue light. The data are predictions derived from a regression model with an unbalanced design (Exp 1.1 and 1.2). The error bars represent averages of standard error of difference (SED). Different letters indicate significant difference of means according to Fisher's unprotected LSD test at P=0.05. (Exp 1.1 and 1.2)

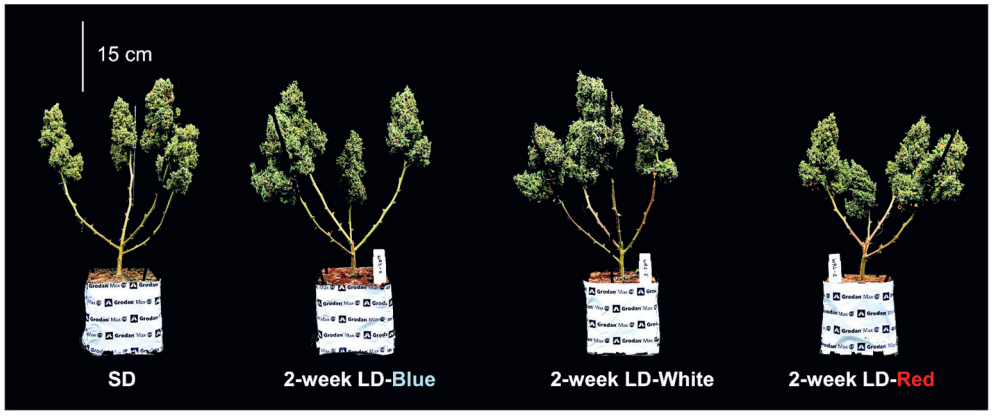

Figure 4. Plant architecture of *Cannabis sativa* L. 'White Russian' after 8 weeks of generative phase (at final harvest). During the last 2 weeks before harvest, the plants were grown under SD, or extended photoperiod with blue, white, and red light. LD means 12 h of 600 µmol $m^{-2}s^{-1}$ white light (SD), extended by either blue (LD-Blue), white (LD-White), or red (LD-Red) for 6 h at 250 µmol $m^{-2}s^{-1}$. For visualization purpose, the fan leaves at the lower part of the canopy were removed. (Exp 1.2)

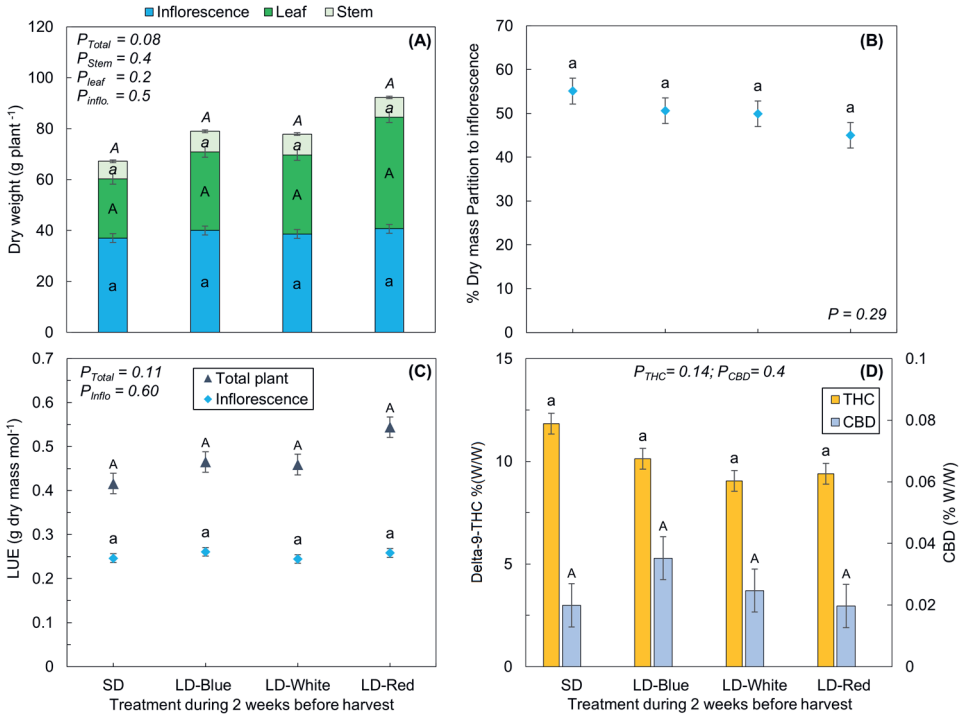

Figure 5. Effect of extended photoperiod with blue, white, or red light for 2 weeks before harvest on *Cannabis sativa* L. 'White Russian' plant dry mass (A), dry mass partition to inflorescences (B), light use efficiency of inflorescence and total plant dry mass per cumulative incident light (C), and concentrations of THC and CBD in inflorescences (D). Data are averages of 2 blocks (n=2; in total 18 plants per treatment). During 8 weeks of generative phase, the plants were grown under 8 weeks of SD, or 6 weeks of SD followed by 2 weeks of LD before harvest. LD means 12 h of 600 µmol $m^{-2}s^{-1}$ white light (SD), extended by either blue (LD-Blue), white (LD-white) or red (LD-Red) for 6 h at 250 µmol $m^{-2}s^{-1}$. The error bars represent standard error of means (SEM). Different letters indicate significant difference of means according to Fisher's protected LSD test at P=0.05. (Exp 1.2)

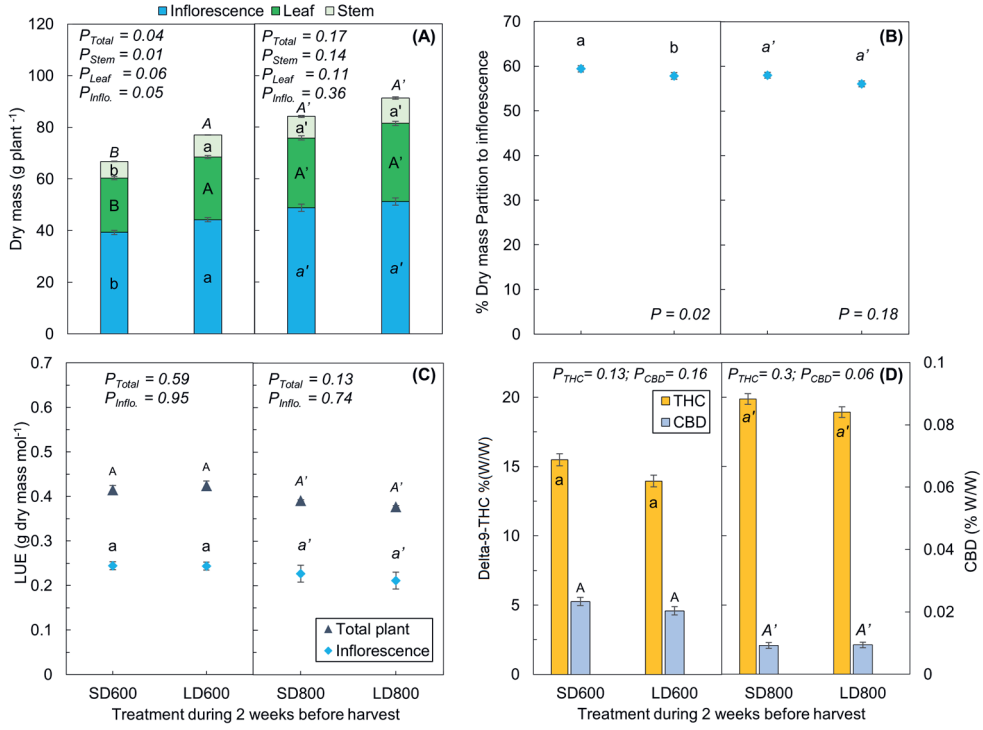

Figure 6. Effect of LD at 600 µmol $m^{-2}s^{-1}$ and 800 µmol $m^{-2}s^{-1}$ for 2 weeks before harvest on *Cannabis sativa* L. 'White Russian' plant dry mass (A), dry mass partition to inflorescences (B), light use efficiency (LUE) of inflorescence and total plant dry mass per cumulative incident light (C), and concentrations of THC and CBD in inflorescences (D). During 8 weeks of generative phase, the plants were grown under 8 weeks of SD at 12 h of 600 µmol $m^{-2}s^{-1}$ (SD600; Exp 2.1) or 800 µmol $m^{-2}s^{-1}$ (SD800; Exp 2.2) white light or under 6 weeks of SD followed by 2 weeks of LD before harvest. LD means 18 h of 600 µmol $m^{-2}s^{-1}$ (LD600; Exp 2.1) or 800 µmol $m^{-2}s^{-1}$ (LD800; Exp 2.2) white light. Data are averages of 3 blocks in Exp 2.1 (n=3; in total 36 plants per treatment) and 2 blocks in Exp 2.2 (n=2; in total 18 plants per treatment). The error bars represent standard error of means (SEM). Different letters indicate significant difference of means within each experiment according to Fisher's protected LSD test at P=0.05. (Exp 2.1 and 2.2)

