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Research Article

Changes in chloroplast ultrastructure, specific and phenomenological energy flux, and proline content in the *Dracocephalum kotschyi* plants during acclimation to combined UVB radiation and high light stress

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Abstract

High light (HL) and enhanced ultraviolet-B (UVB) radiation are major abiotic constraints affecting plant growth worldwide. We studied the effect of HL and UVB on chloroplast ultrastructure, specific and phenomenological energy flux, and proline content in low and high-altitude *Dracocephalum kotschyi* plants. Plants were treated with two levels of light intensity, including 400 and high light (HL, 800 μmol m^{-2} s^{-1}), as well as with two levels of ultraviolet-B irradiation (control, UVB, 15 and 30 kJ m^{-2} d^{-1}) for a further 10 days. We observed that the exposure of high-altitude plants to combined stress (HL+UVB) caused an increase in proline content; however, after exposure of low-altitude plants to stress combination, proline content decreased significantly. Based on present results, trapped energy flux (TR_O/CSm) and electron transport flux (ET_O/CSm) decreased with UVB15+HL because active reaction centers (RCs) are converted into inactive or closed RCs consequently decreasing the trapping efficiency and electron transport from PSII. Interestingly, in high-altitude plants exposed to UVB30+HL, the ratio of total dissipation to the number of active RCs (DI_O/RC) is not very influenced, due to the effective utilization of energy by the active RCs. Ultrastructural analyses of chloroplasts revealed an accumulation of plastoglobules only in high-altitude plants leaves under control conditions. In both low and high-altitude plants, UVB30 alone and combined UVB30+HL treatments caused a significant increase in starch granules in chloroplasts, and those chloroplasts tended to be round, especially in high-altitude plants. Thus, significant variation in chloroplast ultrastructure, specific and phenomenological energy flux, and proline content exists between low and high-altitude *Dracocephalum kotschyi* plants, which is apparently due to their altitudinal distributions.

Keywords: Altitudinal gradient, Starch granules, Trapping efficiency, UVB radiation

Introduction

In nature, plants are continually exposed to abiotic and biotic stresses. Under altitudinal gradients, the environmental change in temperature, radiation, and moisture negatively affects plant growth (Cui *et al*., 2019). Among all the environmental factors that change with the altitude, high radiation (Rau and Hofmann, 1996) is the most important factor limiting the plant growth. Plants have developed diverse morpho-physiological and biochemical adaptations to avoid the inhibitory effects of the higher intensities of light radiation (Shi *et al*., 2022; Lingwan *et al*., 2023; Wu *et al*., 2023). It has been demonstrated that the change in the position of the chloroplasts and the leaf blade results in reduced absorption of light energy (Lingwan *et al*., 2023). Similar to the high-light stress, higher dosages of UV radiation on plants lead to accumulation of ROS and DNA damage associated with the changes in metabolites (Zhu *et al*., 2021). To combat the negative effects of imposed UVB stress, plants attempt to regulate their photosynthetic and antioxidant systems (Singh *et al*., 2023).

Leaf chlorophyll *a* fluorescence transient (O-J-I-P) is a commonly used method to detect plant stress conditions in plant research, frequently in association with other morphological, chemical, and physiological variables (Singh *et al*., 2023). Also, it provides detailed information about changes in photosynthetic efficiency, heat dissipation, and quantitative assessment of oxygenic photosynthesis. The JIP test explores the information about the energy flow in thylakoid membranes, the structure and function of the photosynthetic apparatus, and the trapping of excitation energy and electron transport, to detect plant stress

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*Corresponding Author, Email: ghabibi@pnu.ac.ir conditions (Habibi, 2021).

In addition, electron microscopy images are analyzed to investigate whether oxidative stress affected the ultrastructure of chloroplasts (Saeid Nia *et al.*, 2023). Since oxidative stress in chloroplasts is accompanied by an accumulation of plastoglobules (Austin *et al.*, 2006), we analyzed the effects of a combination of UVB radiation and high light stress on the regulation of ultrastructure of chloroplasts and accumulation of plastoglobules in the medicinal *Dracocephalum kotschyi* plant. In addition, whether UVB radiation and high light (HL) can up-regulate proline production in *Dracocephalum kotschyi*, has not previously been examined. Furthermore, we hypothesized that the significant variation in chloroplast ultrastructure, specific and phenomenological energy flux, and proline content exists between low and high-altitude plants in relation to their altitudinal distributions.

Materials and methods

Plant growth and stress treatments: High and low-elevation ecotypes of *Dracocephalum kotschyi* were collected from high (36°13′N, 51°27′W; 3,300 m) and low (36°13′N, 52°32′W; 2,600 m) elevation sites, located in Mazandaran (in the adjacent Central Alborz mountain range, in the central-northern part of Iran). Three to five seeds of *Dracocephalum kotschyi* were transferred and sown on the surface of the cylindrical plastic pots (30 cm in diameter and 40 cm in depth) containing sandy soil mixed with peat moss and perlite for 12 weeks, and were irrigated with distilled water every 7 days. Plants were grown at 25°C day/14°C night temperatures, with 16 h photoperiods and a daytime photon flux density of 400 μmol m^{-2} s^{-1} (measured by a quantum sensor attached to the leaf chamber of the gas exchange unit) in an environmentally controlled growth chamber for a period of three months prior to the start of experiments. Following the 3-month acclimation period, when the plants had developed 4–5 pairs of leaves, independent pots were selected randomly and assigned to measurements. The mature plants were then exposed to irradiation with UVB and high-intensity light stress. Thereafter, plants were treated with two levels of light intensity, including 400 and high light (HL, 800 µmol m^{-2} s^{-1}), as well as with 3 levels of ultraviolet-B irradiation (control, UVB, 15 and 30 kJ m^{-2} d^{-1}) for a further 10 days. For UV radiation treatments, in addition to the photosynthetically active radiation (PAR, 400-700 nm) supplied by cool white fluorescent lamps throughout the daytime, UVB fluorescent lamps (40 W, Philips, German) were used. The spectral outputs of the three lighting conditions were measured with a calibrated spectrophotometer, and biologically effective UV doses employed were 15 and 30 kJ m^{-2} d^{-1} calculated according to Caldwell (1971).

