Cannabinoids accumulation in hemp (*Cannabis sativa* L) plants under LED light spectra and their discrete role as a stress marker

Md. Jahirul Islam

Kangwon National University

Md Obyedul Kalam Azad

Kangwon National University

Byung Ryeol Ryu

Kangwon National University

Md Hafizur Rahman

Kangwon National University

Jung Dae Lim

Kangwon National University

Eun Ju Cheong

Kangwon National University

Young-Seok Lim (✉ potatoschool@kangwon.ac.kr)

Kangwon National University

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Abstract

Hemp adaptability through physiological and biochemical changes was studied under 10 LED light spectra and natural light in a controlled aeroponic system. Light treatments were imposed on 25 days aged seedlings for 16 hours daily ($300 \mu\text{mol m}^{-2}\text{s}^{-1}$) for 20 days. Plant accumulated highest Cannabidiol (CBD) in R7:B2:G1 light treatment, with relatively higher photosynthetic rate and lower reactive oxygen species, total phenol content, total flavonoid content, DPPH radical scavenging capacity, and antioxidant enzymatic activities. Tetrahydrocannabinol (THC) also accumulated higher in white, R8:B2, and R7:B2:G1 light with less evidence of stress modulated substances. These results indicated that CBD and THC have no or little relation with light-mediated abiotic stress in hemp plants. On the contrary, Tetrahydrocannabinolic acid (THCA) was accumulated higher in R6:B2:G1:FR1 and R5:B2:W2:FR1 light treatment along with lower photosynthetic rate and higher reactive oxygen species, total phenol content, total flavonoid content, DPPH radical scavenging capacity, and antioxidant enzymatic activities. However, Cannabidiolic acid (CBDA) was accumulated higher in R6:B2:G1:FR1 light treatment with higher stress modulated substances and lower physiological traits. CBDA was also accumulated higher in R8:B2 and R7:B2:G1 light treatments with less evidence of stress modulated substances. Besides, Greenlight influenced in CBD and CBDA synthesis where FR and UV-A (along with green) play a positive and negative role in this process, respectively. These results indicate that the role of THCA as a stress marker is more decisive in hemp plant than other cannabinoids under attributed light-mediated stress.

Introduction

Light is the primary source of energy for plant growth and development through photosynthesis. This growth and developmental process depend on light spectral quality, intensity, compositions, duration, and direction¹. A small irradiance of light can bring changes in several compositions in growing plants². These processes come through light interaction with species and cultivars, which mainly depends on its irradiation, which can enhance stressful or non-stressful events for plants³. Despite energy sources for photosynthesis, light can simultaneously act as a stress factor as plant response to light mainly depends on the lighting environment, genotypes, cultivation practices, etc.⁴. Excess light increases evaporation and photoinhibition, resulting in dehydration in leaf tissue, causing reduced photosynthetic production⁵.

Plants respond differently to each spectral band of light. Plants use the wavelength of red light to accumulate carbohydrates and nutrients^{6,7}, red and blue for electron excitation in photosynthesis^{1,8} and, blue and UV to get carotenoids and anthocyanins^{9,10}. Evidence showed that high intensities of UV-B radiation cause stress to plant by inducing DNA damage, photoinhibition, lipid peroxidation, and finally, growth retardation¹¹. When plants are exposed to such light intensities or any abiotic stress condition, the demand for metabolic processes in carbon fixation increases for energy supply and reduces power by involving photosystems and electron transport chains^{12,13}. This asymmetry generates reactive oxygen species (ROS), which have both signaling and toxic (oxidative damage by inducing lipid peroxidation) effect on cells^{1,14}. Light intensity below the compensation point also will result in a net loss of

photosynthetic products, and more light after saturation point has no or negative effect on photosynthesis^{7,15}.

Bioactive compounds are collectively known as primary and secondary metabolites, which gave aroma, color, taste even provide resistance against external biotic and abiotic stress¹⁶. Evidence proved that different forms of external stress help plants produce bioactive compounds^{17–22}. Previous studies suggested that the R:FR ratio, the blue and UV light photoreceptors (CRYs, PHOTs, and UVR8), can alter signaling pathways. These changes may affect phytohormone-mediated regulation of growth, development, physio-biochemical pathways, finally, plant root architecture^{23–25}, which may create partial water stress to plant.

It was reported that despite the negative effect on quantum yield, plants attained a higher photosynthetic rate and biomass accumulation under supplemental UV-A radiation. This higher photosynthetic rate was due to an increase of stomatal conductance (gs) instead of the ratio of intracellular to ambient CO₂ content (C_i/C_a)²⁶. Besides these, ultraviolet light has a crucial role in plant response to several morphological, physiological, and secondary metabolites production, which are combinedly termed as plant photomorphogenic response^{27–30}. These photomorphogenic responses mainly controlled by UVR8 by regulating gene expression relate to hypocotyl elongation inhibition, DNA repair, antioxidative defense, and phenolic compounds production³¹. On the other hand, far-red (710-850 nm) may have an essential role in photosynthetic purposes in leaves³². A high or low R and FR light ratio change the mode of action in phytochromes, converting the Pr into Pfr, or vice versa²⁰. This conversion may bring changes in gene expression related to photomorphogenesis^{33–35}. However, the role of UV-A and FR with the combination of other spectral bands relate to plant physiology and morphological changes remain poorly understood.