Table 2. Plant height and main stem diameter measured at final harvest in each experiment

Experiment	Treatments	Plant height (cm)	Main stem diameter (mm)
1.1	SD	45 a	10 a
	2 weeks LD-Blue	45 a	10 a
	4 weeks LD-Blue	54 b	12 b
	6 weeks LD-Blue	76 c	20 c
	SEM	0.4	0.6
	P-value	0.001	0.005
1.2	SD	45 a	10 a
	2 weeks LD-Blue	45 a	10 a
	2 weeks LD-White	44 a	10 a
	2 weeks LD-White	44 a	10 a
	SEM	0.4	0.4
	P-value	0.13	0.80
2.1	SD (at 600 µmol $m^{-2}s^{-1}$)	42 a	10 b
	2 weeks LD (at 600 µmol $m^{-2}s^{-1}$)	43 a	11 a
	SEM	0.4	0.1
	P-value	0.13	0.04
2.2	SD (at 800 µmol $m^{-2}s^{-1}$)	42 a	12 a
	2 weeks LD (at 800 µmol $m^{-2}s^{-1}$)	44 a	12 a
	SEM	0.5	0.1
	P-value	0.15	0.54

4. Discussion

4.1. Extended photoperiod after flower initiation causes reversion to vegetative growth

We aimed to improve the production of cannabis inflorescences by providing extended photoperiod (LD), consequently, higher DLI, after a period of SD needed for flower initiation. Our study reveals that cannabis plants—whose flowers had even been visibly initiated under SD, meaning a group of flowers visible at the apex (Fig S2)—reversed to vegetative growth when exposed to blue extended photoperiod for longer than 2 weeks. Chrysanthemum and some other SD species perceive blue extended photoperiod as SD, and this does not disrupt the flowering process (Park and Jeong, 2020; SharathKumar et al., 2021; 2024a). These SD species seem to perceive blue light as darkness during photoperiodic flowering (SharathKumar et al., 2024), whereas cannabis seems to perceive the blue of extended photoperiod as actual LD. Interestingly, cannabis also responds to low PPFD (1-10 µmol $m^{-2}s^{-1}$) of white light during a 6-hour photoperiod extension following a 9-hour SD, resulting in incomplete inflorescences (Park et al., 2023). Five days of SD are sufficient to initiate inflorescence at the apex (Duchin et al., 2020), however, continued SD is required to maintain inflorescences development and intense branching of flowers at the apex (Spitzer-Rimon et al., 2019). In our study, extended photoperiod reversed the plants from generative to vegetative growth, as evidenced by the production of new leaves on top of initiated inflorescences and enlarging plant height and stem diameter. In contrast, continuous SD typically stopped increasing height and stem diameter after 3 weeks of the generative phase (Fig S5) (Naim-Feil et al., 2021). This phenomenon parallels findings in SD-soybean, where flower reversion occurred when LD was applied after flowering (Wu et al., 2006). The photoperiod-regulated mechanism of flowering in soybean, mediated via phytochromes, works similarly before and after flowering (Han et al., 2006).

4.2. Timing of extended photoperiod matters to maintain flower development

Although extended photoperiod after flower initiation caused reversion to vegetative growth in cannabis, this was not observed in plants exposed to a 2-week LD just before harvest. We noted that the flower development stage in plants subjected to a 2-week LD was comparable to that of plants continuously treated with SD (Fig 4, Fig S3). This timing response was independent of the light spectrum and intensity during the photoperiod extension. A possible explanation for the absence of reversion to vegetative growth under

two weeks of LD just before harvest could be that although the cannabis plants gradually started reversing back to vegetative growth at the apical meristem, this was not observed as a 2-week period was too short. When the plants which received LD during the last 2 weeks were retained one more day (15 days in total) under LD, the emergence of small leaves started to become visible (Fig S4). Additionally, plants that were exposed to fewer weeks of SD showed an earlier return to vegetative growth, indicated by the presence of leaves on top of the inflorescences. Six weeks of SD, followed by two weeks of LD seems to be a critical timepoint that the floral meristem had not yet visibly reversed to vegetative development. It is important to note that this timepoint can differ between cultivars. This may parallel with the critical photoperiod threshold (i.e., light hour per day required to flowering), which varies among cannabis cultivars (Ahrens et al., 2023) and is often influenced by the region of origin of the cultivar (Zhang et al., 2021). In addition, due to the breeding of commercial cultivars, tracking these critical photoperiods becomes more complex. Therefore, it is essential to approach the study with an understanding of the specific cultivars involved.

4.3. Quantity of light used in extended photoperiod matters to improve yield

The inflorescence yield increased under two weeks of LD just before harvest, compared to SD. This suggests that when the additional light was sufficiently available, it could be used to produce inflorescence, resulting in an increase in inflorescence yield (Fig 6A). Although the increase in inflorescence yield was not statistically significant in some cases, it was proportional to the increase in light (i.e. DLI) and closely aligned with the 1% rule of thumb, stating that 1% more light results in 1% more yield (Marcelis et al., 2006). In our study, 1% increase in light (cumulative incident light during the 8 weeks of the generative phase) applying 250 µmol $m^{-2}s^{-1}$ during two weeks extended photoperiod resulted in a 1.4% rise in average inflorescence dry mass (accumulated over the whole cultivation cycle), regardless of light spectrum, compared to SD. The use of 600 µmol $m^{-2}s^{-1}$ of light for 2-week LD resulted in 1% increase in both cumulative light and inflorescence yield. Nonetheless, the use of 800 µmol $m^{-2}s^{-1}$, the observed increase in inflorescence yield was limited to 0.4%, despite a 1% increase in cumulative light by 2-week LD.

All other studies on extended photoperiod to promote growth in SD species were conducted at lower light intensities (40-100 µmol $m^{-2}s^{-1}$) (Jeong et al., 2014; Park and Jeong, 2020; SharathKumar et al., 2024a, 2021), while our study employed 250-800 µmol $m^{-2}s^{-1}$. The high PPFD (above 500 µmol $m^{-2}s^{-1}$) used in cannabis is common in

commercial facilities (Chandra et al., 2017a) and cannot be compromised to obtain a reasonable yield (Chapter 4). Moreover, our work demonstrated that limited additional light, as obtained from low light intensity, did not lead to a significant increase in inflorescence yield.

4.4. Concentrations of cannabinoids tend to decrease by extended photoperiod

Concentrations of cannabinoids in inflorescences, obviously THC as a dominant compound in 'White Russian' decreased when an extended LD lasted longer than two weeks before harvest, compared to SD. This decline was possibly a consequence of the presence of undeveloped inflorescences due to photoperiod extension that applied to soon and too long (Fig S3 C-D). Concentrations of THC and CBD were found to be lower in undeveloped inflorescences, compared to fully developed inflorescences (Aizpurua-Olaizola et al., 2016). Despite previous reports suggested that blue enriched light enhances THC accumulation (Magagnini et al., 2018; Morello et al., 2022), our results did not show an increase in cannabinoids in plants exposed to blue light. This could imply that either blue light had no effect, or the effect was minimal and potentially diluted by the extension of the photoperiod. However, a 2-week LD just before harvest did not statistically reduce the THC concentration (Fig 3, 5, 6C) and yield (Fig S6), irrespective of light spectrum and intensity. This may indicate well-developed inflorescences comparable to those in SD plants. It was hard to prove the effect of photoperiod on CBD since the amount was limited in this studied genotype.

