

Article

Hyperspectral Detection of Spectral Responses to Acute High-Irradiance Blue Light in Five Microgreen Species

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Abstract

High-intensity blue light is a powerful regulatory signal for plants, but its excess induces oxidative stress requiring rapid diagnosis. This study evaluated the potential for early detection of changes in the spectral characteristics of microgreen canopy cover caused by high-intensity blue light (PPFD 2500 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 12 h) in five species of microgreens (*Helianthus annuus*, *Pisum sativum*, *Eruca sativa*, *Hordeum vulgare*, *Raphanus sativus* ‘Sango Purple’) using hyperspectral imaging (450–950 nm) and machine learning. A Random Forest model trained on 85 vegetation indices classified light stress with high accuracy (Accuracy > 93%, Kappa > 0.87, F1-score > 93%) and detected spectral changes characteristic of light stress as early as 1–3 h of exposure. SHAP analysis identified carotenoid-sensitive indices (PRI, CCI, PRICI2) and chloroplast movement and stress indices (CMI, LSI_{Red} , LSI_{Norm} , Carter5) as the most informative predictors. It is assumed that the primary mechanism underlying early spectral changes was chloroplast avoidance rather than pigment degradation, as confirmed by the reversibility of canopy bleaching, rapid recovery of maximum quantum yield of photosystem II, and unchanged chlorophyll and carotenoid contents. Sunflower and radish were the most sensitive species to high-dose blue light, while barley was the least sensitive. LSI_{Red} was identified as a reliable qualitative marker of light stress that does not require a control sample, simplifying its use in automated monitoring systems. These findings demonstrate the effectiveness of hyperspectral phenotyping for non-invasive, rapid diagnosis of light stress in microgreens, providing a tool for optimising lighting regimes in controlled environment agriculture.

Keywords: diagnosis of light stress; chloroplast avoidance response; photoprotective mechanisms; vegetation indices; Random Forest



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1. Introduction

Light serves not only as a source of energy for photosynthesis, but also as a key regulatory signal that modulates a wide range of physiological and biochemical processes [1].

Within the photosynthetically active radiation range (400–700 nm), blue light (400–500 nm) plays a special role due to its activation of specialised photoreceptors—cryptochromes and phototropins—which trigger reactions that control photomorphogenesis, chloroplast movement, stomatal opening and stress responses [2,3]. The high energy of blue light and its intense absorption by chlorophyll in the upper layers of the leaf mesophyll mean that this spectral range influences the photosynthetic apparatus. For most

crops, the optimal proportion of blue light in the spectrum is 10–30 per cent of the total photosynthetically active radiation (PAR) at an intensity of $150\text{--}400\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ [4–7].

Exceeding these ranges induces the development of oxidative stress. This can occur in two scenarios: (1) when there is a high proportion of blue light (over 50%), even at moderate intensities, which is particularly pronounced in sensitive crops; (2) at high irradiance (above $800\text{--}1000\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), regardless of the spectral composition [6,8,9].

Excess blue light energy leads to an overload of the chloroplast electron transport chain and the generation of reactive oxygen species (ROS), such as the superoxide anion (O_2^-), singlet oxygen ($^1\text{O}_2$) and hydrogen peroxide (H_2O_2) [3].

During their evolution, plants have developed a range of mechanisms to counteract excessive light exposure. The absorption of light energy is limited by the movement of chloroplasts towards the anticlinal cell walls—a phenomenon known as the chloroplast avoidance response [10,11]. Such chloroplast movement induced by blue light can cause leaf bleaching, which is visible to the naked eye [12]. There is an increase in the synthesis of coloured phenolic compounds and their transport into the cytoplasm to reflect light [13,14]. Furthermore, photoprotection is achieved through fluorescence quenching, photochemical quenching and non-photochemical energy quenching (NPQ) [15,16]. The NPQ fast component, energy-dependent quenching (qE), is based on the interconversion of pigments in the xanthophyll cycle (violaxanthin $\leftrightarrow$ antheraxanthin $\leftrightarrow$ zeaxanthin), which ensures the safe dissipation of excess light energy in the form of heat [17–19]. Reactive oxygen species (ROS) are also neutralised via enzymatic and non-enzymatic mechanisms. During photorespiration, NADPH is consumed, which protects the photosynthetic electron transport chain [20]. When stress is excessively intense or prolonged, defence mechanisms become depleted, leading to the photoinactivation of photosystem II (PSII), damage to chloroplast membranes and cell death [21].

Chronic exposure to light at low, non-stressful intensities may lead to a process of acclimatisation—a state of long-term adaptation in which elevated levels of protective metabolites are accompanied by a normalisation of ROS levels and the maintenance of the functional activity of the photosynthetic apparatus [22]. The effect of acclimatisation to blue light has been observed, for example, following prolonged (12-day) exposure to $100\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in vanilla: the plants exhibited increased antioxidant enzyme activity, reduced accumulation of reactive oxygen species (ROS) and increased tolerance to subsequent light exposure [23].

The acclimatisation effect is of interest for microgreen production, as it enables the production of crops with a high content of beneficial compounds without any loss of yield or the risk of toxic oxidation products accumulating [24,25]. Microgreens, which are the young shoots of vegetables, herbs and certain other agricultural crops, are the subject of intensive research as a component of a healthy diet due to their high content of bioactive compounds [26,27]. The rapid growth rate and metabolic activity of microgreens make them sensitive to light conditions. The application of blue light under controlled conditions on farms and in greenhouses is regarded as a promising agronomic technique for managing the accumulation of valuable secondary metabolites, such as anthocyanins and flavonoids [3,24].

However, the fine line between a stimulatory effect and the induction of damaging stress calls for the development of reliable methods for the rapid and non-invasive diagnosis of light-induced stress.

The key laboratory methods for diagnosing stress in plants are:

- spectrophotometric determination of the activity of antioxidant enzymes—superoxide dismutase, catalase, ascorbate peroxidase and guaiacol peroxidase [28];

- assessment of the levels of lipid peroxidation products, primarily malondialdehyde, using thiobarbituric acid [29–31];
- quantification of the pool of ROS, including H_2O_2 and O_2^- , using spectrophotometric and histochemical methods [32,33].

In addition, High-Performance Liquid Chromatography and mass spectrometry are used to analyse metabolomic profiles, including glutathione, ascorbate and polyphenols, for a comprehensive assessment of oxidative stress [34].

Although these methods remain the standard for verifying the physiological condition of plants, their application is limited to laboratory conditions. They require qualified specialists, are labour-intensive and time-consuming, and result in the destruction or damage of the sample. Therefore, laboratory methods are of limited suitability for continuous monitoring in production or field conditions.

In contrast to laboratory methods, hyperspectral imaging (HSI) enables information on the condition of plants to be obtained rapidly, non-invasively and over a wide spatial range, whilst ensuring a high level of automation [35–37].

The combination of hyperspectral data with machine learning and deep learning techniques has emerged as a powerful tool for the early and highly accurate detection of various biotic and abiotic stresses in agricultural crops [38–40].

Carotenoid content (Car) and the ratio of chlorophyll to carotenoid content (Chl/Car) may serve as predictors of light stress in spectral phenotyping [41]. To date, a whole range of vegetation indices (VIs) has been developed to assess Car in plant leaves and canopies, as well as vegetation indices (Vis) to assess the Chl/Car ratio. It has been shown that the photochemical reflection index (PRI) is effective at identifying light stress by recording the diurnal and seasonal interconversions of xanthophyll cycle pigments associated with the plant's response to excess light energy [42,43]. A modified photochemical reflectance index ($\text{modPRI}_{515/550}$) has been developed for the diagnosis and assessment of combinations of light stress with other abiotic stresses [44]. The Chlorophyll/Carotenoid Index (CCI) has also proved to be an informative index for classifying winter light stress in conifers [45].

However, changes in the Car and Chl/Car values (typically an increase), which are reliably detected by spectral phenotyping methods, are also characteristic of other types of abiotic stress. Consequently, these indicators are not strictly specific markers of light stress. In this regard, the chloroplast avoidance response—the first rapid defence reaction to excess light—is of particular interest. By studying the chloroplast avoidance response in *Nicotiana benthamiana* and *Arabidopsis thaliana* under blue light, Hermanowicz & Łabuz [12] proposed the Chloroplast Movement Index (CMI), which is capable of distinguishing between chloroplasts in accumulation and avoidance positions. The light stress indices LSI_{Norm} and LSI_{Red} , developed for *Ficus elastica*, also require further testing [46].

Thus, despite significant progress in understanding the physiological mechanisms of light stress and in developing spectral methods for its diagnosis, the following questions remain unresolved: (1) What are the spectral markers of acute light stress, induced by high intensity of blue light, in various species of microgreens? (2) How informative are existing indices, including CCI, PRI, CMI, LSI_{Red} and LSI_{Norm} , for the early diagnosis of stress in microgreens? (3) What are the dynamics of spectral changes during the development of stress and recovery once it has ceased? Answers to these questions are essential for the development of rapid methods for diagnosing light stress and for optimising light regimes for microgreen cultivation.

The aim of this study is to assess, both qualitatively and quantitatively, the stress induced by high doses of blue light in microgreens from various agricultural crops, using hyperspectral imaging techniques.

To verify the hyperspectral data, the content of photosynthetic pigments (Chl and Car) is determined in parallel using a spectrophotometric method, and the maximum quantum yield of photosystem II (F_v/F_m)—a widely recognised indicator of photoinhibition and stress damage—is measured [47]. This is one of the first studies to use hyperspectral imaging to diagnose light stress caused by high-intensity blue light. The results obtained will enable the identification of correlations between hyperspectral signatures and physiological parameters, which could form the basis for developing precise methods for diagnosing light stress in microgreens and optimising light regimes in controlled plant cultivation technologies.