Living hemp plants contain cannabinoids as carboxylic acid like THCA and CBDA that decarboxylate during storage and heating transform to neutral cannabinoids such as THC and CBD^{36–39}. Although secondary metabolites in cannabis are mostly controlled by selecting genotypes and their phenotypic characteristics; however, some horticultural techniques, including photoperiod, lighting intensity, and quality, can change among them^{36,40–42}. Earlier in a study, the increment of THC in cannabis was described when it was treated under controlled UV-B radiation⁴⁰. Ning et al. showed that UV-A and UV-B could increase secondary metabolites in *Lonicera japonica* medicinal plants⁴³. For this reason, the plant treated with low dose UV mediated stress is crucial from a biotechnological and pharmaceutical point of view to increase valuable compounds⁴⁴. Previous studies revealed that under long-time UV treatment, all types of cannabinoids did not respond equally^{40,45}. It is also essential to find out the LED combination that can manipulate different targeted cannabinoids compounds by bringing in a metabolic system change in hemp plants. Besides, it is not clear which cannabinoids are directly involved with a light stress environment identified as stress markers in the hemp plant. Therefore, the objectives of the study were to determine the suitable LED combination for the higher accumulation of medicinal cannabinoids and select the stress markers of cannabis plants under light-mediated stress conditions.

Results And Discussions

Photosynthetic gas exchange

Photosynthetic parameters varied concerning different light treatments (**Figure 1**). In the current study, photosynthetic rate, transpiration rate, stomatal conductance and water use efficiency ranged from $0.397 - 6.23 \mu\text{mol m}^{-2} \text{s}^{-1}$, $0.587 - 3.942 \text{ mol m}^{-2} \text{s}^{-1}$, $0.023 - 0.1833 \text{ mol m}^{-2} \text{s}^{-1}$ and $0.101 - 4.647 \mu\text{mol mol m}^{-2} \text{s}^{-1}$, respectively. The higher photosynthetic rate was observed in L4, while the transpiration rate and stomatal conductance were higher in L8 and L2 light treatments. On the other hand, higher water use efficiency was recorded in L11 treatment.

Photosynthesis can be affected by the stomatal density, distribution, and opening status as it regulates diffusion of water vapor and the uptake of carbon dioxide in plants. Besides, many factors can influence stomatal behavior, including light, CO_2 concentration, and temperature⁴⁶. Some previous studies suggested that light intensity can enhance stomatal conductance in plants⁴⁷⁻⁴⁹. Simultaneously, the photosynthetic rate and stomatal conductance can be reduced under both lower and excessive light⁴⁹. It has also been known that the photosynthetic rate depends on chlorophyll content, and it can be affected by any change in it^{50,51}. Our study also gave a similar pattern of the result as under the treatments L1, L5, L6, L7, L8, and L9 plant attain lower chlorophyll content and lower photosynthetic rate. We also observe a similar pattern of results between photosynthesis and water use efficiency, and stomatal conductance and transpiration rate.

Both photosynthetic rate and water use efficiency were increased under all light treatments except L5 and L8. On the other hand, both transpiration and stomatal conductance significantly increased under all light spectra compare to natural light, except L10 and L11. Plant attained a higher photosynthetic rate and water use efficiency and lower transpiration rate and stomatal conductance under L3, L4, and L11. On the other hand, photosynthetic rate and water use efficiency and higher transpiration rate and stomatal conductance were recorded lower in L5 and L8 treatments.

Influence of LED on lipid peroxidation and hydrogen peroxide

Both malondialdehyde (MDA) and H_2O_2 level were considerably influenced by different light treatments (**Figure 2**). Higher MDA was recorded in L6, followed by L1, L5, and L8, while lower MDA was observed in L2, L3, and L4 treatments. On the other hand, plants accumulated higher H_2O_2 in L7, followed by L6, L5, L2, and L9, while lower H_2O_2 was observed in L8 and L11 treatments.