4.5. Application of extended photoperiod

In medicinal cannabis cultivation extended photoperiod can be applied, but this extension should not exceed two weeks before harvest to prevent reduction in inflorescence yield and cannabinoid content. However, the additional light provided has to be substantial to clearly increase inflorescence yield. Notably, responses to photoperiod are genotype-dependent (Zhang et al., 2021), emphasizing the need to fine-tune the timing for each cultivar. The information is relevant not only for indoor growers, suggesting that designing photoperiod can improve yield, but also for outdoor growers who may benefit by keeping blackout curtains open during the summer, exposing plants to longer days to increase yield. Cultivators should also be aware that maintaining a prolonged photoperiod for more than two weeks during the generative phase could reduce quantity (biomass) and quality (maturity and specialized metabolites) of flowers. Conversely, the reversion of flowering mother plants to vegetative plants, can be achieved with a longer LD period than two weeks. In conclusion, the timing of extended

LD and the amount of light must be appropriate and sufficient to expect an improvement in the harvested yield.

5. Conclusion

Extending photoperiod after flower initiation caused reversion to vegetative growth. However, last 2 weeks of longday just before harvest (6-8 weeks from start of shortday) can increase inflorescence yield without negative effects on cannabinoid concentration.

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Supplementary material

Supplement 1 Rooting method, following the protocol described in the study by Sae-Tang et al. (2024) (Chapter 2)

The stem cuttings of medicinal cannabis cultivar 'White Russian' were cut from 3-6 months old mother plants grown in a glasshouse (Wageningen University and Research, Greenhouse Horticulture, Bleiswijk, the Netherlands). The lower end of the stem cuttings was dipped into 0.25% IBA powder (Rhizopon, the Netherlands) and rooted in 3.6 x 3.6 x 4 cm stonewool plugs (Grodan, the Netherlands). The cuttings were placed in an enclosed transparent plastic tent at 100 µmol $m^{-2}s^{-1}$ PPFD, consisting of 11% blue (400-500 nm), 6% green (500-600 nm), and 83% red light (600-700 nm) provided by a mixture of red-blue-white LEDs (GreenPower Dynamic LED 2.0, Philips, Eindhoven, the Netherlands) with GrowWise Control System. The photoperiod was 18/6 h (light/dark). The rooting took 21 days. During the rooting phase, the climate setting was at 28/28 °C air temperature, 80% relative humidity (RH) and ambient CO_2. Irrigation was done every third day by soaking the plugs in 1.5 dS m^{-1} (EC) nutrient solution (1.25 mM NH_4^+; 6.2 mM K^+; 1.9 mM Ca^{2+}; 0.9 mM Mg^{2+}; 10.5 mM NO_3^-; 0.85 mM SO_4^{2-}; 0.85 mM PO_4^{3-}; 60 µM Fe^{2+}; 20 µM Mn^{2+}; 3 µM Zn^{2+}; 20 µM B^{2+}; 0.5 µM Cu^{2+}; 0.5 µM Mo^{2+}).

Table S1. Averages of realized air temperature and relative humidity in each experiment and averages over 4 experiments ± SE. The measurement was done at plant level, recorded every 10 minutes by dataloggers (Easylog USB-1-LCD, Lascar electronics, Wiltshire, UK).

Week of generative phase	Exp 1.1		Exp 1.2		Exp 2.1		Exp 2.2		Average	
	Temp	RH	Temp	RH	Temp	RH	Temp	RH	Temp	RH
1	29.3	74.3	28.7	78.0	28.6	76.8	28.3	80.0	28.7±0.4	77.3±2.4
2	29.1	73.2	28.9	76.7	29.1	80.0	28.4	80.0	28.9±0.3	77.5±3.2
3	*N.D.**	*N.D.**	28.7	72.7	28.0	76.5	28.3	73.0	28.3±0.4	74.1±2.1
4	28.9	63.0	28.6	71.9	27.8	69.0	28.6	73.1	28.5±0.5	69.3±4.5
5	29.1	56.9	28.9	60.8	28.1	60.5	28.6	63.8	28.7±0.4	60.5±2.8
6	29.5	55.8	29.2	53.1	28.3	55.7	28.8	54.5	29.0±0.5	54.8±1.3
7	28.9	56.7	28.5	54.7	28.4	55.1	28.5	55.1	28.6±0.2	55.4±0.9
8	29.4	50.1	28.6	54.5	28.2	54.8	28.8	55.0	28.8±0.5	53.6±2.3

**N.D. = No data*

Table S2. Averages of realized light intensity measured at plant canopy in each treatment ±SE from replicated experiments.

Exp	Treatment	Light intensity during 12 h (µmol $m^{-2}s^{-1}$)	Light intensity during 6 extended h (µmol $m^{-2}s^{-1}$)
1.1	SD	591.8 ± 4.4	-
	2 weeks Blue-LD	594.2 ± 7.2	250.3 ± 0.5
	4 weeks Blue-LD	604.2*	251.8*
	6 weeks Blue-LD	608.1*	251.2*
1.2	SD	588.7 ± 8.6	-
	2 weeks Blue-LD (LD-Blue)	593.5 ± 2.3	250.0 ± 0.8
	2 weeks White-LD (LD-White)	583.0 ± 1.6	248.9 ± 1.3
	2 weeks Red-LD (LD-Red)	604.9 ± 5.8	249.0 ± 2.5
2.1	SD at 600 (SD600)	607.2 ± 1.0	-
	2 weeks LD at 600 (LD600)	608.8 ± 2.4	608.8 ± 2.4
2.2	SD at 800 (SD800)	790.2 ± 4.7	-
	2 weeks LD at 800 (LD800)	786.6 ± 4.5	786.6 ± 4.5

**The treatment has one repetition, therefore no SE.*

Table S3. Number of repetitions and tested plants in each experiment

Exp	Number of repetitions	Number of replicate plants per treatment in each repetition
1.1	1	18
1.2	2 (repeated by time)	9 , 9
2.1	3 (repeated once by time and once in parallel)	18 , 9 , 9
2.2	2 (repeated in parallel)	9 , 9

Table S4. Number of real replicates in each treatment as used in statistic test, indicated in Fig 3, 5, and 6

Fig	Treatment	Number of real replicates (n)	Conducted Experiment	Statistical test
3	SD	3	1.1, 1.2	One-way ANOVA in unbalanced design with blocks
	2 weeks Blue-LD	3	1.1, 1.2	
	4 weeks Blue-LD	1	1.1	
	6 weeks Blue-LD	1	1.1	
5	SD	2	1.2	One-way ANOVA with blocks
	2 weeks Blue-LD (LD-Blue)	2	1.2	
	2 weeks White-LD (LD-White)	2	1.2	
	2 weeks Red-LD (LD-Red)	2	1.2	
6	SD at 600 (SD600)	3	2.1	One-way ANOVA with block
	2 weeks LD at 600 (LD600)	3	2.1	
	SD at 800 (SD800)	2	2.2	One-way ANOVA with blocks
	2 weeks LD at 800 (LD800)	2	2.2	

Figure S1. Plant architectures after 8 weeks of flower induction phase, at the final harvest. The plants were grown under 8 weeks of SD at 12 h of 600 µmol $m^{-2}s^{-1}$ (SD600; Exp 2.1) or 800 µmol $m^{-2}s^{-1}$ (SD800; Exp 2.2) white light or under 6 weeks of SD followed by 2 weeks of LD before harvest. LD means 18 h of 600 µmol $m^{-2}s^{-1}$ (LD600; Exp 2.1) or 800 µmol $m^{-2}s^{-1}$ (LD800; Exp 2.2) white light. For visualization purpose, the fan leaves at the lower part of the canopy were removed.

Figure S2. Group of flowers at the apex (A), top of canopy (B) and side of canopy (C) of the plant under 12 h (SD) of 600 µmol $m^{-2}s^{-1}$ white light for 2 weeks (24 DAT) when the first treatment of extended photoperiod started.