2. Results

2.1. Detection of Light Stress in Microgreens

Changes in the F_v/F_m ratio, measured before and after exposure to blue light, indicate the presence of light stress in various microgreen crops (Figure 1).

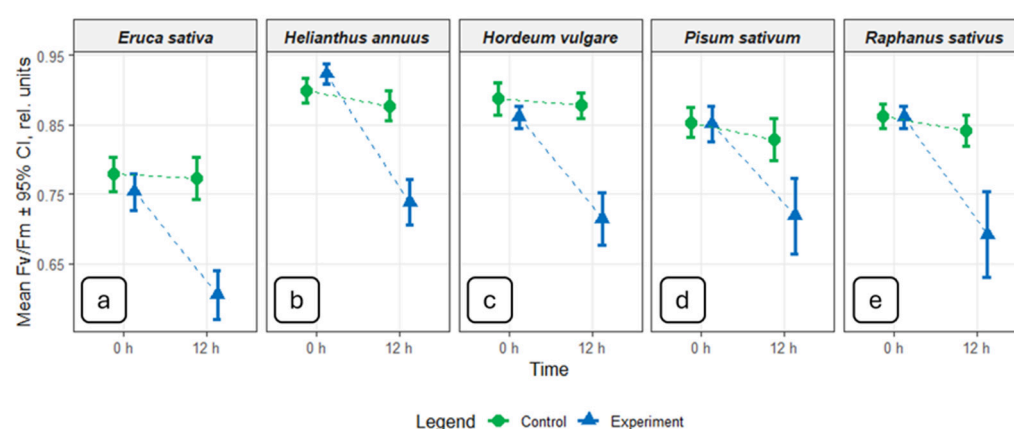


Figure 1. Mean F_v/F_m values with confidence intervals at $p < 0.05$ for the microgreen canopy of five crops before and after 12 h of exposure to blue light (photosynthetic photon flux density (PPFD) $2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). (a)—rocket/arugula (*Eruca sativa* Mill.), (b)—sunflower (*Helianthus annuus* L.), (c)—barley (*Hordeum vulgare* L.), (d)—pea (*Pisum sativum* L.), (e)—leafy red radish (*Raphanus sativus* L., ‘Sango Purple’).

After 12 h of exposure to blue light at PPFD $2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, the F_v/F_m value decreased significantly and consistently in all crops. The greatest decrease was observed in *Eruca sativa* (from 0.82 to 0.61, $\Delta = 0.21$), while the smallest was in *Hordeum vulgare* (from 0.81 to 0.71, $\Delta = 0.10$). This confirms the development of photoinhibition and justifies the use of the 13th HSI (12 h of exposure to high-intensity blue light) as a reference for the ‘stress’ class. Visually, the irradiation caused a clearly observable, reversible paling of the canopy (Figure 2), which may indicate chloroplast avoidance.

A Random Forest (RF) model was used to assess the spectral similarity of microgreen canopies at different time points during blue light exposure, compared with a reference stress condition. From the third hour of exposure to high-intensity blue light, the spectral signatures of the samples began to be classified as ‘stress’ with an accuracy of $>80\%$, a Kappa coefficient of >0.6 and an F1 score of $>75\%$, indicating their similarity to the reference stress state (Supplementary Table S1, Figure 3a).



Figure 2. Change in the colour of the microgreen canopy. (A)—before exposure to blue light whilst under a fluorescent lamp with a PPFD of $5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. (B)—after 12 h exposure to blue light at $2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. 1—rocket/arugula (*Eruca sativa*), 2—sunflower (*Helianthus annuus*), 3—barley (*Hordeum vulgare*), 4—pea (*Pisum sativum*), 5—leafy red radish (*Raphanus sativus* ‘Sango Purple’).

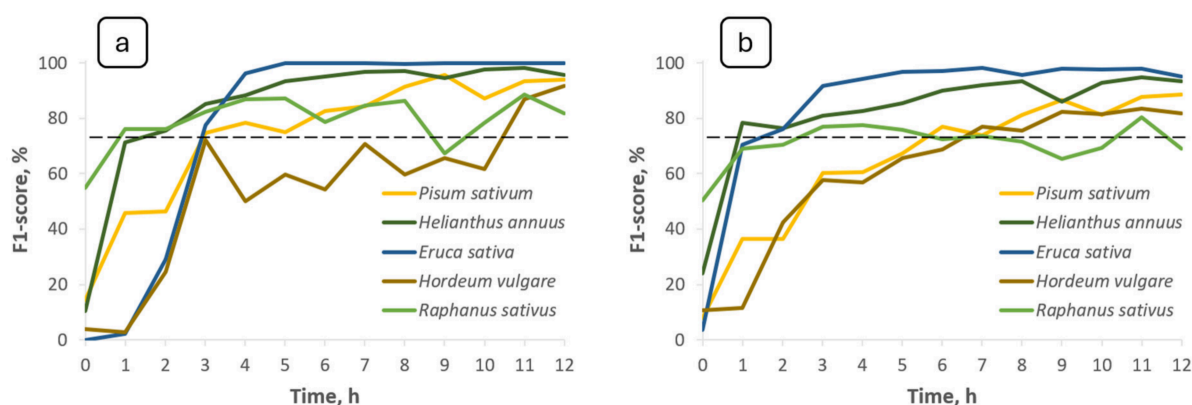


Figure 3. Changes in the F1 score for classifying microgreens as being in a ‘light-stress’ or ‘normal’ state using the RF model. The dotted line indicates an F1-score $\geq 75\%$ —the stress threshold. (a)—a ‘complex’ model, based on 85 VIs; (b)—a ‘light’ model based on 9 VIs.

This means that hyperspectral signatures reflect early changes in the canopy of microgreens. Sunflower and radish proved to be the most sensitive crops (significant spectral changes were detected after just two hours of irradiation), peas and rocket showed moderate sensitivity (significant spectral changes were detected after three hours of irradiation), whilst barley was the least sensitive or exhibited a more complex response to stress (Kappa reached the 0.60 threshold only at 10 h, although the Accuracy and F1 thresholds were reached earlier).

The test for the persistence of the ‘light stress’ state showed that 24 and 48 h after the cessation of irradiation, light stress was not identified in barley microgreens—Accuracy 50.55%, Kappa 0.01, F1-score 2.18% and Accuracy 54.10%, Kappa 0.08, F1-score 17.45% respectively (Figure 4).

This result further confirms the effectiveness of the trained RF model in predicting states.

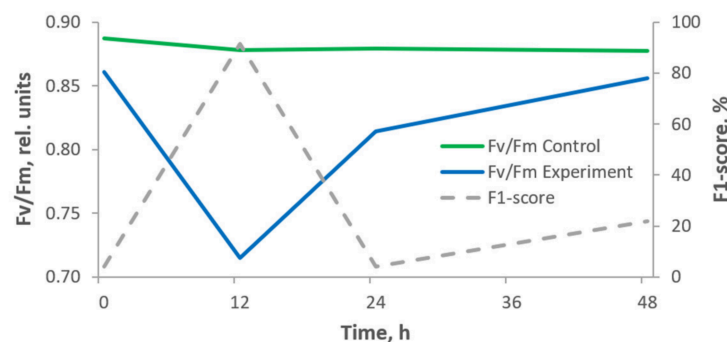


Figure 4. Results of the test to assess the persistence of the light-stress effect in a canopy of barley microgreens.

2.2. Identification of Significant Variables for the Detection of Light Stress

SHapley Additive exPlanations (SHAP) were applied to identify the VIs most relevant for RF-based classification of light stress. The SHAP summary plot (Figure 5) highlights the top 30 most informative VIs.

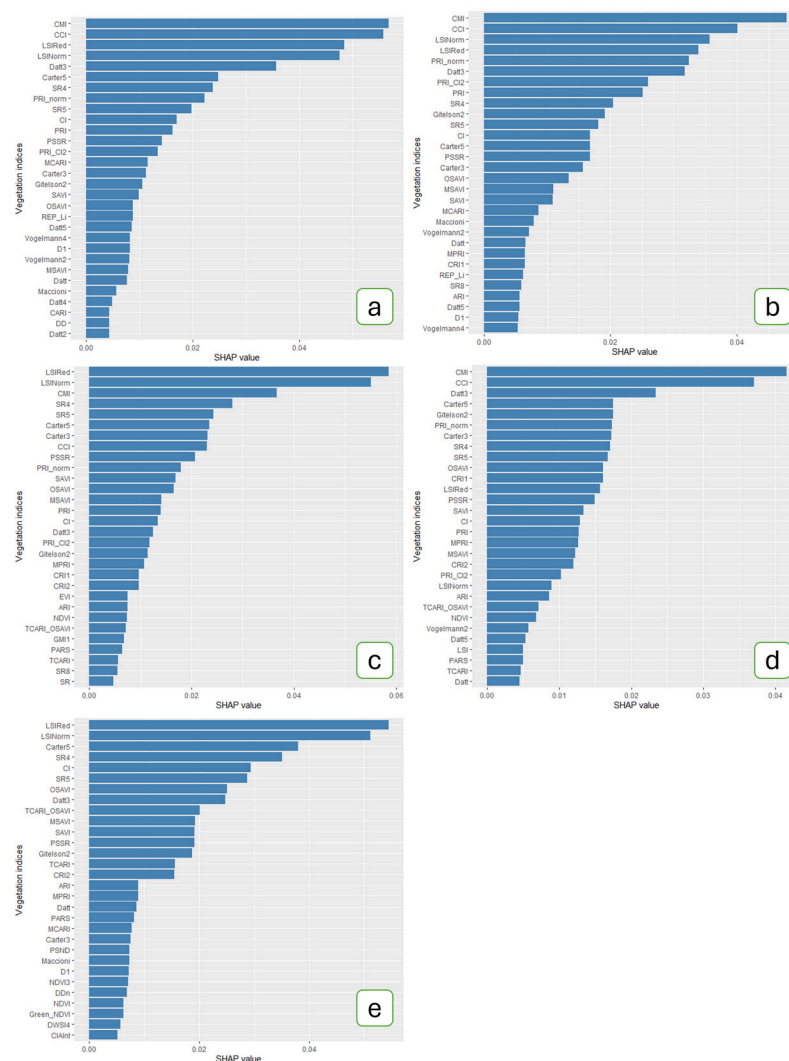


Figure 5. Results of the distribution of VI values according to their impact on the effectiveness of RF classification of the ‘normal’ and ‘light stress’ states in microgreens. (a)—pea (*Pisum sativum*), (b)—sunflower (*Helianthus annuus*), (c)—rocket/arugula (*Eruca sativa*), (d)—barley (*Hordeum vulgare*), (e)—leafy red radish (*Raphanus sativus*, ‘Sango Purple’).