In the presence of light, chloroplasts and peroxisomes act as leading ROS producers in plants⁵². Thylakoids are the membrane-bound compartments inside chloroplasts that harbors the efficient light for light-dependent photosynthesis reactions by PS I and PS II^{53,54}. Light energy at the over-saturation point is responsible for photoinhibition by reducing the light-induced photochemical activity in PS II. These negative changes in the photosynthetic electron transport system are mainly responsible for the

generation of ROS^{12,13,55}. In these connections during overexcitation of chlorophyll, $^1\text{O}_2$ and $\text{O}_2^{\cdot-}$ produce from O_2 in PS II (during electron transfer to O_2 through Q_A and Q_B) and PS I (Mehler reaction), respectively^{56–58}. Peroxisomes can generate H_2O_2 by the activities of flavin oxidase, while $\text{O}_2^{\cdot-}$ and H_2O_2 may be generated in mitochondria of the cell by reduction of O_2 near the electric transport chain^{59–61}. In the present experiment, under the light treatments, L5, L6, L7, and L9 accumulated higher H_2O_2 with a lower photosynthetic rate indicating an active production of ROS resulting in photoinhibition and/or overexcitation of chlorophyll. To scavenge the excess ROS produced in the electron transport system plant uses various antioxidative defense mechanisms, including enzymatic and non-enzymatic scavenging procedures, which work synergistically and interactively with each other^{62,63}.

Lipids and proteins are the primary victims of oxidative damage by ROS accumulated in plant cells⁶⁴. Lipid peroxidation, considered as an indicator of determining the lipid damage extent, occurs in every organism by the oxidative decomposition of polyunsaturated lipids in the plasma membrane under severe condition^{65–67}. However, constant stress for plant generates redundant ROS that cannot be entirely homeostated by the scavenging system of the cell and exert some physiological actions like lipid peroxidation, nucleic acid oxidation, protein denaturation, enzyme activity inhibition and finally lead to programmed cell death^{57,64,68}. In the present study, under the light treatments L1, L5, L6, and L8 produced higher MDA along with lower photosynthetic rate and water use efficiency, indicating severe lipid damage in the plasma membrane of the plant cell.

Effect of LED spectra on antioxidant enzymes activities

From our study, the highest and lowest SOD activity was recorded in L7 and L5 treatments, respectively (**Figure 3**). However, a higher increment was observed from L7 (9.1%), followed by L11 (6.76%), L6 (6.58%), L3 (5.94%), and L9 (5.89%) respectively compared to natural light. Higher CAT was recorded in L11 followed by L5, L10, L3, L6 and L7 with 62.88%, 45.49%, 42.66%, 39.11%, 38.49% and 34.9% increment (compare to natural light), respectively. Higher APX was recorded in L6, followed by L9, L5, L3, and L11, with 81.12%, 30.77%, 27.97%, 12.59%, and 5.59% increment respectively with comparison to natural light. On the other hand, a higher reduction of APX activity was also observed in L4 (26.57%) and L8 (25.17%). However, higher activity of GPX was observed in L8, L10, L6, L11, L5, and L7 with 92.6%, 91.6%, 70.32%, 44.3%, 42.8%, and 39.4% increment, respectively compare to natural light.

ROS accumulated under stress conditions can act as signaling molecules and trigger a signal transduction pathway. It is also crucial that despite causing programmed cell death, ROS is inevitable to confer the resistance to stress. Notably, the activated response created by ROS should be rapid and decay as long as the stress disappeared⁶³. The main antioxidant enzymes that play a vital role in detoxifying the ROS are SOD, CAT, and APX. SOD converts $\text{O}_2^{\cdot-}$ to O_2 and H_2O_2 , while CAT, APX, and other peroxidase convert H_2O_2 to H_2O and O_2 ^{19,69}. In the present experiment in L6 and L7 light treatment, both H_2O_2 accumulation and SOD activity was higher, indicating an active mode of stress and plant response to mitigate the ROS compound. Under the light treatment, L1, L5, L6, L8, L10, and L11 plant accumulated

higher MDA indicated a secondary damage occurrence is running by lipid peroxidation in the plant cell. At the same time, higher activity of CAT in L3, L5, L6, L10, and L11, higher APX activity from L3, L5, L6 and L9, higher GPX activity from L5, L6, L8 and L10 light spectra were recorded. On the other hand, activity of SOD was found lower in L1, L5, L8 and L10 treatments. Earlier a decreased amount of SOD and increased APX activity with the increasing of MDA accumulation under drought stress was reported⁷⁰. These results indicate that lipid peroxidation may be activated with the lower activity of SOD and higher activity of peroxidases. Generally, elevated oxidative stress stimulates the production of H₂O₂ and provokes the increase of antioxidant enzyme activities, which help minimize the negative effect of abiotic stress⁷¹. Findings from a previous study stated that a higher irradiance of far-red and red light treatment plants produces higher MDA than that of lower irradiance²⁰. Higher MDA from L5, L6, L8, L10, and L11 compare to other LED spectra in the present study may be due to the presence of far-red light in those spectra.

Effect of LED spectra on antioxidant activities

Total polyphenol (TPC) and total flavonoid (TFC) varied with the spectral variation (**Figure 4**). Higher TPC was recorded in L6, while both TFC and DPPH free radical scavenging activity (%) was recorded higher in L7 treatment. Results also showed that both TPC and TFC increased under L2, L6, L7, L8, L9, and L10 treatments compare to natural light, while DPPH free radical scavenging activity (%) increased under L2, L6, L7, L8, and L9 treatments.