Figure S3. Inflorescences of *Cannabis sativa* 'White Russian' after 8 weeks of generative phase. During 8 weeks of generative phase, the plants were grown under 8, 6, 4, or 2 weeks of SD followed by 0 (SD; control), 2, 4, and 6 weeks of blue extended photoperiod (LD) respectively. LD means 12 h of 600 µmol $m^{-2}s^{-1}$ white light (SD), extended with 6 h of 250 µmol $m^{-2}s^{-1}$ blue light. (Exp 1.1)

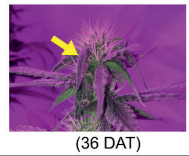
Treatment (*Exp 1.1*)	Days after LD that leaves started to emerge on top of inflorescences	10 days after LD started	14 days after LD started	15 days after LD started
2-week LD-Blue	15 days	 (63 DAT)	 (67 DAT)	 (68 DAT)
4-week LD-Blue	14 days	 (48 DAT)	 (52 DAT)	 (53 DAT)
6-week LD-Blue	10 days	 (36 DAT)	 (40 DAT)	Not available

Figure S4. The emergence of leaves on top of inflorescences after LD treatment (Exp 1.1). The yellow arrows indicate the emerging leaves. Days after transplant (DAT) indicated below the picture. For the treatment of 2-week LD-blue the picture on 15 days after start of LD was based on spare plants in this treatment.

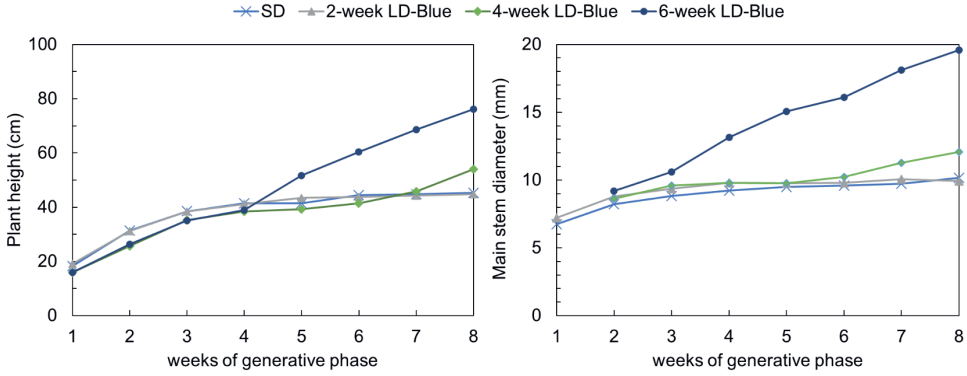


Figure S5. Plant height and stem diameter over 8 weeks of generative phase. The plants were grown under 8, 6, 4, or 2 weeks of SD followed by 0 (SD; control), 2, 4, and 6 weeks of blue extended photoperiod (LD-Blue) respectively. LD-Blue means 12 h of 600 µmol $m^{-2}s^{-1}$ white light (SD), extended with 6 h of 250 µmol $m^{-2}s^{-1}$ blue light. (Exp 1.1)

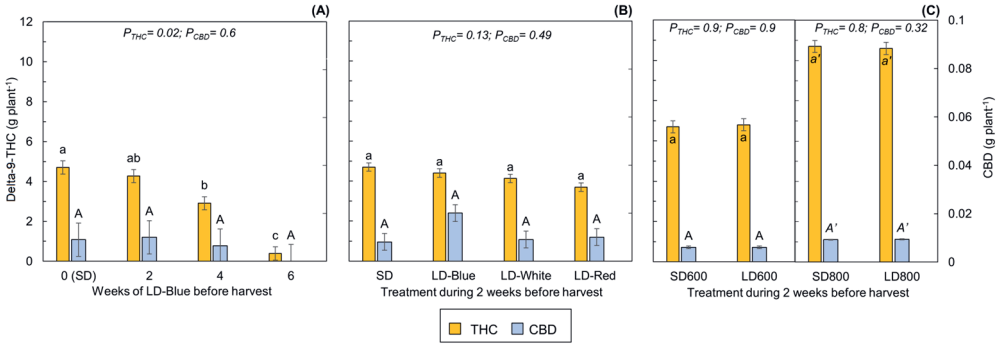


Figure S6. Yield of cannabinoids in dry inflorescences per plant. The yield was calculated based on the concentration in Fig 3D (A), 5D (B), and 6D (C). The error bars represent standard error of means (SEM). Different letters indicate significant difference of means within each experiment according to Fisher's protected LSD test at P=0.05.

Chapter 6

General discussion

General discussion

Medicinal cannabis is a high-value crop, which is often cultivated indoors (Chandra et al., 2017a), as this allows precise control over growing conditions, thereby obtaining high and consistent plant growth and product quality. In indoor cultivation, light intensity, light spectrum, and photoperiod can be controlled, and all three play pivotal roles in influencing plant development and productivity (SharathKumar et al., 2020). Using light to manipulate cannabis plants has the potential to maximize total yield and specialized metabolites accumulation by shaping desired plant architecture, accelerating growth cycle, or influencing the biosynthesis of metabolites. However, the light strategies are often kept confidential among growers, with limited scientific evidence available to support.

In this thesis, I aimed to explore the roles of light on cannabis in order to obtain lighting strategies for cultivation that can improve the yield which is defined as high inflorescence dry mass and high concentrations of specialized metabolites at desired ratios. I investigated the potential underlying physiological and morphological responses to light. A series of experiments in climate rooms was conducted involving various light spectra, light intensities, and photoperiods. The effects of lights were studied for critical developmental processes such as rooting of stem cuttings, plant growth, flower induction and development, dry matter production, and, finally, the accumulation of specialized metabolites. In this chapter, I discussed the overall effects of light on cannabis (Fig 1), recommend further research, and propose implementations for cultivation practices.

1. Light spectrum as a tool to shape plant morphology but not always in cannabis

Light spectrum plays a critical role as an environmental signal, influencing plant morphology through a process called photomorphogenesis, mediated by a variety of photoreceptors which perceive different wavelengths (Kami et al., 2010). Light within 400-700 nm is defined as photosynthetically active radiation (PAR) and absorbed by spectrum-specific photosynthetic pigments, providing energy for photosynthesis (McCree, 1971) and thereby determining plant growth and biomass production.

Blue (400-500 nm) is essential to be present with **red** (600-700 nm) to maintain photosynthesis capacity and regular plant architecture (Boccalandro et al., 2012; Hogewoning et al., 2010). High fraction of blue promotes plant compactness (Cope et al., 2014; Ying et al., 2020), which may have a negative impact on biomass production

(Hernández and Kubota, 2016). Conversely, additional **far-red** (700-800 nm) has been demonstrated to increase yield in several crops, for example, tomato, lettuce, and basil (Ji et al., 2020; Jin et al., 2021; Larsen et al., 2020). This increase is associated with shade avoidance responses such as internode elongation and leaf expansion. A number of studies have demonstrated the impacts of light, which can be used to formulate optimal light spectra for indoor production, particularly for leafy vegetables (Goto, 2012; Neo et al., 2022; Stamford et al., 2023). However, the research in cannabis remains rather limited.

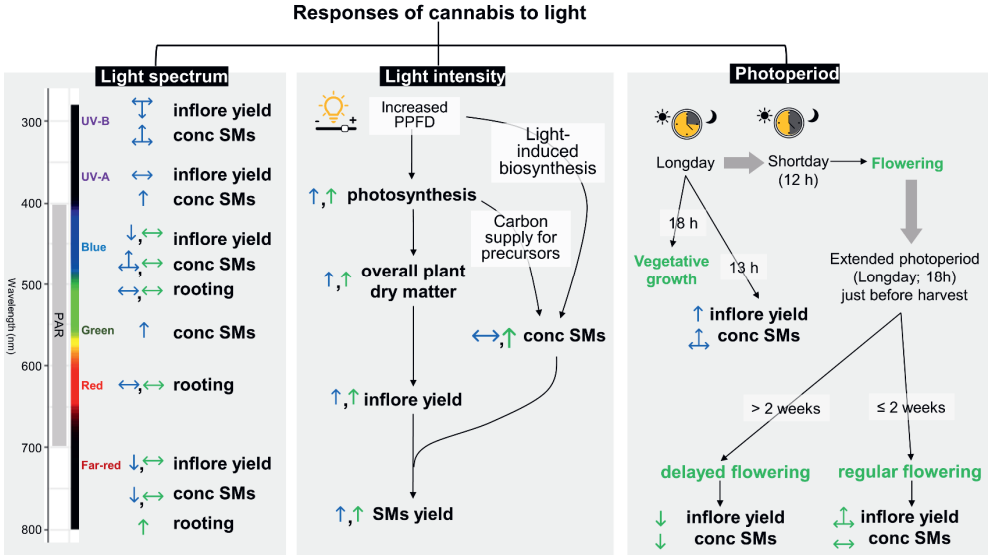


Figure 1. Effects of light on cannabis rooting, inflorescence yield, and concentration of specialized metabolites (SMs). The arrow icons indicate the direction of the responses, including ↑: denotes an increase, ↓: a decrease, ↔: no change or no response, ↥: either an increase or no change, and ↧: either a decrease or no change. **Blue arrow icons** represent responses reported by published research, while **green arrow icons** indicate responses found in the experiments in this thesis

Previous studies on cannabis, together with the findings of this thesis, have shown inconsistent responses to light spectrum. For instance, high blue light (25-47%) caused shorter plants, compared to 4-18% blue light (Danziger and Bernstein, 2021), whereas using blue light within the range of either from 4% or 8% to 20% did not observe this effect (Kotiranta et al., 2024; Westmoreland et al., 2021, Chapter 3). Moreover, the increase in blue light from 4% to 20% was found to reduce inflorescence yield in some studies (Westmoreland et al., 2021).