For all microgreen crops, the most informative VIs were those sensitive to carotenoid content (Datt3, CCI, PRI, PRI*CI2, PRI_{norm}) and stress indices (CMI, LSI_{Red}, LSI_{Norm}, Carter5). Of these VIs CMI, LSI_{Red} and LSI_{Norm} were specifically developed to identify light stress.

A ‘light’ RF model was constructed, using the nine most informative features—as identified by SHAP—as independent variables. The model demonstrated high performance, comparable to that of the ‘complex’ model (Figure 3b, Supplementary Table S2). The reduction in the ‘light’ model’s Accuracy and F1-score values by filming time averaged just 3.2 per cent and 2.1 per cent, respectively. This provides further confirmation of the SHAP results regarding the assessment of feature informativeness.

2.3. Dynamics of Vegetation Indices in Microgreens Under High-Intensity Blue Light Exposure

The dynamics of informative VIs for RF classification are shown in Figures 6–10.

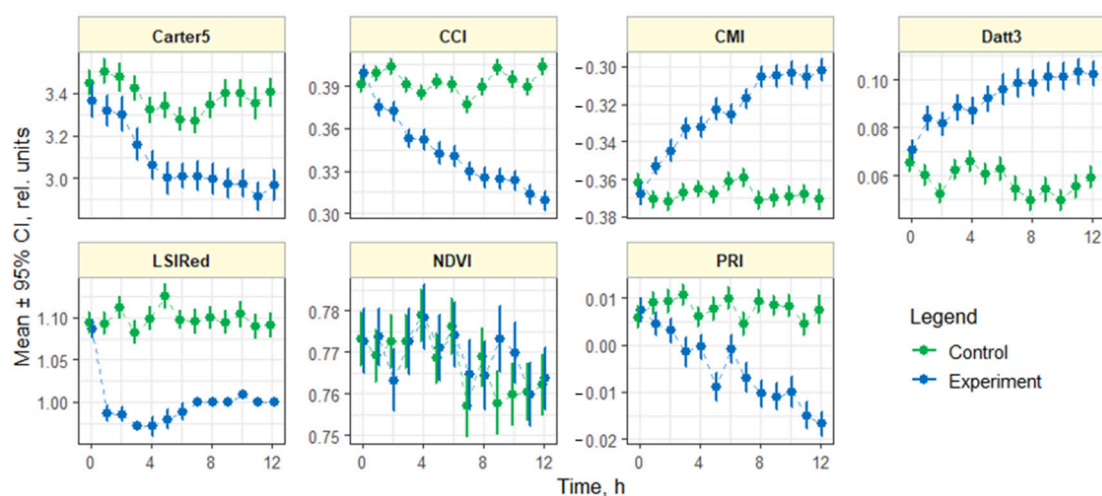


Figure 6. Changes in average VI values as a function of the duration of blue light exposure to the canopy of *Pisum sativum*.

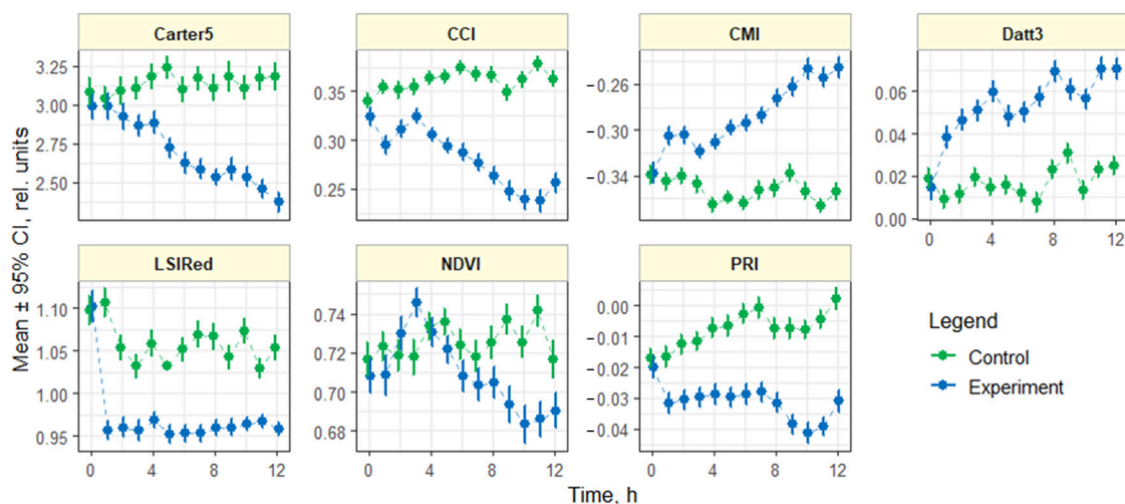


Figure 7. Changes in average VI values as a function of the duration of blue light exposure to the canopy of *Helianthus annuus*.

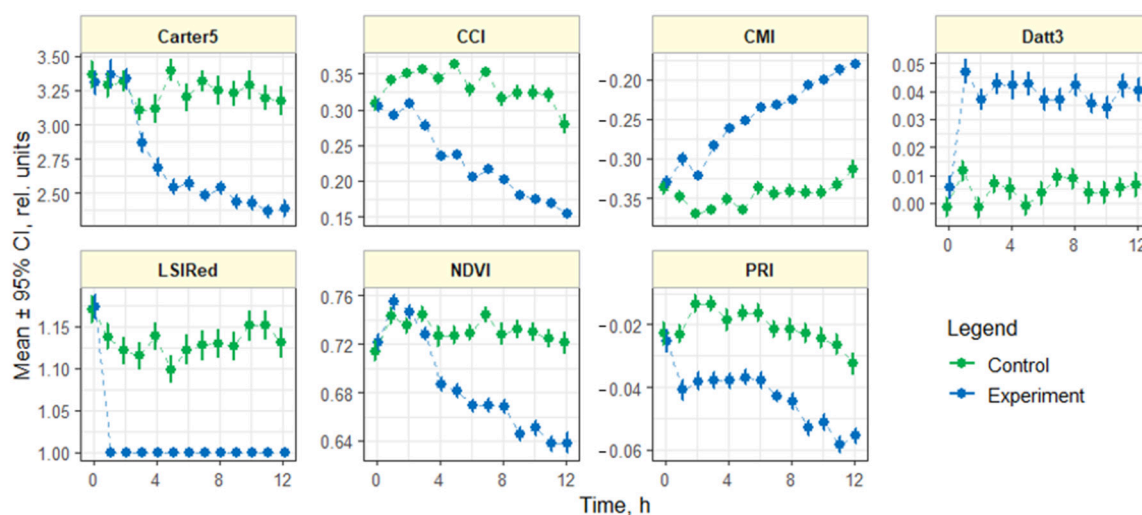


Figure 8. Changes in average VI values as a function of the duration of blue light exposure to the canopy of *Eruca sativa*.

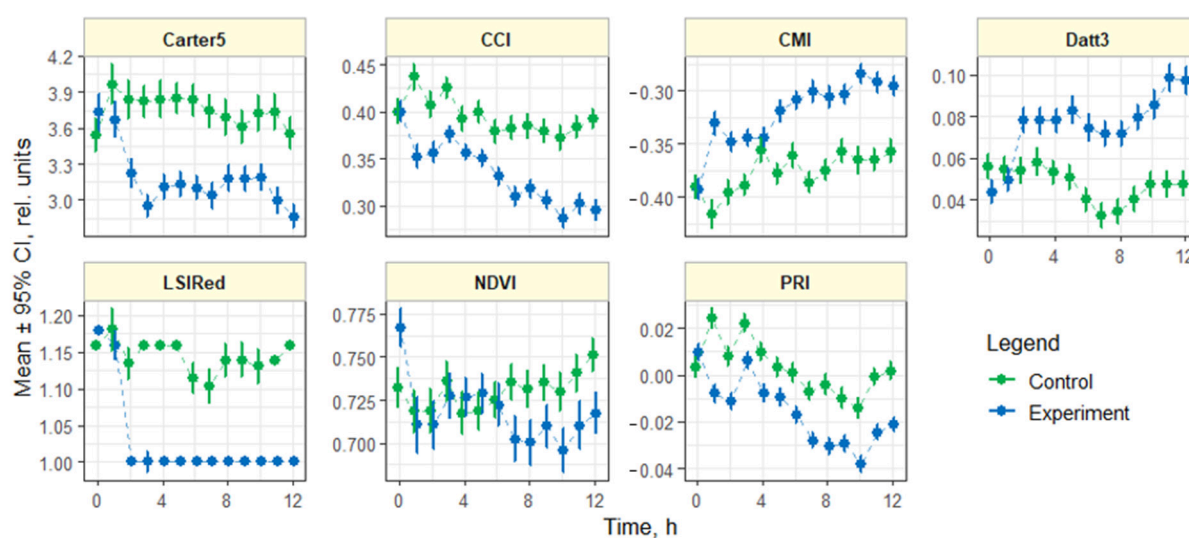


Figure 9. Changes in average VI values as a function of the duration of blue light exposure to the canopy of *Hordeum vulgare*.