Generally, the cytokinin level increased by a red light that can stimulate the synthesis of phenolics compound, where far-red helps increase the plants' antioxidant capacity^{72,73}. A previous study of both phenolic compound and antioxidant capacity decreased under a combination of red and blue compared to monochromatic red, blue, and natural light¹⁸. The intensity of red light and its ratio with other light sources may contribute to secondary metabolites production. Further, secondary metabolites and antioxidant capacities can vary with the light intensities and ratio of monochromatic light sources^{74–76}. In our study, both TPC and TFC decreased at $\geq 70\%$ and increased at 50-60%, while it turned to dropped at $< 40\%$ red light sources compare to natural light. Supplementary UV radiation can increase flavanols and other secondary metabolites that act as a stress response to protect plants from radiation^{17,77}. In our study, UV A radiation was observed prominent with a 60% red light source. Artificial blue LED and far-red light enhance secondary metabolites, and the nutritional quality of crops, including ascorbate, total phenolic compounds, total flavonoid contents, and antioxidant activity, have been reported^{76,78}. We also found an increment of secondary metabolites with the addition of FR light, but the effect of FR light was found prominent with 50-60% red light sources. A previous study stated an increasing intensity of red to blue increased plant flavonoid, which was found best at 7:3 ratio⁷⁵. Our research also produces higher flavonoids at L6, L7, L8, and L9 treatments with similar red and blue ratios.

Effect of LED spectra on THC, THCA, CBD, and CBDA

Significant ($p < 0.05$) variations in the Tetrahydrocannabinol (THC), Tetrahydrocannabinolic acid (THCA), Cannabidiol (CBD), and Cannabidiolic acid (CBDA) were observed under different LED spectra (**Figure 5**).

Plant accumulated higher CBD in L4, L5, and L8 while higher THC in all light spectra compare to natural light. Notably, CBD and THC showing a positive relationship in L4, L5, and L8 spectra where both CBD and THC increased significantly. On the other hand, an opposite relationship was observed in L2, L3, L7, L9, L10, and L11 spectra where THC and CBD showed an increasing and decreasing trend, respectively. Higher CBDA was accumulated under all spectra except L7, and higher THCA was accumulated under all spectra except L10 compared to natural light. Interestingly L7 produced quite an antagonistic relationship while others produced an almost positive relationship between THCA and CBDA accumulation.

In general, CBGA produces by alkylation of two precursors olivetolic acid and geranyl pyrophosphate with the help of geranyl pyrophosphate:olivetolate geranyl transferase^{79,80}, which further can convert to THCA by THCA synthase^{81,82} and CBDA by CBDA synthase⁸³ in the oxidation process. In this connection, during oxidation of CBGA, it produces hydrogen peroxide and THCA in THCA synthase reaction⁸⁴, which may play a role in the self-defense of *Cannabis* plants⁸². Light quality may play an essential role in cannabinoid synthesis as light intensity influences cannabis yields strongly^{40,85}. We observed both higher THCA and H₂O₂ accumulation in L6 and L7 spectra in the present study, but we did not find any clear relation between THC and H₂O₂ from this observation. THCA also showed a positive relationship with antioxidant activities and antioxidant enzymes in L6, L7, L8, and L9 treatments.

On the other hand, it showed a negative relationship with the photosynthetic rate in the above four treatments. In the present study, except L11, THC accumulation was most prominent in L2 (white), L3 (R₈B₂), and L4 (R₇B₂G₁) spectra, where we can assume very little influence of FR and UV A light on THC accumulation in cannabis plants. It was also reported that Cannabis plants were grown under blue, and synergy between UV-A and blue light improved cannabinoid and cannabigerol accumulation, respectively⁸⁶. We also found higher THCA, CBDA, and THC concentrations under UV-A mediated spectral combinations.

On the other hand, CBD and CBDA accumulated higher in L4, L5, L6, and L8. From these results, we can see that green light has a significant role in CBDA synthesis and its conversion to CBD. Notably, FR light also influences CBDA and CBD accumulation along with green light, where white and UV-A play a negative role in this process. In some previous studies, the role of green light was shown negative for THC accumulation^{7,87}, but its role in CBD and CBDA synthesis was not clear. Despite having some shreds of evidence in the previous studies^{45,88,89}, the complex functions of cannabinoids relate to the defensive role toward biotic and abiotic stresses are not clear. Among the cannabinoids, THC and CBD were most discussed for having their antioxidant properties⁹⁰. Earlier increasing of THC, THCA, CBD, and CBDA were predicted as stress indicators along with some other secondary metabolites in hemp plant under controlled drought stress⁸⁹. It was also reported that THCA induces necrotic cell death in Cannabis cells and leaves⁹¹. The increasing cannabinoids in the present study also indicated a stress response of the cannabis plant under some controlled LED light spectral treatments.