Adding far-red during the generative phase resulted in elongated plants, but surprisingly decreased inflorescence yield in cannabis (Jähne et al., 2020; Kotiranta et al., 2024). In Chapter 2, additional far-red during the rooting phase also enhanced stem elongation of stem cuttings. However, in Chapter 3, adding far-red during the last half of the generative phase did not result in plant elongation or influenced yield. This no-response suggests that the timing of the far-red application in Chapter 3 was when the plant had already reached final plant height.

Furthermore, manipulating light spectrum can be used to regulate specific plant developmental processes. For example, adventitious rooting of stem cuttings is enhanced by additional far-red light, as observed in several ornamental crops (Christiaens et al., 2019; Park et al., 2022), and also cannabis (Chapter 2). Substituting blue light for red light did not affect rooting of cannabis stem cuttings (Moher et al., 2023, Chapter 2). Besides, light spectrum influences flowering in some plants, for example, far-red advanced flowering in shortday amaranth and rice, potentially caused by inactive phytochrome (Jähne et al., 2020). To the best of my knowledge, the time to flower cannabis was not affected by the light spectrum (Kotiranta et al., 2024; Rodriguez-Morrison et al., 2021b; Westmoreland et al., 2021, Chapter 3). Additionally, during night break or photoperiod extension the light spectrum has been used to control flowering, a topic further discussed in section 3.

Overall, the effects of light spectrum on plant morphology can vary depending on factors such as dosage (amount of photons), timing of application, species, and even cultivars within species (Ouzounis et al., 2015; Zhang et al., 2021b). The effects of light spectrum on cannabis are still inconclusive. It is important to note that many studies have derived the effects of different amounts of blue or far-red light by comparing different light sources, including HPS, fluorescence, and LEDs lamps. Consequently, not only the targeted light regions were varied, but other wavelengths were also differed. Interestingly, this thesis and some previous studies have found that the effects of light spectrum on cannabis are relatively limited compared to classical theories or common findings in other crops. In the next sections, potential reasons are discussed.

1.1. Diminished effect of light spectrum under high light intensity

Studies on the effect of light spectrum in cannabis have often been conducted at relatively high light intensities (>500 µmol $m^{-2}s^{-1}$ PPFD), which is typical in cannabis cultivation practices. In contrast, most studies in other crops have been carried out under much lower light intensities (<300 µmol $m^{-2}s^{-1}$ PPFD). Under such high light intensity, the

impact of light spectrum may be diminished. This was found in basil that the effect of blue was less pronounced under high light level (Larsen et al., 2020). An interaction between amount of blue and total light intensity has been reported (Cope and Bugbee, 2013). It might be possible that photoreceptors could receive a signal of light spectrum only up to a certain PPFD level, although this is still unknow. Consequently, the expected photomorphogenesis regulated by light spectrum may fail under high light intensity.

1.2. Cannabis is a species rather irresponsive to light spectrum

Another potential explanation for the diminished effect of light spectrum on cannabis is the plant itself. Cannabis is a sun-loving plant that can grow at high levels of light. Cannabis, is also a fast-growing crop, has features similar to weeds, thriving under a variety of environments (Malík et al., 2021). This might give plasticity, to grow well under a range of light spectra. Moreover, cannabis leaves contain flavonoids, sterols, terpenoids, and cannabinoids (Jin et al., 2020) which potentially act as photo-protectants to against excess light (Agati and Tattini, 2010; Desaulniers Brousseau et al., 2021), these compounds may diminish the light reception of photoreceptors. More research is needed to understand how cannabis photoreceptors perceive the light spectrum with the presence of these specialized metabolites.

2. Cannabis can utilize very large amount of light and has high photosynthetic capacity

Increasing the amount of light, expressed as the daily light integral (DLI), either by increasing light intensity or extending photoperiod, often leads to higher yields up to saturating levels (Poorter et al., 2019). Notably, cannabis inflorescence yield linearly increases with an increase in light intensity up to the highest levels studied (Chapter 4 and 5, Eaves et al., 2020; Llewellyn et al., 2022; Rodriguez-Morrison et al., 2021b). To my knowledge, the point at which cannabis yield saturates under increased light intensity remains unknown, with reported maximum PPFD reaching 1800 µmol $m^{-2}s^{-1}$ while yield continued to increase (Rodriguez-Morrison et al., 2021b). Beside raising DLI through higher light intensity, extending photoperiod, when flowering is not impaired, also increases cannabis yield (Chapter 4, Ahrens et al., 2024).

Remarkably, the light use efficiency (LUE) of cannabis remains constant (Chapter 4), while LUE of most species decreases with increasing light intensity (Fu et al., 2012; Heuvelink et al., 2002; Pennisi et al., 2020). This suggests that cannabis possesses a high photosynthetic capacity capable of utilizing extremely high light intensities. This

likely makes cannabis a so-called photosynthetic extremophile, i.e. an organism thriving in extreme environmental conditions. For example, grey mustard (*Hirschfeldia incana* L.) has been presented as a model of photosynthetic extremophile due to its regular photosynthesis at high radiation levels (Taylor et al., 2023).

Beside high light intensity, cannabis appears to be tolerant to drought stress. Cannabis plants that were exposed to drought stress by maintaining 30-50% field capacity of substrate during the generative phase showed inflorescence yield comparable to non-stressed plants (Morgan et al., 2024). Similarly, withholding fertigation until reaching plant water potential at the stem of -1.5 MPa (3% moisture content of substrate) during the last two weeks of the generative also did not affect yield (Caplan et al., 2019). Cannabis appears to effectively maintain biological processes, even under extreme conditions, yet the underlying mechanism is unknown. This resilience could be attributed to unique patterns of leaf tissue anatomy or high enzymatic activity, which allow for efficient light use. Furthermore, specialized metabolites distributed throughout the plant may act as antioxidants, alleviating oxidative stress caused by high light.

3. Flowering of cannabis strongly responds to photoperiod rather than light spectrum

Most cannabis cultivars are classified as shortday plants, requiring long nights for flower initiation and development (Zhang et al., 2021a). However, in some studies, cannabis is also described as day-neutral since solitary flowers can form under both long and short days (Spitzer-Rimon et al., 2022, 2019). Still, continuous shortdays are necessary for inflorescence bud formation (Spitzer-Rimon et al., 2019). Therefore, a 12-hour photoperiod as shortday is commonly used to induce flowering and maintained inflorescence development until harvest (Chandra et al., 2017). Nevertheless, this limits DLI, which could limit photosynthesis and biomass productivity, especially in cannabis cultivation where high light intensity is typically utilized and increasing higher light intensity can be challenging due to lamp limitations.