For all microgreen crops, the pattern of change in informative VI values under blue light exposure indicated changes in the content and proportions of photosynthetic pigments and the localisation of chloroplasts.

The CMI value for all microgreen crops in the experimental treatment increased relative to the control treatment and stabilised after eight to ten hours of exposure. This may reflect a process of chloroplast avoidance, which takes place over 8–10 h depending on the crop. This is confirmed by the reversible fading of the canopy of the microgreen crops (Figure 2).

The CCI value began to decline steadily after the first hour of blue light exposure. The difference between the control and experimental treatments depended on the microgreen crop. The pattern of change in the CCI value may indicate a gradual increase in the proportion of carotenoids within the pool of photosynthetic pigments.

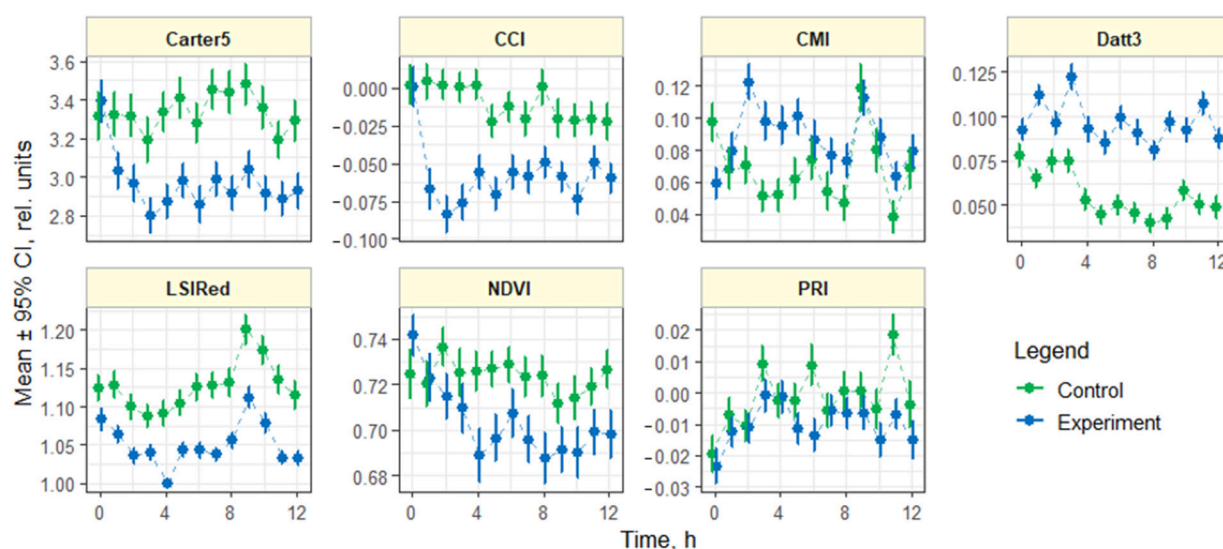


Figure 10. Changes in average VI values as a function of the duration of blue light exposure to the canopy of *Raphanus sativus* ‘Sango Purple’.

The change in the PRI value followed a similar pattern to that of the CCI. However, this index did not ‘work’ for the leafy red radish microgreen canopy. The PRI trend may indicate activation of the xanthophyll cycle and an increase in carotenoid content within the microgreen canopy.

The Carter5 value, depending on the crop, begins to decrease after 1–4 h of exposure. A decrease in the values of this index may indicate the development of abiotic stress.

The NDVI value for the microgreen canopy of peas remains unchanged throughout the entire irradiation period; for rocket, it decreases after 4 h of irradiation, and for barley and sunflower, after 5 h of irradiation. For radish, this occurs after 2 h of irradiation. This may be due either to the chloroplast avoidance effect or to a reduction in chlorophyll content.

The Datt3 value begins to rise as early as one hour after exposure, which may indicate an increase in chlorophyll content. The exception is barley microgreens, where the increase in Datt3 is only slight. The trend in this VI, which indicates an increase in chlorophyll content, does not correspond with the trend in the NDVI.

The LSI_{Red} value drops to one after the first hour of exposure for all crops. Thereafter, the LSI_{Red} level fluctuates slightly around one. This is due to the fact that the spectral profile, with a maximum absorption at 660 nm, changes shape from asymmetric to symmetric within the 654–674 nm range (Supplementary Figures S1–S5). As a result, the LSI_{Red} value tends towards one. The dynamics of this VI indicate that it provides a good qualitative identification of stress, answering the question with a ‘yes’ or ‘no’, but does not quantify it.

An analysis of the correlation between the thirty most informative VIs for RF classification (Figure 5), using Spearman’s correlation coefficient (r), showed that most of them are not collinear (Figure 11, Supplementary Table S3). The $CMI = (R_{635} - R_{555}) / (R_{635} + R_{555})$ and $CCI = (R_{528} - R_{645}) / (R_{528} + R_{645})$ VIs correlate with each other with a strong negative correlation ($r = -0.81$). These VIs use closely spaced spectral bands; in the numerator, they are mirror images of one another, which accounts for the negative correlation coefficient. Furthermore, CMI correlates significantly ($r = 0.82$) with $Datt5 = R_{672} / R_{550}$ and ($r = 0.68$) with $modPRI = (R_{515} - R_{550}) / (R_{515} + R_{550})$.

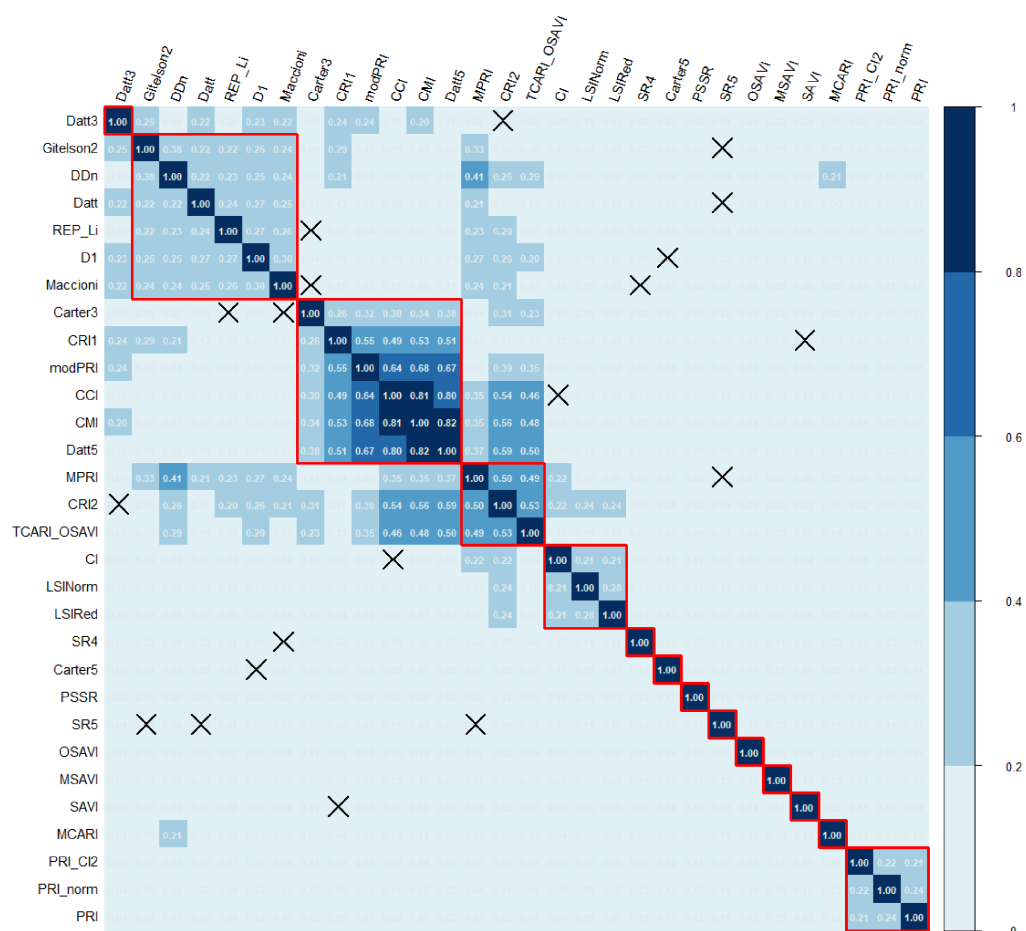


Figure 11. Correlation matrix of 30 informative VIs. The correlation coefficient is given in absolute terms. A statistically insignificant correlation is marked with a cross. Threshold value $|r| > 0.7$. The red frame indicates groups of VIs obtained as a result of hierarchical clustering.

2.4. The Nature of Changes in the Spectral Profiles of Microgreen Canopy Under High-Intensity Blue Light

When the canopy of microgreens is exposed to high-intensity blue light, there is a gradual increase in its reflectance. The changes occur in the visible and near-infrared ranges (450–760 nm), with the greatest difference between the control and the experimental group observed at a wavelength of 734 nm (Figure 12). The exception is red-leaved radish, whose canopy absorbs light in the green spectrum, with all changes occurring in the 600–690 nm range (Supplementary Figures S1–S5).

The part of the spectrum most sensitive to high doses of blue light lies within the range 574–654 nm, where differences become noticeable after just 1–2 h of exposure (Figure 13). As the exposure time increases, the differences in reflectance extend across the entire range of photosynthetically active radiation—from 450 to 690 nm. The greatest differences in the visible light range are observed between 590 and 614 nm. At the same time, the shape of the spectral profile changes around 646 nm, with an additional weak inflexion point forming. As a result, the bend in the spectral profile, with a maximum absorption at 660 nm, changes shape from asymmetric to symmetric in the range 654–674 nm (these changes occur rapidly—after 1–2 h of exposure).