Hierarchical clustering and heatmap unveiled the connections between variables and treatments

The values of all physiological and biochemical parameters of different light treatments were employed to construct the hierarchical clustering and a heatmap (**Figure 6**). Hierarchical clustering grouped all the variables into two clusters (cluster-A and cluster-B). Hierarchical clustering and heatmap revealed that all the parameters characterized cluster-A relate to abiotic stress, such as MDA, H₂O₂, SOD, CAT, APX, GPX, TPC, TFC, DPPH, and THCA. All the cluster-A variables showed minimal values at the natural light, white, R8B2, and R7B2G1, which indicated low comparative stress for hemp, whereas R₆:B₂:G₁:FR₁ and R₅:B₂:W₂:FR₁ treatments increased this trend. On the other hand, cluster-B represents all photosynthetic attributes (P_n, E, g_s, and WUE) and cannabinoids like CBD, CBDA, and THC. All cluster-B variables showed maximum values at R₇:B₂:G₁ followed by R₅:B₂:G₁:FR₁:UV₁, W, and R₈:B₂. This result is indicating that CBD and THC have a negligible relationship with stress-producing compounds. On the other hand, CBDA has a small extent of the relationship with stress compounds as it increased a little at stress-producing light like R₆:B₂:G₁:FR₁. Interestingly, the treatment RBG exhibited minimum and maximum values of almost all cluster-A and cluster-B parameters, respectively.

Conclusions

Higher CBD was accumulated in L4 (R₇:B₂:G₁), L5 (R₇:B₂:FR₁), and L8 (R₅:B₂:G₁:FR₁:UV₁), while higher THC in all light spectra compare to natural light. On the other hand, higher CBDA synthesis was recorded in L3 (R₈:B₂), L4 (R₇:B₂:G₁), L6 (R₆:B₂:G₁:FR₁), and L8 (R₅:B₂:G₁:FR₁:UV₁) treatments. The treatment L4 (R₇:B₂:G₁) produced all cannabinoids (CBD, CBDA, THC, and THCA) in higher concentration with lower stress response compounds like reactive oxygen species, antioxidants, THCA, and enzymatic activities. Besides this, the treatments L6 (R₆:B₂:G₁:FR₁) showed a lower CBD and THC and higher THCA and CBDA accumulation with higher activities of all other stress response compounds. On the other hand, L7 (R₅:B₂:W₂:FR₁) produced lower CBD, THC, and CBDA with a higher accumulation of all other stress-responsive compounds, including THCA. Besides, Greenlight has a significant role in CBD and CBDA synthesis where FR and UV-A (along with green) play a positive and negative role in this process, respectively. From our result, THCA showed a significant role as a stress marker followed by CBDA. On the other hand, THC and CBD showed a negligible response as stress response compounds to such conditions.

Material And Methods

Experimental design and treatment

Hemp seeds (*Cannabis sativa* cultivar India) were sown in sixty cells plug tray filled with commercial soil mixture (Bio-soil No. 1, Heungnong Agricultural Materials Mart, Korea) in a glasshouse. Before sowing, the seeds were sterilized [70% (v/v) ethanol, 0.1% (w/v) HgCl₂ and 0.2% (w/v) thiram] and soaked in water for 24 h at room temperature to facilitate the germination. The environmental conditions such as temperature, relative humidity (RH), and photoperiod were recorded at 30/25 °C (day/night), 60-70 %, and 12 h, respectively. The seedlings were irrigated daily using tap water to the field capacity level. After three

weeks, the seedlings were transferred to the steel made chamber (80 cm × 60 cm × 80 cm) equipped with different LED light (Bisol LED light Co. Korea) combination (**Figure 7; Table 1**). The photosynthetic photon flux density (PPFD), photoperiod, and temperature of the chamber were 300 $\mu\text{mol m}^{-2}\text{s}^{-1}$, 16h (6.00 AM to 10.00 PM), and 23 to 27 °C, respectively. The plant chamber was designed for an aeroponic system where nutrient formulated water (**Table 2**) was sprayed to the plant root zone for twenty seconds every two minutes. After 20 days, the youngest completely formed leaves were collected as samples from each treated plant subjected to further analysis.