Using light spectrum to extend the photoperiod in order to control flowering in indoor cultivation has been explored in photoperiodic ornamental crops, such as, chrysanthemum, petunia, and calibrachoa (Jeong and Park, 2022; Meng and Runkle, 2017). While extending the photoperiod with blue light has successfully led shortday chrysanthemum and also other shortday plants to flower under longdays (Park and Jeong, 2020; SharathKumar et al., 2024, 2021), this approach was ineffective for cannabis (Chapter 5). Despite flowers had been initiated under shortday of at least two

weeks, subsequent exposure to a long day period (i.e., an 18-hour photoperiod) for more than two weeks caused the plants to revert to vegetative growth, regardless of the light spectrum (Chapter 5). Thus, cannabis strictly relies on photoperiod cues for flowering. We found that six-weeks of shortday followed by two-weeks of longday tended to increase inflorescence yield compared to eight weeks of shortday (Chapter 5). Some studies have shown that a 13-h photoperiod increased inflorescence yield in some of the studied cultivars compared to a 12-h photoperiod (Ahrens et al., 2024, 2023). This suggests that the critical daylength for cannabis can vary within a small range, depending on the cultivar,

4. Challenges in concluding the impact of light on specialized metabolites

About 565 specialized metabolites were identified in cannabis accumulating in glandular trichomes found predominantly on female inflorescences (ElSohly et al., 2017). These metabolites, previously called secondary metabolites, are hypothesized to serve as protective agents against UV light, pathogens, and pest insects (Gülck and Møller, 2020). Their accumulation could be influenced by both genetic factors and environmental conditions (Vásquez-Ocmín et al., 2021). The composition of these metabolites is important in several aspects. Cannabis cultivars are often categorized based on their metabolite profiles, particularly THC and CBD levels (Pacifico et al., 2006). It defines medical properties, especially entourage effect, the postulated synergistic action of various compounds (Desaulniers Brousseau et al., 2021). Moreover, the concentrations of THC and CBD are crucial for legal compliance; for example, in some regions, only cannabis with THC levels below 0.3% is permitted for cultivation (Mead, 2017).

High light intensity was found to increase specialized metabolites in many crops, improving their quality (Min et al., 2023). In Chapter 4, I found that high light intensity strongly increases the concentration of both terpenoid and cannabinoids, while Hawley et al., (2018) found increased THC and Rodriguez-Morrison et al. (2021b) reported small increased terpenoids. The increase in light might increase carbon supply via photosynthesis and these can be used as a precursor in the biosynthesis pathway, including the biosynthesis of terpenoids (Saadat et al., 2023). Additionally, light can induce the methyl-D-erythritol phosphate (MEP) pathway, which produces iso-pentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are precursors that can be transformed into terpenoids and cannabinoids (Tholl, 2015).

The first study on the impact of light spectrum on cannabis, conducted by Lydon et al., (1987) found that UV-B radiation increased THC levels. Supplemental UV-A was found to

increase some cannabinoids (Desaulniers Brousseau et al., 2021; Jenkins, 2021; Magagnini et al., 2018). However, the later studies by (Kotiranta et al., 2024; Llewellyn et al., 2022; Rodriguez-Morrison et al., 2021b; Westmoreland et al., 2023) did not observe this positive effect of UV-A and UV-B. These various responses may be due to differences in doses, genetics, timing, and background lights (Contreras-Avilés et al., 2024). Subsequently, blue light has gained interest for its potential to enhance specialized metabolite production, as it is closely related to UV-A and shares perception though photoreceptors cryptochrome, phototropin and ZTL/FKF1/LKP2 family (Huché-Thélier et al., 2016). Blue light has been shown to increase the levels of compounds such as carotenoids and anthocyanins in several plants (Hoffmann et al., 2016; Huché-Thélier et al., 2016; Liu et al., 2022). Initial studies in cannabis claimed that LEDs lighting increased cannabinoid content compared to traditional HPS lighting systems (Amrein et al., 2020; Magagnini et al., 2018; Wei et al., 2021), possibly due to the higher fraction of blue in the LEDs spectra. Nonetheless, varying the fraction of blue light using LEDs from 8-21% had no difference in cannabinoids (Chapter 3, Westmoreland et al., 2021). The research on the effects of green light is still limited. It has been reviewed that green light can increase THC and some monoterpenoids (Contreras-Avilés et al., 2024; Desaulniers Brousseau et al., 2021). Far-red tends to reduce specialized metabolites and make plants more susceptible to disease (Courbier et al., 2020). This caution should be taken into account when adding far-red in cannabis, as it also reduced some cannabinoids (Kotiranta et al., 2024). However, adding far-red only during the last half of the generative phase did not affect specialized metabolite concentrations (Chapter 4). In addition, extended photoperiod (i.e., longday) did not influence specialized metabolites when flowering was not hampered (Chapter 5, Ahrens et al., 2024).

Many diverse responses have been observed, which can be related to differences in plant species, cultivars, growing conditions, and also timing of sampling or measurement in each study. These pose challenges in elucidating the effects of light on specialized metabolites. Given the current understanding, it is not easy to draw clear conclusions.

5. Further research

In the past, cannabis research was very limited by legalization restrictions, relying largely on insights from experienced growers. However, in recent years, including during the timeframe of this thesis, there has been a surge in cannabis-related studies in many countries. While my thesis represents only a small part of this progress, numerous unexplored topics offer potential for further investigation. Advancement in cannabis

research not only benefits the cannabis industry but also contributes valuable insights to the broader field of plant science.

5.1. How cannabis can utilize a huge amount of light

This thesis, along with several studies agrees that cannabis, a C_3 plant, can effectively cope with high light intensity, as more light results in better growth and yield. Exploring mechanisms such as light-harvesting systems, electron transport, carbon dioxide fixation enzyme efficiency, and specialized leaf structures could provide insights into how cannabis effectively utilizes very high light levels. Additionally, cannabis can be used as a model plant for extremophiles to improve photosynthesis of other important commercial crops.

Besides the leaf level, exploring photosynthesis at the canopy level could be interesting. During the generative phase, the cannabis plant is largely covered by flowers on top. The green flowers are structured as floral bracts, which are modified leaves (Romero et al., 2020) that can potentially photosynthesize. This might contribute to the whole canopy photosynthesis and, consequently, the whole plant's growth. Understanding this process could help optimize a more efficient light supply.

5.2. Cannabis under low light intensity

Although cannabis is a sun-loving plant which naturally grows in open fields (Clarke and Merlin, 2016), there has been a significant move towards indoor cultivation. The use of extremely high light intensities (500-1500 µmol $m^{-2}s^{-1}$ PPFD) for cultivation is becoming more common (Chandra et al., 2017) thanks to advancements in lighting technology. However, it may be worth reconsidering the necessity of this approach from an energy-saving perspective. While high light intensity can indeed increase yield, cannabis plants demonstrate resilience by thriving under lower light intensities (<300 µmol $m^{-2}s^{-1}$ PPFD) (Wei et al., 2021). LUE is rather similar under higher and lower light intensity. Low light intensity from fewer lamps produces less heat emission, and potentially lowers cooling demand. This presents an opportunity for exploring the optimal light intensity for both economic profitability and sustainability of indoor cultivation practices for cannabis.

5.3. Multi-omics analysis to elucidate effects of light spectrum on specialized metabolites

The impact of light spectrum on specialized metabolites in cannabis presents a puzzle, with conflicting findings across studies. To clarify these effects, further research is

needed, not only focusing on the spectrum of interest but also carefully controlling other factors and investigating their interactions with light. To improve our understanding of the complex responses of cannabis to the light spectrum, I proposed employing multi-omics approaches. This integrative method combines and analyzes data from several omics technologies, allowing for a holistic view of the biological processes (Rai et al., 2017). Metabolomics can offer a comprehensive view of changes in both primary and specialized metabolites due to light spectrum effects. Proteomics can elucidate gene translation, especially to enzymes related to biosynthetic pathways, while transcriptomics can provide insights into overall gene regulation patterns which are potentially influenced by light. Finally, these omics data can be integrated with physiological data, efficiently measured using high-throughput phenotyping technology. All the steps of plant responses will be covered with these insights, providing a complete picture how light spectrum influence specialized metabolites via biological processes.

5.4. Interaction of lights and other environmental factors

This thesis primarily focused on the effects of light, covering several aspects of light, and often varying only one of them at a time. For a better understanding, future research could look into the interactions of various light factors, for instance, studying how different light spectra interact with light intensity. Moreover, it is important to acknowledge that other environmental factors also influence growth and yield, and these factors can interact with light. Future studies could look into how combinations of factors such as light and CO_2 enrichment, light and temperature, and light and nutrients affect cannabis growth, dry matter production, and specialized metabolites.

5.5. Breeding

Cannabis has a long history of human use, not only as medicine, but also as food, oil, and textile (Xie et al., 2023). As a result, it has been bred for various purposes over time (Kovalchuk et al., 2020). Despite the abundance of strains available, tracing them back to their genetic origins can be challenging, as many have been often bred by small-scale home growers (Rahn et al., 2016). On the other hand, modern agricultural practices focus on specific profitable traits and rely on clone propagation, which could reduce genetic diversity (Ren et al., 2021). This highlights the need for comprehensive genetic datasets to support better breeding selection.