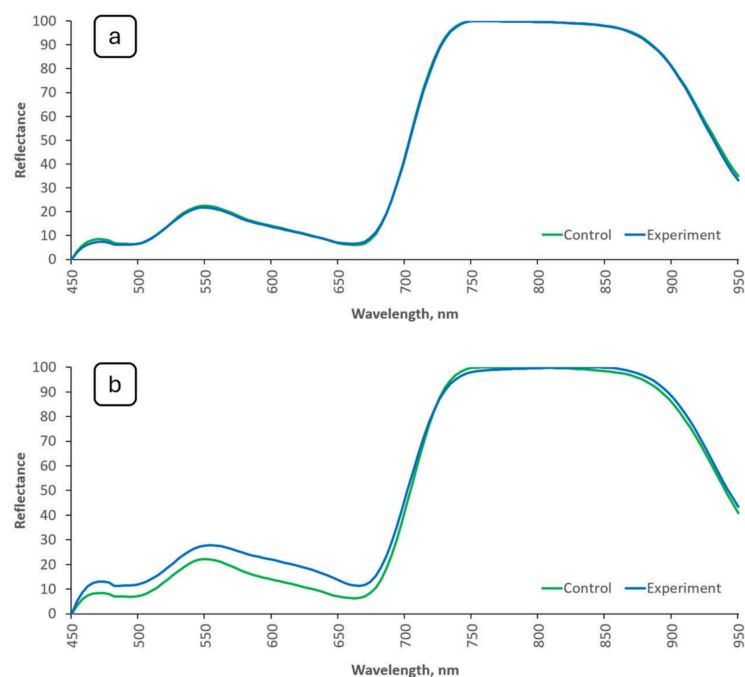


Figure 12. Spectral profiles of rocket/arugula leaves before (a) and after (b) twelve hours of exposure to blue light.

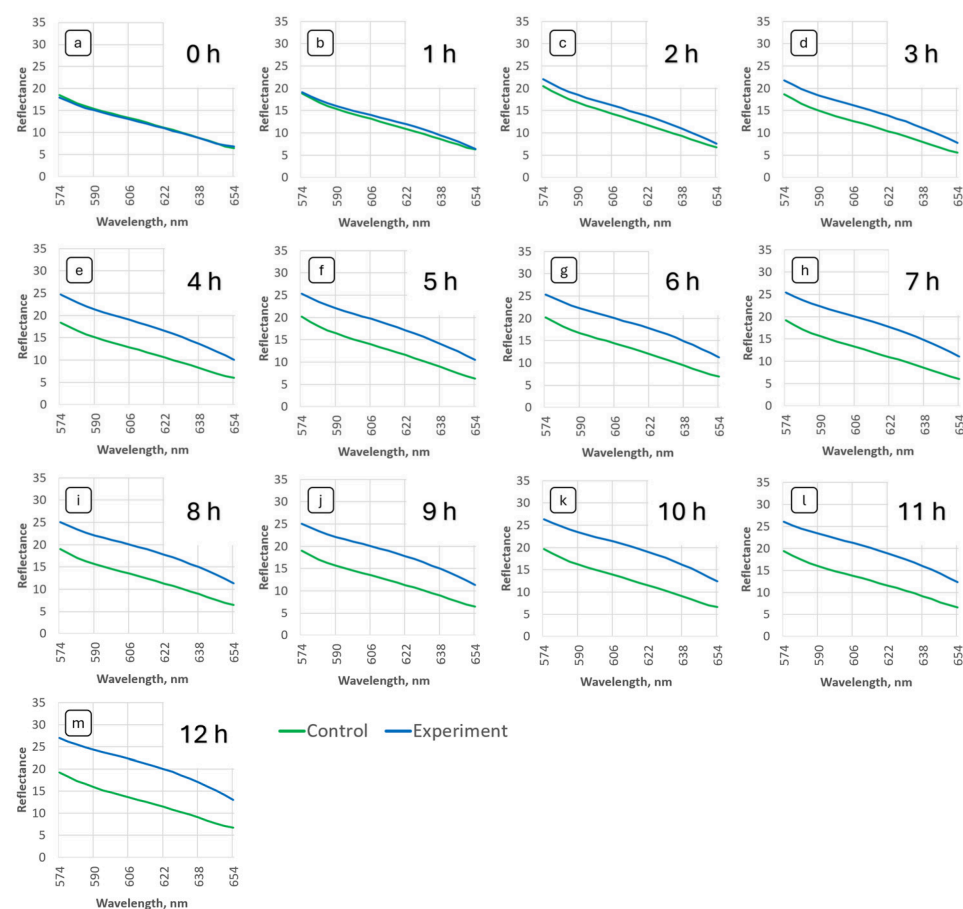


Figure 13. The pattern of change in the reflectance of rocket/arugula leaves in the 574–654 nm range under high-intensity blue light of varying exposure duration. (a) 0 h of exposure; (b) 1 h of exposure; (c) 2 h of exposure; (d) 3 h of exposure; (e) 4 h of exposure; (f) 5 h of exposure; (g) 6 h of exposure; (h) 7 h of exposure; (i) 8 h of exposure; (j) 9 h of exposure; (k) 10 h of exposure; (l) 11 h of exposure; (m) 12 h of exposure.

In pea microgreens, the change in spectral profiles as a function of exposure duration is not clearly defined. This may be due to the characteristics of the plant's canopy, which is formed by the plant's tendrils (Figure 2).

2.5. Changes in the Content of Photosynthetic Pigments in the Canopy of Microgreens Under High-Intensity Blue Light

To understand the nature of the observed spectral changes in the canopy of microgreens, spectrophotometric measurements of Chl (*a+b*) and Car were carried out in the control and experimental treatments before the start of exposure (0 h), after 12 h under control conditions, and after 12 h of exposure to blue light at $2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The results are shown in Figure 14.

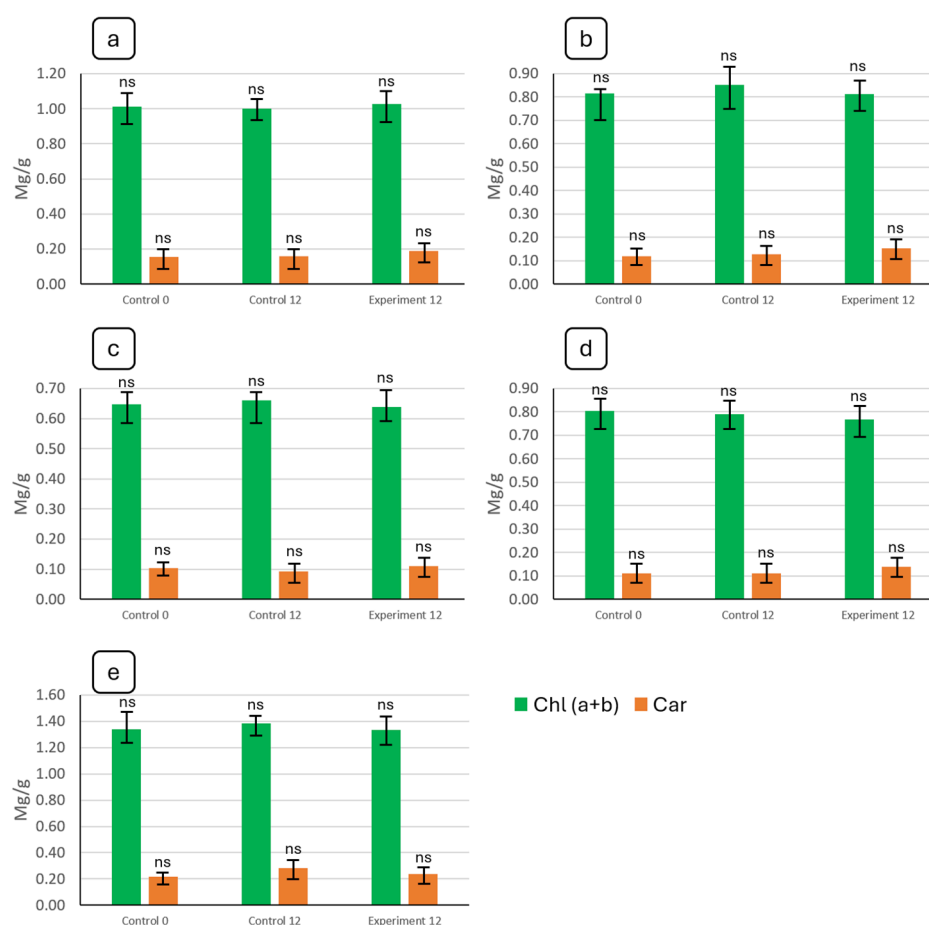


Figure 14. The mean value and confidence interval at $p < 0.05$ for the Chl (*a+b*) and Car content in the canopy of microgreens before the start of exposure (Control 0), after 12 h under control conditions (Control 12) and after 12 h of exposure to blue light at $2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Experiment 12). Statistical comparisons (one-way ANOVA with Tukey's HSD post hoc test) were performed separately for Chl (*a+b*) and Car content. For both pigments, no statistically significant differences were found between any of the conditions for all species ($p > 0.05$). Same letters (ns) above bars indicate no significant differences within each pigment group. (a)—rocket/arugula (*Eruca sativa*), (b)—pea (*Pisum sativum*), (c)—sunflower (*Helianthus annuus*), (d)—leafy red radish (*Raphanus sativus*, 'Sango Purple'), (e)—barley (*Hordeum vulgare*).