Table 1: LED light composition

Spectrum combinations	Ratio (%)	Intensity ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	Code name
Natural light	-	-	L1
White	100	300	L2
R+B	80:20	300	L3
R+B+G	70:20:10	300	L4
R+B+FR	70:20:10	300	L5
R+B+G+FR	60:20:10:10	300	L6
R+B+W+FR	50:20:20:10	300	L7
R+B+G+FR+UV	50:20:10:10:10	300	L8
R+B+FR+UV	60:20:10:10	300	L9
R+B+W+FR+UV	40:20:20:10:10	300	L10
R+B+G+W+FR+UV	20:20:20:20:10:10	300	L11

Table 2: Nutrient solution

	A Tank(50L)*	B Tank(50L)
Ca(NO ₃)	1.5 kg	
KNO ₃	3.79 kg	3.79 kg
(NH ₄) ₂ HPO ₄		1.6 kg
MgSO ₄		4.3 kg
K ₂ SO ₄		
Fe-EDTA	460 g	
MnSO ₄		30.8g
H ₃ BO ₃		57.2 g
ZnSO ₄		3.6 g
CuSO ₄		1.3 g
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O		0.4 g

*Solution of Tank A and Tank B were subjected to mixed to maintain a E.C. range between 1.2-1.7 (dSm⁻²)

Leaf gas exchange measurement

The net photosynthetic rate (A, $\mu\text{mol m}^{-2} \text{s}^{-1}$), transpiration rate (E, $\text{mmol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$) were measured on well-developed leaves (3rd node from the top) of six plants under each treatment using an ADC BioScientific LCpro gas analyzer. The level of A, g_s , E, and WUE was measured at the ambient environmental condition. The measurements of gas exchange were carried out at the mid-day between 10 AM and 3.00 PM. The photosynthetic water use efficiency (WUE) was calculated as the ratio A/E.

Measuring malondialdehyde and H₂O₂ concentration

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in the leaves of hemp. MDA was measured by following the method of Heath and Packer⁹² with slight modification. The freeze-dried leaf sample (25 mg) was macerated in 0.1% trichloroacetic acid (5 mL). The homogenate was centrifuged at 10000×g for 10 min at 4 °C and stored at 4 °C for analysis. After that, 4 mL of TCA (20%) containing thiobarbituric acid (0.5%) was added to 1 mL aliquot of supernatant in a test tube. The test tube was placed in a water bath and warmed at 95°C for 30 min, followed by cooled quickly on an ice bath. The resulting mixture was centrifuged again at 5000 rpm for 15 min, and the absorbance was taken

at 532 nm. For avoiding unspecific turbidity, absorbance at 600 nm was subtracted from 532 nm, and an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to calculate the concentration.

The estimation of H_2O_2 was done, according to Singh et al.⁹³. Freeze-dried leaves (25 mg) were extracted in an ice bath with 5 mL of 0.1% (w/v) TCA and centrifuged at $12,000 \times g$ for 15 min. For every 0.5 mL of the aliquot of the supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI was added, followed by incubation in darkness for 1 h. The absorbance was measured at 390 nm, where 0.1% TCA was used as blank prove in place of leaf extract. A standard H_2O_2 curve was prepared to calculate the concentration of H_2O_2 in the sample.

Activities of antioxidant enzymes

For the analysis of antioxidant enzymes, leaves sample were collected and immersed immediately in liquid nitrogen and stored at -80°C until use. A 200 mg sample was homogenized in 5 mL of 50 mM sodium phosphate buffer solution (pH 7.8) using a pre-chilled mortar and pestle, then centrifuged at $15000 \times g$ for 20 minutes at 4°C . After collecting supernatant, the enzyme extract was stored at 4°C for analysis.

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was estimated by the method described by Islam et. al.⁹⁴. A reaction mixture was prepared containing 50 mM sodium phosphate buffer with 0.1 mM EDTA, 12 mM methionine, 75 μM NBT, 50 mM Na_2CO_3 . Then, a 100 μL enzyme extract or 100 μL buffer was used in the sample or blank, respectively. After that, 300 μL of 0.1 mM Riboflavin was added to the reaction mixture to make 2 mL of the final volume. The tubes were shaken and irradiated under the fluorescent light (15 W) for 15 minutes. The absorbance of each solution was measured at 560 nm by a spectrophotometer. One unit of SOD represented as the quantity of enzyme that caused 50% inhibition of NBT reduction under the experimental conditions⁹⁵.

The Guaiacol peroxidase (POD; EC 1.11.1.7) and catalase (CAT; EC 1.11.1.6) activities were determined according to Zhang⁹⁶. The 3 mL reaction mixture for POD consisted of 100 μL enzyme extract, 100 μL guaiacol (1.5%, v/v), 100 μL H_2O_2 (300 mM), and 2.7 mL 25 mM sodium phosphate buffer with 2 mM EDTA (pH 7.0). The increased rate of absorbance was measured spectrophotometrically at 470 nm ($\epsilon = 26.6 \text{ mM cm}^{-1}$). The assay mixture for CAT contained 100 μL of enzyme extract, 100 μL of H_2O_2 (300 mM), and 2.8 mL of 50 mM phosphate buffer with 2 mM EDTA (pH 7.0). The decreased rate of absorbance was measured spectrophotometrically at 240 nm ($\epsilon = 39.4 \text{ mM cm}^{-1}$).