By using genomic information in breeding programs, researchers can establish diverse parental lines as the basis for developing hybrids. These hybrids can aim to enhance

profitable traits, such as high yield and high targeted metabolites, and also resilience to environmental conditions. Genome editing techniques such as, CRISPR/Cas9, have been successfully applied in some crops, including rice, tomato, and soybeans (Wada et al., 2020), to generate new lines by precisely altering nucleic acids in specific regions of the genome. In cannabis, gene editing technology has been limited due to the complexity of the genome and inefficient transformation techniques (Guo et al., 2022). Nevertheless, the CRISPR/Cas9 holds promise for further enhancing the variety of breeding programs in cannabis within a shorter timeframe.

6. Recommendation for lighting in cannabis cultivation

I strongly recommend growers to base their growing conditions on scientific studies, and results that are based on experiments with good statistical design. Here, we present recommendations for lighting, drawing from the results found in this thesis and recent publications. These recommendations aim to assist cannabis growers in optimizing their practices for yield and quality improvement.

6.1. Far-red at the beginning of rooting phase improve rooting success.

Stem cutting, also known as cloning, is a widely-use method of asexual propagation because it preserves genetic identity, including the sex and metabolite profile of desired cultivars, while remaining cost-effective and time-efficient. To improve rooting success, synthetic auxins such as 0.25% IBA (Indole-3-butyric acid) are commonly used to dip the stem end. This method was found effective especially cultivars that are difficult to root (Chapter 2 Exp 1, Blythe et al., 2007).

Alternatively, far-red can be used to replace synthetic chemical treatments, particularly in some regions where synthetic compound is not allowed due to food safety concerns. We propose using far-red for the first 7 days of the rooting phase. This increases rooting success while avoiding excessive plant elongation (Chapter 2 Exp 2), which could cause problems when transplanting.

6.2. Use of high light intensity has to be done with cautions.

Chapter 4-5 and several studies confirm that increasing light intensity could increase inflorescence yield (Eaves et al., 2020; Llewellyn et al., 2022; Rodriguez-Morrison et al., 2021). However, other growth factors must be adjusted accordingly to maximize the

benefits. Elevated CO_2 concentration is required, typically ranging from 800 to 1000 ppm (Zheng and Llewellyn, 2022). Furthermore, because high light intensity can emit a substantial amount of heat, and plants definitely need sufficient water, temperature and water availability must be carefully monitored. Additionally, high light intensity leads to dense and thick inflorescences which are thought to have a high market value while being easily infected by fungi. Thus, there is a trade-off of high light intensity.

6.3. Flexibility to chose light spectrum

During the generative phase, this thesis and some other studies have shown minimal effects of light spectrum manipulation, for example, fractions of blue (Chapter 3). Therefore, there is flexibility in the use of light spectrum in cultivation. Growers may prioritize the lamp selection with cost, size, and intensity over light spectrum. For instance, using LEDs with a high fraction of red and relatively low blue is energy-saving, as red LEDs have the higherst photon efficacy (Kusuma et al., 2020). In addition, white light (red and blue LEDs with a small addition of white LEDs) may be preferred for crop management as it facilitates tasks such as scouting of plant, disease, and pest, as well as being worker-friendly during plant maintenance activities like pruning or harvesting.

6.4. Additional light treatment may not be needed for the last period

Initially I tested several light treatments on cannabis throughout the generative phase. I noticed that when the treatments were applied during the last half of the generative phase, only marginal effects were observed (Chapter 3 and 5). During this period, leaf senescence begins, and photosynthetic rates drop noticeably (Chapter 4). Treatments given at the end of production (EOP) could be used to improve quality, as seen in some other crops (Min et al., 2023). However, the EOP will be less effective in cannabis. Additional EOP treatments, while not harmful to the plant, may be unnecessary because plants may not use them efficiently. Exploring the light treatment at earlier stages could be an option worth considering. It also suggests that light intensity during the EOP can be reduced to save energy, while more research is needed to ensure that this has no negative influence on yield or metabolites.

6.5. Photoperiodic control timing of flowering

A long photoperiod (> 16 h of light per day) is necessary to maintain vegetative growth such as, leaf production and stem elongation while preventing flowering. After the plants reach a certain size, the photoperiod must switch from long to short (typically 12 h of

light per day) to initiate flowers and maintain their development (Chandra et al., 2017). This phase lasts 8-10 weeks until harvest. However, exposing the plants to longday (a 18-h photoperiod) during the last two weeks before harvest does not disturb yield and its quality (Chapter 5). Yet, the benefit of intentionally extending the photoperiod to increase yield is not substantial (Chapter 5). If necessary, a blackout curtain in greenhouse production can be left open during periods of long daylight hours. Surprisingly, there is a potential to apply 'longer' shortday, some cannabis cultivars were found to still flower under 13 h of light per day, resulting in substantially higher inflorescence yield compared to the standard 12-h photoperiod (Ahrens et al., 2024). Therefore, this photoperiod optimization to be slightly longer than 12 h can be attempted with specific cultivars.

7. Conclusions

- Cannabis can utilize relatively high light intensity and has a high photosynthetic capacity, leading to increased yield at high light intensity.
- The photomorphogenic response of cannabis to light spectrum is relatively limited; possibly this is not only due to species-specific traits but also to the high light intensity used in cannabis cultivation.
- Far-red light promotes the rooting of stem cuttings in some cases, but not always. Adding far-red only in the initial stage could be sufficient to improve rooting without stem elongation, serving as an alternative to synthetic auxin application.
- Photoperiod extension (i.e., longday) can be applied during the last phase of the generative phase without negative impact on yield and cannabinoids, regardless of light spectrum.
- Light intensity has a greater impact on specialized metabolites than light spectrum.

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Summary

Medicinal cannabis (*Cannabis sativa* L.) is a high-value crop containing a number of specialized metabolites with medical properties. Major compounds include cannabinoids and terpenoids, primarily accumulated in female inflorescences. Medicinal cannabis is often cultivated indoors as it allows precise control over growing conditions, ensuring high and consistent quality and yield. Artificial light in indoor cultivation can be customized regarding light spectrum, light intensity, and photoperiod, which play a critical role in plant growth and development and potentially alter the concentrations of specialized metabolites. Despite some research, the knowledge of the effects of light on cannabis remains limited. Moreover, light strategies used for cultivation are often kept confidential among growers, with limited scientific evidence available to support them. This thesis aims to understand the responses of medicinal cannabis to light during crucial processes at different developmental stages: rooting of stem cuttings, plant growth during the vegetative phase, flower induction, and development during the generative phase. These determine the production of inflorescence yield and levels of specialized metabolites. Additionally, the thesis aims to discuss the potential underlying physiological and morphological mechanisms of light responses. The roles of light on cannabis discovered in this thesis are expected to support the optimization of light strategies based on scientific evidence, enhancing indoor medicinal cannabis production.

Chapter 1 introduces the background knowledge of the cannabis plant and its specialized metabolites. The cultivation of medicinal cannabis is described as starting from rooting of stem cutting, vegetative, and generative phases. This chapter also reviews the role of light in plant growth and development based on previous cannabis research, and highlights what is known and unknown in cannabis. Lastly, this chapter outlines the scope of this thesis, which focuses on the impacts of light on the cannabis plant throughout its whole growing cycle.

The first step of cultivation is plant propagation. The most common propagation method of medicinal cannabis is stem cuttings, which produce large numbers of genetically identical plants. The light spectrum has the potential to determine the success of the rooting of stem cuttings. For instance, far-red light regulates the biosynthesis of auxin, a crucial hormone in the rooting process, while blue light plays a contrasting role. Chapter 2 investigated the influence of different fractions of far-red and blue light during the adventitious rooting phase and whether these effects are related to changes in

endogenous auxin and carbohydrates. Two separate experiments were conducted in climate chambers with sole LEDs lighting (blue, red, far-red) using two cannabis cultivars. One of two experiments showed a positive effect of far-red to improve rooting. It was observed that adding 50 µmol $m^{-2}s^{-1}$ of far-red to either red:blue (90 µmol $m^{-2}s^{-1}$) or sole red (45 µmol $m^{-2}s^{-1}$) background promoted the rooting of stem cuttings, compared to only red:blue light (90 µmol $m^{-2}s^{-1}$). In addition, when adding far-red had a positive effect on rooting, it was sufficient to apply this only during the initial stage of rooting (i.e., the first seven days), and in this case it did not result in excessive stem elongation. The presence or absence of blue did not significantly affect rooting. Although the positive effects of far-red on auxin and carbohydrate concentrations in stem cuttings are a likely explanation for the observed effects of far-red on rooting, we did not find a correlation between auxin or carbohydrates and rooting. Both cultivars responded similarly. However, the two experiments differed in growth conditions such as substrate, light intensity, and air temperature which could influence the effect of far-red.