For all microgreen crops, no changes in Chl (*a+b*) were observed following a 12 h exposure to high-intensity blue light. The carotenoid content in the experimental treatments showed a tendency towards a slight increase (by 5–27 per cent, depending on the crop); however, these differences are likely to be random.

3. Discussion

The study demonstrated the possibility of early (within 2–3 h) detection of spectral changes characteristic of light stress, caused by high-intensity blue light ($2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), in five microgreen cultures using hyperspectral imaging. It was shown that high-intensity blue light causes changes in the reflectance of the leaves in the region of photosynthetic pigment absorption (450–690 nm) and in the near-infrared range (750–760 nm), which is consistent with literature data on changes in leaf spectral profiles under abiotic stress [48,49]. This effect can be explained by the degradation of chlorophylls within cells under the influence of high light doses, resulting from photooxidation, chloroplast vesiculation or autophagy [50,51]. However, the most likely cause of the observed spectral effect is the chloroplast avoidance response. At high light intensities, chloroplasts migrate towards the anticlinal cell walls, aligning themselves parallel to the incident light [52]. This movement can reduce the amount of light absorbed by the leaf by approximately 15 per cent. This interpretation is indirectly supported by the findings of the present study: (i) reversible fading of the canopy of microgreen crops (Figures 6–10), (ii) the rapid recovery of barley following stress (Figure 4), (iii) the absence of changes in the content of photosynthetic pigments before and after exposure, as determined by spectrophotometry (Figure 14) and (iv) the dynamics of a specialised CMI developed to detect chloroplast movement.

The absence of chlorophyll degradation, coupled with the presence of pronounced spectral changes (increased reflectance in the visible range, changes in vegetation indices), suggests that the observed spectral effects are due to the redistribution of pigments within the cell, rather than their degradation. This is consistent with the hypothesis that the chloroplast avoidance response is the primary mechanism of the early spectral response to high-intensity blue light. It should be noted, however, that we did not observe significant changes in reflectance at 660 nm, although it is precisely this wavelength that is used to detect the chloroplast avoidance effect photometrically [53].

The arrangement of chloroplasts along the anticlinal cell walls, one below the other, leads to a reduction in the area of the regions containing photosynthetic pigments that are ‘visible’ to a hyperspectral camera. In a simple model that does not account for structural changes in the canopy, changes in the values of the indices would be expected to indicate a reduction in the content of photosynthetic pigments. In other words, structural indices should respond to the avoidance of chloroplasts in the same way as they respond to a reduction in the leaf area index of the vegetation canopy. However, changes in indices such as PRI, CCI and Datt3 do not fit this simple scheme (Figures 6–10). Table 1 summarises the expected and observed changes in key VI indices and their interpretation in the context of two main processes: chloroplast movement and pigment degradation.

Changes in the PRI reflect not only the amount of Car, but also the state of the xanthophyll cycle. A decrease in the PRI is a widely recognised indicator of its activation (the de-epoxidation of violaxanthin to zeaxanthin) in response to an excess of light energy [42]. The authors of the CMI index [12] also note that, in the presence of a chloroplast-avoidance effect, the PRI value decreases. Since the xanthophyll cycle is localised in chloroplasts, for it to be maintained during chloroplast avoidance, the effect of xanthophyll cycle activation must significantly outweigh the effect of the reduction in the localisation area of Car.

The observed decrease in the CCI value can be explained by the localisation of secondary carotenoids outside the chloroplasts [54] when the area of Chl localisation is reduced due to the chloroplast localisation effect.

Table 1. Expected and observed changes in vegetation indices under high-intensity blue light.

Vegetation Index	Expected Change Under Chloroplast Avoidance	Expected Change Under Chlorophyll Degradation	Observed Change in the Experiment
PRI	Increase (decrease in the area of carotenoid localisation)	Stable (carotenoid content does not change under chlorophyll degradation)	Decrease (activation of the xanthophyll cycle)
CCI	Stable (constant Chl/Car ratio, independent of pigment localisation area)	Decrease (increase in the proportion of carotenoids in chloroplasts)	Decrease (accumulation of carotenoids outside chloroplasts)
NDVI	Decrease (decrease in the area of chlorophyll localisation)	Decrease (chlorophyll degradation)	Decrease (decrease in the area of chlorophyll localisation)
Datt3	Decrease (decrease in the area of chlorophyll localisation)	Decrease (chlorophyll degradation)	Increase
CMI	Increase (capture of chloroplast avoidance effect)	Stable (does not respond to chlorophyll degradation)	Increase (capture of chloroplast avoidance effect)
LSI _{Red}	Decrease (light stress)	Decrease (light stress)	Decrease (light stress)
Carter5	Decrease (abiotic stress)	Decrease (abiotic stress)	Decrease (abiotic stress)

Changes in Datt3 indicate an increase in Chl concentration [55], which is not corroborated by other indicators. It is possible that the changes in spectral profiles caused by chloroplast avoidance are specific and do not correspond to the changes associated with the accumulation of Chl in eucalyptus leaves, on which this VI was developed. Datt3 is calculated as the ratio of reflectance in narrow bands (754 nm and 704 nm) and has been found to be sensitive to a specific change in the shape of the spectral curve in the ‘red edge’ region, which occurs when microgreens are exposed to high-intensity blue light, as demonstrated in this study (Figure 12b). Nevertheless, Datt3 changes predictably for all microgreen crops during irradiation and is therefore informative for the RF model when classifying the states ‘light stress’ and ‘normal’.

The change in CMI effectively identifies and confirms the hypothesis of chloroplast avoidance and detects the completion of this process. Importantly, CMI revealed significant differences between the control and experimental groups after a 12 h exposure, provided that the concentrations of Chl and Car in the canopy remained unchanged. However, it would be advisable to test this VI on datasets with a wide range of Chl and Chl/Car values, as it correlates closely with Datt5 and CCI. This could confirm the specificity of CMI with regard to chloroplast movement.

LSI_{Red} responds rapidly to high-intensity blue light, stabilising at a value of one within the first hour of exposure and remaining unchanged thereafter. This makes LSI_{Red} a convenient index for the accurate diagnosis of light stress right from the start of exposure. Furthermore, it may not require a control sample: a value of one unequivocally indicates the presence of stress.

Our results show that high-intensity blue light induces rapid and reversible spectral changes in microgreens, presumably linked to chloroplast avoidance. These changes, manifested as an increased reflectance in the visible range (particularly at the ‘green peak’) and a shift at the ‘red edge’, are detectable within 1–2 h of exposure. To avoid misinterpretation of pigment content estimates, the light history of the plants must be taken into account. The processes we have observed can significantly influence the spectral

characteristics of plants depending on the time of day, the season and the plants' shade tolerance [11,56].

The circadian rhythm of photosynthetic pigment levels is the result of two processes: their synthesis/degradation and physical redistribution (chloroplast movement). It has been shown that hyperspectral sensors are sensitive to circadian changes in chlorophyll. Pan et al. [57], using multispectral imaging, described the circadian rhythm in soya, identifying key wavelengths—the Chl absorption regions (470 nm, 660 nm). The authors demonstrated that Chl peaks occur during daylight hours. De Souza et al. [58] demonstrated that VI values depend on the time of measurement. The most stable indices were found to be the NIR/Red edge ratio, REIP (red edge position) and the Water Index. The greatest fluctuations were observed in the simple ratios NIR/Red, NIR/Green and Red edge/Red. The period of least variation in the VIs lies between 09:00 and 16:00. Padilla et al. [59], using the SPAD-502 sensor, observed a diurnal decline in SPAD values in peppers and linked this to chloroplast movement.

The relationship between chloroplast movement and the seasons can be observed in conifers in temperate and boreal climate zones. The effect of seasonal chloroplast redistribution has been described in *Picea obovata*, *P. pungens* and *Pinus sylvestris* [60,61]. In spruce trees, when cold weather sets in, the chloroplasts—which in summer were located along the cell walls—migrate to the central part of the cell, forming cytoplasmic conglomerates. In Scots pine, chloroplasts aggregate in the folds of the mesophyll cells.

The relationship between light history and the spectral characteristics of plants becomes particularly important when conducting long-term observations or when comparing specimens from different habitats with varying light conditions. In particular, it is known that light-loving plants growing under conditions of high insolation exhibit a faster and more pronounced response to high light doses than shade-tolerant plants [56,62]. Kihara et al. [63] demonstrated that *Oryza sativa* and its ancestral species *O. rufipogon*, typically grown in full sun, exhibited much more active chloroplast movement than *Oryza* species usually grown in shade or partial shade (*O. officinalis*, *O. eichingeri* and *O. granulata*).

A methodological implication of these observations is the need to standardise lighting conditions when conducting hyperspectral studies. To ensure the reproducibility of results, it is necessary to record and, where possible, control the light history of the plants: specifying the light intensity during the growth period (PPFD), the spectral composition, the photoperiod, and the time of the last exposure prior to imaging. Ideally, prior to taking measurements, all samples should undergo a dark adaptation procedure or adaptation to standard background lighting to minimise the influence of previous light conditions. Ignoring this factor may be one of the reasons for the lack of reproducibility of results across different laboratories and during different seasonal measurements.

It is important to note that all experiments were carried out under laboratory conditions. In real-world production environments—such as greenhouses and vertical farms—spectral signatures may be influenced by additional factors, such as fluctuations in ambient light, and variations in temperature and humidity [64]. These factors may introduce additional variability into the hyperspectral data and potentially reduce classification accuracy. Therefore, before the developed model is implemented in commercial monitoring systems, further calibration is required using data obtained under conditions approximating those in production environments, taking into account the aforementioned disturbing factors.