The activity of Ascorbate peroxidase (APX; EC 1.11.1.11) was assayed by the method developed by Nakano and Asada⁹⁷. A 3 mL reaction mixture consisted of 25 μL enzyme extract, 100 μL ascorbate (7.5 mM), 100 μL H_2O_2 (300 mM), and 2.775 mL of 25 mM sodium phosphate buffer with 2 mM EDTA (pH 7.0). The oxidation of ascorbate was determined by the decrease of absorbance at 290 nm ($\epsilon = 2.8 \text{ mM cm}^{-1}$).

Determination of Total phenolic content, total flavonoid content, and antioxidant capacity

The freeze-dried (25 mg) sample was dissolved in 10 mL of ethanol (80%, v/v in water) followed by sonication at 35 °C for 60 min. Afterward, the extracts were filtered (Advantech 5B filter paper, Tokoyo Roshi Kaisha Ltd., Saitama, Japan) and kept in a refrigerator (4 °C for further analysis).

The Folin-Ciocalteu method was performed to estimate the total phenolic content (TPC) of the sample⁹⁸. A reaction mixture contained 1 mL of sample, 200 µL of phenol reagent (1N), and 1.8 mL of distilled water. The mixture was vortexed, and 400 µL of Na₂CO₃ (10 %, v/v in water) was added after 3 min. After that, 600 µL of distilled water was added to get the final volume (4 mL) and left for 1 h incubation at room temperature. The absorbance was taken at 725 nm, and the phenolic acid was calculated from a standard calibration curve of Gallic acid and expressed as µg g⁻¹ dry weight.

The total flavonoid content (TFC) was estimated following the method described by Ghimeray et al.⁹⁹. In brief, 500 µL of the extract was mixed with 100 µL of Al(NO₃)₃ (10%, w/v) and 100 µL of potassium acetate (1 M) solution, and finally, 3.3 mL of distilled water was added to adjust the volume up to 4 mL. The reaction mixture was vortexed and left at room temperature for incubation for 40 min, and the absorbance was measured at 415 nm by a UV-Vis spectrophotometer. The total flavonoid was calculated as mg/g of Quercetin equivalent on a dry weight basis.

DPPH (2,2-diphenyl-1 picryl hydrazyl) was used to assess the antioxidant capacity of cannabis leaf extract following the method described by Braca et al.¹⁰⁰. Firstly, DPPH powder (5.914 mg) was dissolved in methanol (100 mL) to prepare a stock solution, and the absorbance range was maintained between 1.1 and 1.3 by a spectrophotometer. After that, 1 mL of extract was mixed with 3 mL of DPPH solution followed by shaking vigorously and kept in the dark room for 30 min at room temperature. The blank sample was produced by mixing distilled water (1 mL) with DPPH solution instead of extract. The absorbance was taken at 517 nm by a UV-Vis spectrophotometer (UV-180 240 V, Shimadzu Corporation, Kyoto, Japan). The scavenging capacity of the samples was calculated by using the following formula, and results were expressed as a percentage (%):

$$\text{Inhibition (\%)} = [(\text{blank sample} - \text{extract sample}) / \text{blank sample}] \times 100$$

Determination of Tetrahydrocannabinol (THC), Tetrahydrocannabinolic acid (THCA), Cannabidiol (CBD), and Cannabidiolic acid (CBDA)

The freeze-dried (100 mg) sample was dissolved in 5 mL of methanol (100%) followed by sonication for 20 mins at room temperature. The solution was filtered through a syringe filter (0.45 µm, Millipore, Bedford, MA, USA) and kept in a refrigerator (4 °C for further analysis). The HPLC system (Shimadzu LC-20AT) with a UV-VIS detector and a reverse phase Zorbax SB-C₁₈ column (4.6 mm × 100 mm, 3.5 µm) was used. The mobile phase was 70% acetonitrile containing 0.1% phosphoric acid with isocratic elution

mode. The sample (10 µl) was injected with a flow rate of 1.5 ml min⁻¹ where the oven temperature was 27 °C, and the detection wavelength was 275 nm.

Statistical Analysis

All results were expressed as mean ± SE (standard error), and the one-way analysis of variance was performed using Statistix 10 (Tallahassee, FL 32312, USA). Different letters indicate the statistically significant differences between treatments at $p < 0.05$, according to the least significant differences (LSD). The heatmap and clustering analysis were prepared from normalized mean values using the MetaboAnalyst 4.0 (www.metaboanalyst.ca)¹⁰¹.

Declarations

Author Contributions: M.J.I. conceived and designed the experiments, carried out all the experimental works, collected, analyzed the data, and drafted the final manuscript. B.R.R. and M.H.R. contributed to collecting data and preparing experimental works. J.D.L., M.O.K.A., and E.J.C. improved the manuscript. Y.-S.L. validated all the protocols and supervised the study. All authors read and approved the final manuscript.