After transplantation, the cannabis plant is first subjected to longdays favorable for vegetative growth and then induced into flowering by shortdays during the generative phase. Adjusting the light spectrum during these phases can influence plant morphology and potentially enhance biomass production and the accumulation of specialized metabolites. Chapter 3 aimed to investigate the influence of blue and far-red light on plant growth and the concentrations of terpenoids and cannabinoids in inflorescences. Two separate experiments were conducted involving different fractions of blue light and the addition of far-red light. Blue light fractions ranging from 8% to 21% as a replacement for red light in white light at 690 µmol $m^{-2}s^{-1}$ PPFD during both the vegetative and generative phases had no significant impact on plant morphology and dry matter production. Adding 200 µmol $m^{-2}s^{-1}$ of far-red light to 800 µmol $m^{-2}s^{-1}$ PPFD of white light during the final four weeks of the generative phase did not significantly alter plant morphology, dry matter production, or inflorescence size. Furthermore, neither the blue light fraction nor the additional far-red light affected the concentrations of terpenoids and cannabinoids. This chapter suggests that the effect of the light spectrum in cannabis is rather limited.

Next, the focus is on the amount of light given to the cannabis plant, specifically the daily light integral (DLI) during the generative phase. This phase is crucial as it is when the plant develops its inflorescences. Chapter 4 studied the effects of DLI varying the light intensity. Since this phase typically requires shortdays to induce flowering and maintain flower development, it is common in commercial cultivation to use extremely high light

intensity to achieve a higher DLI. This chapter aims to highlight the impact of light intensity ranging from 600 to 1000 µmol $m^{-2}s^{-1}$ PPFD and the underlying components of the responses. The results indicate a linear increase in the concentration of both cannabinoids and terpenoids with increasing light intensity. A 1% increase in light intensity resulted in a 1% increase in inflorescence yield with a constant light use efficiency (LUE) of inflorescences. The higher inflorescence yield was attributed to an increase in total plant dry matter, driven by a high photosynthetic rate. Additionally, the photosynthesis light response curve showed that the rate was not saturated even at 3000 µmol $m^{-2}s^{-1}$ PPFD. This suggests that cannabis has a high photosynthetic capacity and can utilize high light intensities effectively.

The other approach to increasing DLI is extending the photoperiod (i.e., longer hours of light). This allows plants to receive more light without increasing the already high light intensity used in cannabis cultivation, without additional lamp installation. Despite cannabis being classified as a short-day plant, an extended photoperiod might disrupt flowering. Extended photoperiods with blue light have been successful in maintaining flowering in short-day Chrysanthemum; this approach has proven to be ineffective for cannabis. In Chapter 5, we explored alternative approaches to extend the photoperiod from 12 h (shortday) to 18 h (longday), thus after flower induction under shortday. When the photoperiod was extended by 250 µmol $m^{-2}s^{-1}$ of blue light, even after the flowers were induced, the plants returned to vegetative growth. Interestingly, the plants exposed to the extended photoperiod by blue for the last two weeks, after six weeks of shortday, had comparable growth and development to the plants under shortday. Subsequently, the photoperiod was extended for the last two weeks by 250 µmol $m^{-2}s^{-1}$ of various spectra: red, blue, or white light. There was no difference among light spectra. The increase in light from the extended photoperiod treatment may have been limited by the low light intensity. Therefore, in the last experiments, the photoperiod was extended for the last two weeks with a constant light intensity of either 600 or 800 µmol $m^{-2}s^{-1}$ of white light. Inflorescence yield increased with the extended photoperiod for the last 2 weeks, although a significant increase was found only at 600 µmol $m^{-2}s^{-1}$, while concentrations of cannabinoids in inflorescence were not influenced. These findings suggest extending the photoperiod only during the last two weeks before harvest and with a substantial light increase in inflorescence yield without a negative effect on cannabinoids.

In Chapter 6, a general discussion is presented, summarizing the findings on the effects of light in this thesis and linking them with other studies on cannabis and other plant

species. The discussion revisits these findings and explores potential underlying mechanisms regarding plant responses to light. The inconsistent effects of light on cannabis, as have been reported, were discussed along with potential reasons. Further research is proposed to minimize the knowledge gap regarding cannabis responses to light. These aim to gain more insights into how light influences cannabis in order to improve the cultivation of not only medicinal cannabis but also other crops. Lastly, suggestions derived from the findings in this thesis for using light in cannabis cultivation are presented.

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About the authors

Wannida Sae-Tang was born on June 2, 1993, in Bangkok, Thailand. In 2014, she graduated with a Bachelor's degree in Science (Botany) from Kasetsart University, Thailand. Her passion for plants and sciences, along with her curiosity about the world, inspired her to pursue further studies in agriculture abroad. From 2015 to 2017, Wannida earned her Master's degree in Agricultural Science from Kyoto University, Japan. She then returned to Thailand, working as a research assistant in the physiology of ornamental plants laboratory at Kasetsart University for two years.

Thanks to a scholarship from the Thai government, Wannida moved to the Netherlands to pursue her PhD at Wageningen University and Research. After completing her PhD, she will return to Thailand and work as a researcher with the innovative herbal plant factory research team at the National Science and Technology Development Agency (NSTDA). To catch up with her new adventures, feel free to contact her at wannida.st@gmail.com.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 30 ECTS (= 20 weeks of activities)

Review/project proposal (4.5 ECTS)

- Physiological and morphological responses to light in medicinal cannabis: from rooting to flowering and specialized metabolites

Post-graduate courses (10.1 ECTS)

- 13th International metabolite workshop (2021)
- Common Data Standards and Integrative Analytic Methods for Plant Phenotyping (2022)
- Environmental Signaling in Plants (2022)
- Basic Statistics (2019)
- Design of Experiments (2020)
- Multivariate analysis (2022)
- Tidy data transformation and visualization with R (2023)

Competence, skills and career-oriented activities (4.2 ECTS)

- Efficient writing strategies (2020)
- Presenting with Impact (2020)
- Project and time management (2020)
- Critical thinking and argumentation (2022)
- Reviewing a Scientific Manuscript (2022)

Scientific Integrity/Ethics in science activities (0.3 ECTS)

- Ethics in Plant and Environmental Sciences (2020)

PE&RC Annual meetings, seminars and PE&RC weekend/retreat (1.8 ECTS)

- First Year weekend (2019)
- PE&RC Day (2019)
- Mid Term retreat (2022)

National scientific meetings, local seminars, and discussion groups (6 ECTS)

- FLOP: Frontier Literature on Plant Physiology (2020-2023)

International symposia, workshops and conferences (5.7 ECTS)

- IX International Symposium on Light in Horticulture (2020)
- XXXI International Horticulture Congress (2022)
- International Symposium on New Technologies for Sustainable Greenhouse Systems (2023)

Lecturing/supervision of practicals/tutorials (1.2 ECTS)

- Supervision of practical's in HPP-31806 Advanced Methods for Plant-Climate Research in Controlled Environments (2022)

BSc/MSc thesis supervision (6 ECTS)

- Research topic 1: The effect of blue light and light intensity on photomorphogenesis, growth, and cannabinoid content in medicinal cannabis
- Research topic 2: The effect of extended photoperiod during flowering phase on morphogenesis, floral yield, and chemical profile of medicinal cannabis
- Research topic 3: Effect of extended photoperiod and light spectrum on inflorescence development, floral yield' and cannabinoid concentration in *Cannabis sativa* L.
- Research topic 4: Influence of light spectrum of extended photoperiod on growth, floral yield, and cannabinoid content in medicinal cannabis
- Research topic 5: The effect of light intensity and far-red radiation on the morphology and floral yield of *Cannabis sativa* L.
- Research topic 6: Effect of extending last two weeks of generative shortday photoperiod on cannabinoid content in *Cannabis sativa* L.

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