4. Materials and Methods

4.1. Object of Study

The study used microgreens of five species: rocket/arugula (*Eruca sativa* Mill.), pea (*Pisum sativum* L.), barley (*Hordeum vulgare* L.), sunflower (*Helianthus annuus* L.), and leafy

red radish (*Raphanus sativus* L., ‘Sango Purple’), characterised by dark purple cotyledons. Plants were cultivated in plastic containers on felt mats without fertilisation under fluorescent lighting at a PPFD of $5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, a 10:14 h light:dark photoperiod, and a temperature of approximately 25°C ; relative humidity ranged from 30 to 50 per cent.

Blue light treatment was applied at the following BBCH stages: BBCH 10 for sunflower, arugula, and radish (cotyledons fully expanded); BBCH 12 for pea (two expanded leaves with stipules or two expanded tendrils); and BBCH 12 for barley (two expanded leaves). In both the experimental and control groups, three containers of each culture of microgreens were used.

4.2. Exposing Microgreens to Blue Light

The cuvettes containing the control samples of microgreens were placed under artificial lighting provided by a fluorescent lamp with a PPFD of $5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The cuvettes containing the test samples of microgreens were placed under a blue light lamp. The spectral profile of the lamp is shown in Figure 15.

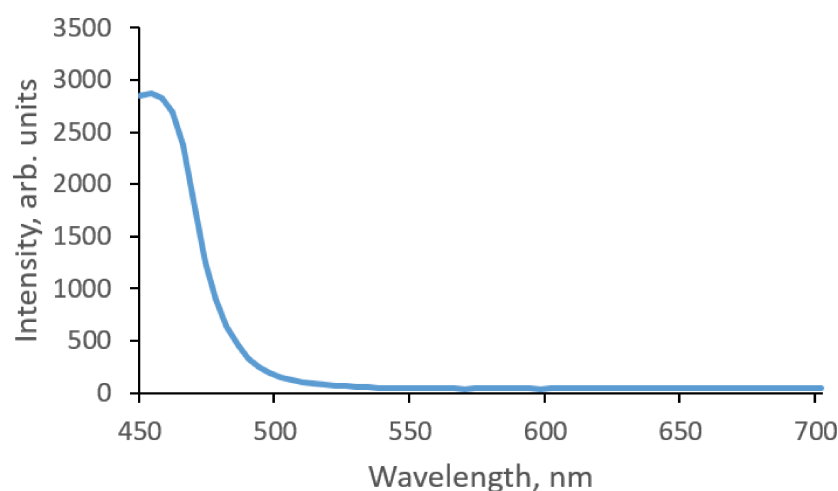


Figure 15. Spectrum of blue LEDs in the range from 450 to 700 nm used in the experiments.

The distance between the lamp and the plants was adjusted so that the PPFD at canopy level was $2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. PPFD was measured using a Growsvet PAR 092 quantummeter. The duration of blue light exposure was 12 h. Air ventilation was provided to prevent the leaves from overheating due to the lamp. The canopy temperature of the microgreens was monitored using a UNI-T Mini Dual K/J Thermometer UT320D (Uni-Trend Technology (China) Co., Ltd., Dongguan, China). The temperature of the microgreen canopy increased by $1.0\text{--}1.5^\circ\text{C}$ when exposed to high-intensity blue light and subjected to simultaneous ventilation.

4.3. Hyperspectral Imaging

A Cubert UHD-185 frame camera (Cubert GmbH, Ulm, Germany) was used for hyperspectral imaging (HSI), with a spectral range of 450 to 950 nm and a spectral resolution of 4 nm. To obtain quantitative, reproducible data and minimise noise, the hyperspectral camera was calibrated. The calibration was carried out in two stages: ‘black’ (1) and ‘white’ (2), immediately prior to each series of measurements, using CubePilot (v. 1.5.7) software. For dark current calibration (‘black’), the camera lens was tightly covered with an opaque cap to prevent any external light from entering. For ‘white’ calibration, a white reference panel was placed in front of the camera lens, completely filling the field of view. To ensure maximum measurement accuracy, calibration was carried out at the same temperature as the main measurements.

Imaging was carried out under laboratory conditions using artificial lighting. Four halogen lamps and a blue LED were used to illuminate the samples during the hyperspectral imaging (HSI), covering the full wavelength range of the hyperspectral camera. The distance from the camera lens to the microgreen canopy was 40 cm. Hyperspectral imaging of the control and experimental samples was carried out before the start of the experiment (0 h), and then at one-hour intervals throughout the exposure period (13 h in total). To carry out HSI, the container of microgreens was moved from beneath the blue light source to the lens of the hyperspectral camera. It took no more than two minutes to image a single container.

4.4. Pre-Processing of Hyperspectral Imaging Data

To reduce noise in the hyperspectral data, the Savitzky–Golay filter with a step size of 15 nm (3–4 spectral bands) and the Random Reflectance method were used [65]. A two-stage segmentation method [66] was used to select the region of interest (ROI) on the hyperspectral image. For subsequent calculations, all spectral profiles (pixels) were selected from the ROI. The number of pixels ranged from 1000 to 1500, depending on the crop. When calculating the VIs, in cases where the required wavelengths for the VI did not exactly match the available bands, we applied the following approaches:

- For wavelengths less than 2 nm away from an available band, the reflectance value of that band was used.
- For wavelengths situated midway between two bands, the reflectance value was calculated as the arithmetic mean of the reflectance values of the two neighbouring bands.

A total of 85 VI were calculated; their abbreviations and formulae are set out in Supplementary Table S4.

4.5. Determination of the Content of Photosynthetic Pigments

The photosynthetic pigments were analysed in all crops before the start of the experiment (control at 0 h) and after its finish (control at 12 h, experiment at 12 h) of the microgreen growth period. For each treatment, three samples (one sample from each container of microgreens) weighing 1.5 g were taken. To extract photosynthetic pigments, cotyledons were taken from rocket, radish and sunflower; tendrils (the top 2 cm of the canopy) from peas; and leaves (the top 2 cm of the canopy) from barley. The pigments were extracted in 96% ethanol [67]. The concentrations of Chl *a*, Chl *b* and Car in mg/g of fresh plant material were determined using a Beckman Du 730 spectrophotometer (USA).

4.6. Measurement of the Maximum Quantum Yield of Photosystem II (F_v/F_m)

Before measuring F_v/F_m , cuvettes containing control and experimental microgreen samples were placed in the dark for 20 min. Measurements were taken before and after exposure to high-intensity blue light using a DivingPAM fluorometer (Walz, Effeltrich, Germany) at 10 different points within the canopy of the microgreens in each of the three containers. To assess the recovery of the canopy of *Hordeum vulgare* microgreens following exposure to high-intensity blue light, F_v/F_m measurements were taken 24 and 48 h after the end of the irradiation.

4.7. Data Analysis

Data analysis was performed using standard statistical methods and the Random Forest (RF) machine learning algorithm [68]. Data processing and preprocessing were performed in the R environment (version 4.5.1) [69] using the RandomForest package [70]. A binary RF model common to five microgreen crops was constructed. It was hypothesised that a model trained on all microgreen crops, unlike models trained on specific crops,

would enable the identification of common stress indicators and allow a comparison of the rate of stress development between crops under equivalent conditions. The model was trained using hyperspectral imaging data following a 12 h exposure to high-intensity blue light at a PPFD of $2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (class ‘light stress’) and a 12 h exposure to a fluorescent lamp with a PPFD of $5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (the ‘normal’ class), for which a state of light stress was recorded based on changes in the F_v/F_m value (Figure 16). A total of 85 VIs were used as independent variables in the model. The number of pixels for each container of each microgreen culture was limited to 1000.

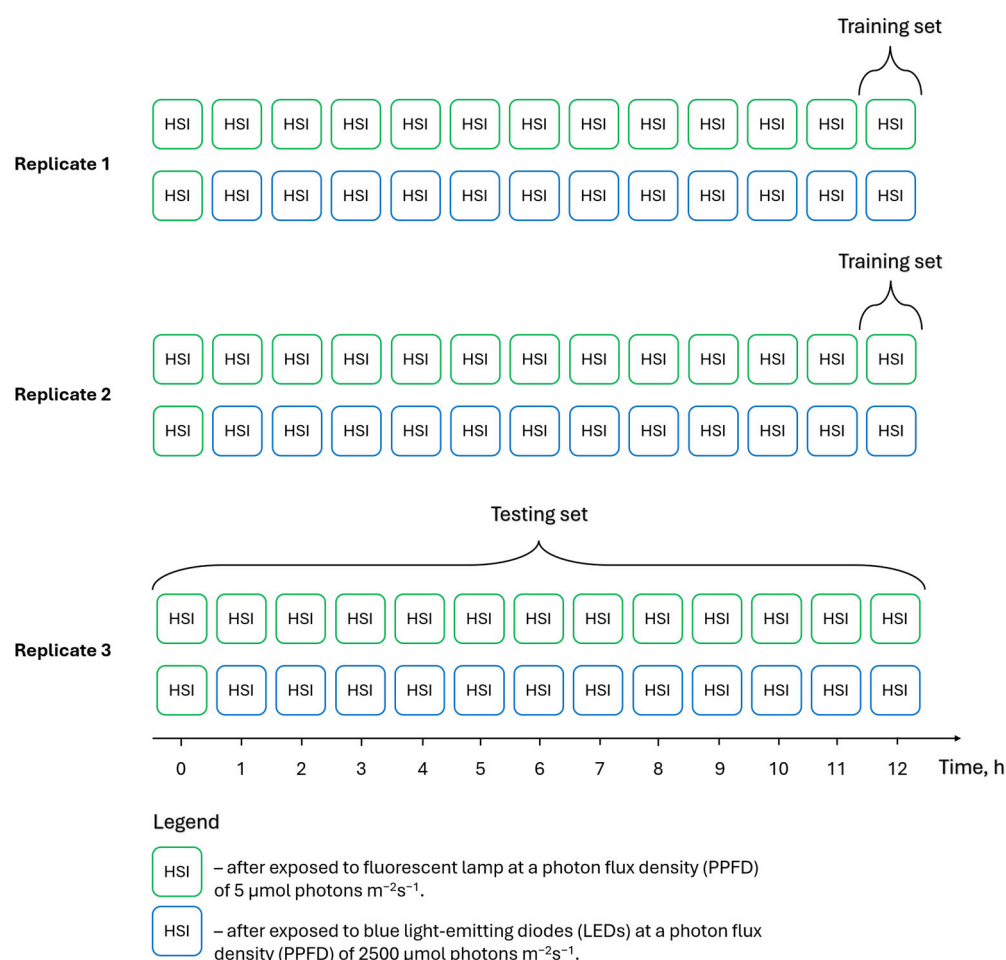


Figure 16. Scheme of training and testing the RF model.