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Figures

Figure 1

Effect of 20 days exposure to different LED spectra on photosynthetic rate (A), Transpiration rate (B), Stomatal conductance (C) and Water use efficiency (D) of hemp seedlings. Here and subsequent figures: L1, Natural light; L2, White; L3, R8:B2; L4, R7:B2:G1; L5, R7:B2:FR1; L6, R6:B2:G1:FR1; L7, R5:B2:W2:FR1; L8, R5:B2:G1:FR1:UV1; L9, R6:B2:FR1:UV1; L10, R4:B2:W2:FR1:UV1; L11, R2:B2:G2:W2:FR1:UV1. All treatments used a photosynthetic photon flux density of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$. Column height indicates mean, vertical bars indicate standard error means ($n = 4$), and different letters indicate significant differences at $p < 0.05$ based on Duncan's multiple range test.

Figure 2

Effect of 20 days exposure to different LED spectra on Lipid peroxidation (MDA) (A) and H₂O₂ (B) of hemp seedlings. Here and subsequent figures: L1, Natural light; L2, White; L3, R8:B2; L4, R7:B2:G1; L5, R7:B2:FR1; L6, R6:B2:G1:FR1; L7, R5:B2:W2:FR1; L8, R5:B2:G1:FR1:UV1; L9, R6:B2:FR1:UV1; L10, R4:B2:W2:FR1:UV1; L11, R2:B2:G2:W2:FR1:UV1. All treatments used a photosynthetic photon flux density of 300 μmol m⁻² s⁻¹. Column height indicates mean, vertical bars indicate standard error means (n = 4), and different letters indicate significant differences at p < 0.05 based on Duncan's multiple range test.

Figure 3

Effect of 20 days exposure to different LED spectra on Superoxide dismutase (SOD) (A), Catalase (CAT) (B), Ascorbate peroxidase (APX) (C) and Guaiacol peroxidase (GPX) (D) of hemp seedlings. Here and subsequent figures: L1, Natural light; L2, White; L3, R8:B2; L4, R7:B2:G1; L5, R7:B2:FR1; L6, R6:B2:G1:FR1; L7, R5:B2:W2:FR1; L8, R5:B2:G1:FR1:UV1; L9, R6:B2:FR1:UV1; L10, R4:B2:W2:FR1:UV1; L11, R2:B2:G2:W2:FR1:UV1. All treatments used a photosynthetic photon flux density of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Column height indicates mean, vertical bars indicate standard error means ($n = 4$), and different letters indicate significant differences at $p < 0.05$ based on Duncan's multiple range test.

Figure 4

Effect of 20 days exposure to different LED spectra on Total polyphenol (TPC) (A), Total flavonoid (TFC) (B), and DPPH radical scavenging activity (C) of hemp seedlings. Here and subsequent figures: L1, Natural light; L2, White; L3, R8:B2; L4, R7:B2:G1; L5, R7:B2:FR1; L6, R6:B2:G1:FR1; L7, R5:B2:W2:FR1; L8, R5:B2:G1:FR1:UV1; L9, R6:B2:FR1:UV1; L10, R4:B2:W2:FR1:UV1; L11, R2:B2:G2:W2:FR1:UV1. All treatments used a photosynthetic photon flux density of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Column height indicates mean, vertical bars indicate standard error means ($n = 4$), and different letters indicate significant differences at $p < 0.05$ based on Duncan's multiple range test.

Figure 5

Effect of 20 days exposure to different LED spectra on Tetrahydrocannabinol (THC) (A), Tetrahydrocannabinolic acid (THCA) (B) Cannabidiol (CBD) (C), and Cannabidiolic acid (CBDA) (D) of hemp seedlings. Here and subsequent figures: L1, Natural light; L2, White; L3, R8:B2; L4, R7:B2:G1; L5, R7:B2:FR1; L6, R6:B2:G1:FR1; L7, R5:B2:W2:FR1; L8, R5:B2:G1:FR1:UV1; L9, R6:B2:FR1:UV1; L10, R4:B2:W2:FR1:UV1; L11, R2:B2:G2:W2:FR1:UV1. All treatments used a photosynthetic photon flux density of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Column height indicates mean, vertical bars indicate standard error means ($n = 3$), and different letters indicate significant differences at $p < 0.05$ based on Duncan's multiple range test.

Figure 6

Hierarchical clustering and heatmap analysis to reveal the treatment-variable association of hemp seedlings grown under different light treatments. At the variable level, two separate clusters (A and B) were recognized. The color scale shows the intensity of the normalized mean values of different parameters.

Figure 7

Hemp plant under treatment in steel made chamber (80 cm × 60 cm × 80 cm) equipped with different LED light. Here and subsequent models: L1, Natural light; L2, White; L3, R8:B2; L4, R7:B2:G1; L5, R7:B2:FR1; L6, R6:B2:G1:FR1; L7, R5:B2:W2:FR1; L8, R5:B2:G1:FR1:UV1; L9, R6:B2:FR1:UV1; L10, R4:B2:W2:FR1:UV1; L11, R2:B2:G2:W2:FR1:UV1. All treatments used a photosynthetic photon flux density of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$.