To prevent spatial autocorrelation and overfitting, a 3-fold cross-validation scheme based on containers (cross-validation with one container excluded) was used in the model: Fold 1—Train (Replicate 1 + Replicate 2), Test (Replicate 3); Fold 2—Train (Replicate 1 + Replicate 3), Test (Replicate 2); Fold 3—Train (Replicate 2 + Replicate 3), Test (Replicate 1). The final accuracy of the model was calculated as the average across the three test sets.

A 5-fold stratified cross-validation procedure was used to tune the hyperparameters. The area under the ROC curve (AUC), which is robust to potential class imbalance, served as the target metric for selecting the optimal combination of parameters. At each cross-validation iteration, the initial sample of 20,000 spectral profiles (10,000 of each class) was divided into five equal parts whilst maintaining the class proportions; four parts were used for training and one for validation. Two key hyperparameters were optimised:

The number of predictors randomly selected for splitting at each tree node (No. of variables tried at each split).

The minimum permissible number of observations in a terminal node (leaf) of the tree (Target node size).

The split rule was set to 'gini' (Gini index), and the number of trees (num.trees) was set to 500 during configuration to speed up computations. The search grid included values for $mtry \in \{5, 9, 13, 20, 30\}$ and $min.node.size \in \{1, 5, 10\}$. The performance of the trained RF model was assessed using the following set of metrics: Overall Accuracy (Accuracy), Cohen's Kappa coefficient (Kappa) and F1-score.

A classifier was considered capable of reliably identifying plant stress conditions when the following threshold conditions were simultaneously met: Accuracy $\geq 80\%$, Kappa ≥ 0.6 , and F1-score $\geq 75\%$. An accuracy value of 80% is a generally accepted empirical indicator of successful classification tasks in plant remote sensing. In particular, in the work of Weksler et al. [71], the average accuracy of classifying abiotic stress in pepper plants using hyperspectral data was 80%. Similarly, in the study of Poornima et al. [72], a CNN model achieved 83.40% accuracy in classifying six levels of crop stress severity. In the work of Park et al. [73], all tested machine learning models demonstrated high classification accuracy in the range of 90–98%. The choice of the Kappa coefficient threshold (≥ 0.61) is based on the Landis and Koch scale [74]. According to this scale, Kappa values in the range of 0.61–0.80 correspond to a “high” (substantial) level of agreement between the model predictions and the true labels, while values of 0.41–0.60 are interpreted as “moderate” (moderate) agreement. An F1 score $\geq 75\%$ is used as an additional criterion to ensure that the model achieves balanced performance in terms of Precision and Recall [75,76].

5. Limitations of the Study

This study has a number of limitations. In terms of its objectives, the methods employed and its overall structure, this study should be classified as a preliminary study.

The study was conducted on five microgreen species (sunflower, pea, arugula, barley, and radish) representing different botanical families. The generality of the identified spectral markers to other microgreen species or to full-sized plants remains an open question.

The experiment was conducted under laboratory conditions. Under real greenhouse or field conditions, variability in background light, temperature, humidity, leaf dustiness, and other factors can significantly affect hyperspectral signatures. Transferring the model to such conditions will require additional calibration and, an expansion of the training set.

The interpretation of spectral changes as a consequence of the chloroplast avoidance response is based on indirect indicators (photosynthetic pigment content, CMI value, rapid recovery of the F_v/F_m value after exposure, and reversible fading of the microgreen canopy). Although these indicators are reliable, further confirmation of the results is required through microscopic examination. The experiment did not employ direct biochemical methods for assessing reactive oxygen species (ROS) and the activity of antioxidant enzymes. The absence of hourly independent physiological measurements (F_v/F_m , NPQ, ROS) for all time points does not allow us to state unequivocally that the 'stress' classification during the early hours corresponds to the onset of physiological stress. Our results should therefore be interpreted as demonstrating that hyperspectral changes associated with stress become detectable long before a significant drop in F_v/F_m .

Overall, whilst these limitations should be borne in mind when interpreting the results, they do not call into question the main conclusion that hyperspectral imaging, combined with machine learning, enables the identification of light stress in microgreens. Addressing these limitations in future work will further strengthen the applicability of the approach.

6. Prospects for Further Research

The obtained results open several avenues for further research.

Validation of spectral markers on a wider range of crops and conditions. This will allow us to evaluate the versatility of the proposed markers and develop species-specific calibration models. Furthermore, it is necessary to test the developed model and the VIs indicative of light stress against other types of abiotic stress (temperature stress, mineral nutrient deficiency, water stress), which are accompanied by changes in the content of photosynthetic pigments. There is a need to expand the range of methods used to assess the response of microgreens to exposure to high-intensity blue light—biochemical methods for assessing ROS and the activity of antioxidant enzymes, and microscopy to provide direct evidence of chloroplast avoidance.

The developed RF model, trained under laboratory conditions, requires further training and validation in real greenhouses and vertical farms, where temperature, humidity, background lighting, and other factors can vary. This requires expanding the training set with data obtained under conditions similar to production conditions, as well as developing algorithms to compensate for external interference.

Further experiments should evaluate the impact of repeated daily stress exposures (e.g., periodic supplemental illumination with high-intensity blue light) on the spectral characteristics, productivity, and nutraceutical accumulation of microgreens. Also of interest is studying acclimation to blue light—a long-term, “mild” stress that can increase plant tolerance without sacrificing yield—which is particularly relevant for the production of functional foods. In practical terms, this means answering the question: “Is it possible to achieve high antioxidant levels in the microgreen canopy without the concurrent presence of ROS?”

7. Conclusions

A RF model has been developed to classify light stress caused by high-intensity blue light ($2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), based on hyperspectral data from five species of microgreens. The model achieved high accuracy (Accuracy > 93%, Kappa > 0.87, F1 > 93% at the 11th hour of irradiation) and detects spectral changes associated with stress as early as 1–3 h after exposure.

Key spectral predictors were identified: carotenoid-sensitive VIs (PRI, CCI, PRI*CI2, PRI_norm) and VIs developed to detect chloroplast movement and stress (CMI, LSI_{Red}, LSI_{Norm}, Carter5).

Species-specific sensitivity to blue light was demonstrated: sunflower and radish were the most sensitive crops (spectral changes associated with stress were detected after 1–2 h); peas and rocket showed moderate sensitivity (3 h); and barley was the least sensitive (7 h according to the Kappa coefficient).

Spectrophotometric analysis revealed no statistically significant changes in Chl and Car content between control and experimental samples after 12 h of irradiation. This may indicate that the early spectral changes are due to the redistribution of pigments rather than their degradation.

The LSI_{Red} index has been identified as a qualitative marker of light stress: its value decreases rapidly to one after the first hour of irradiation and remains stable throughout the entire irradiation period.

The practical significance of this work lies in the following:

The proposed approach can be implemented as a technology based on multispectral or hyperspectral cameras installed above conveyor lines in vertical farms. This will enable the early detection of light stress and the automatic adjustment of lighting regimes, thereby preventing yield losses and a decline in product quality.

Necessary steps for implementing the results:

- Validation under production conditions. Pilot trials of the developed model must be conducted in commercial greenhouses and vertical farms, taking into account variations in ambient light, temperature and humidity.
- Adaptation of the model for multispectral cameras. The transition from hyperspectral to multispectral imaging will reduce equipment costs and simplify data processing, whilst maintaining sufficient classification accuracy.
- Determination of threshold values. Based on the proposed indices, specific threshold values must be developed for automated decision-making.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/stresses6030061/s1>, Table S1: Results of testing the stress state using the RF ‘complex’ model, based on 85 VIs, depending on the duration of exposure of microgreen crops to high-intensity blue light ($2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$); Table S2: Results of testing the stress state using the RF ‘light’ model, based on 9 VIs (Datt3, PRI, CCI, PRI*CI2, PRI_norm, CMI, LSI_{Red}, LSI_{Norm} and Carter5), depending on the duration of exposure of microgreen crops to high-intensity blue light ($2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$); Table S3: Correlation matrix for 85 vegetation indices. Statistically insignificant correlations are marked with a cross. The threshold value is $|r| > 0.7$; Table S4: Vegetation indices calculated in this study [77–125]. Figure S1. Dynamics of changes in the reflectivity of the rocket/arugula (*Eruca sativa* Mill.) microgreen canopy under high-intensity blue light ($2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Figure S2. Dynamics of changes in the reflectivity of the sunflower (*Helianthus annuus* L.) microgreen canopy under high-intensity blue light ($2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Figure S3. Dynamics of changes in the reflectivity of the barley (*Hordeum vulgare* L.) microgreen canopy under high-intensity blue light ($2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Figure S4. Dynamics of changes in the reflectivity of the pea (*Pisum sativa* L.) microgreen canopy under high-intensity blue light ($2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Figure S5. Dynamics of changes in the reflectivity of the leafy red radish (*Raphanus sativus* L., ‘Sango Purple’) microgreen canopy under high-intensity blue light ($2500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

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Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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