Measurements of chlorophyll fluorescence (which were described in the following section) and other analyses were performed on the fully expanded and mature leaves. For the latter analysis, leaf samples were frozen immediately in liquid N_2 and stored until assay.

Determination of soluble sugar concentrations and proline content: Soluble sugar concentrations were measured by the method of Quentin *et al.* (2015). Leaf tissues were extracted with 2.5 mL 80% ethanol in a water bath for 2 h at 30°C. After centrifugation at 3,000 g for 10 min, the supernatants were treated with anthrone-sulfuric reagent, and then the absorbance at 630 nm was determined. Glucose (Sigma) was used for the standard curve.

Proline concentrations were determined as described by Bates *et al*. (1973). Leaf samples from each group were homogenized with 3% (w/v) sulfosalicylic acid at 4°C and the homogenates were centrifuged at 3,000 g for 20 min. The supernatant was boiled with acetic acid and acid ninhydrin, and then the absorbance was read at 520 nm. The standard curve was made using proline (Sigma).

Chlorophyll *a* fluorescence: The chlorophyll *a* fluorescence was done daily on leaves from in vitro cultured shoots using a Pocket-PEA chlorophyll fluorimeter (Plant Efficiency Analyzer, Hansatech Instruments Ltd., King’s Lynn, Norfolk, PE 32 1JL, England) between 09 hours and 11 hours in dark-adapted leaves for at least 30 min. The JIP-test method has been developed for the determination of phenomenological and biophysical parameters, which quantify the PSII and PSI behaviors. The JIP test is based on the rise in polyphasic fast chlorophyll a, and is used for investigating the correlation between light dependent reactions and ChlF. The O–J part of the fluorescence rise reflects the closure of some of the PSII reaction centers in response to the reduction of QA. The J–I part of the curve corresponds to the reduction of the secondary electron acceptor plastoquinone (PQ), and the I–P part is typically related to the reduction of electron transporters (such as NADP) of the PSI acceptor side. To visualize functional and structural changes of photosystem II (PSII), selected parameters were calculated according to Jiang *et al.* (2008) and Kalaji *et al.* (2016), which were described in the following section.

ABS/RC: Absorption flux per RC, which reflects the proportion between chlorophyll *a* molecule amounts in fluorescence-emitting antenna complexes and the active reaction centers.

TR_o/RC: Trapped energy flux per RC.

ET_o/RC: Electron transport flux per RC.

DI_o/RC: Dissipated energy flux per RC.

ABS/CS: Absorption flux per CS; represents the amount of photon energy absorbed by the antenna associated with active and inactive reaction centers of PSII.

TR_o/CS_m: Trapped energy flux per CS.

ET_o/CS_m: Electron transport flux per CS.

DI_o/CS_m: Dissipated energy flux per CS.

RC/CS_m: Amount of active PSII RCs per CS.

φP_o: Maximum quantum yield of primary photochemistry.

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Microscopic analyses: For transmission electron microscope (TEM), the segments of fresh leaves were fixed overnight at 4°C in a solution containing 4% glutaraldehyde and 0.1 M cacodylate buffer (pH 7.4). After post-fixation in the same buffer containing 1% (w/v) osmium tetroxide (OsO_4) for 2 h, the segments were washed using the same buffer and then dehydrated with ethanol via placing the sample in 50% ethanol for 30 min, followed by changes in 30%, 50%, 70%, 90%, and 100% ethanol. Subsequently, the samples were embedded in a fresh resin at 60°C overnight. Ultrathin sections were placed on copper grids and then examined with TEM (Model 912 AB, LEO, UK) as described by Mousavi Kouhi *et al*. (2015).

Statistical analysis: Experiments were carried out as a completely randomized design with four independent replications. All data were subjected to ANOVA to compare the data means at the same time point, and significant differences between means were concluded by the Tukey test ($P < 0.05$). The achieved data on Chl fluorescence were assessed using the PEA Plus ver. 1.10 software.

Results

A significant decrease in the proline content was only detected under UVB+HL in low-altitude plants, suggesting that low-altitude plants were more affected by combined stress compared to individual stress (Figure 1). In high-altitude plants, proline content was not influenced by UVB alone; however, it increased with combined stress (UVB+HL) treatments in comparison to their respective plants under control conditions. Thus, we observed that the exposure of high-altitude plants to stress combination (HL+UVB) caused an increase in proline content; However, after exposure of low-altitude plants to stress combination, proline content decreased significantly (Figure 1).

In high-altitude plants, soluble sugar concentration was not affected by UVB or HL stress, while in low-altitude plants, the UVB30 and HL combination caused higher soluble sugar concentration compared with UVB30 treatment alone (Figure 2).

In the present studies, impacts of UVB radiation and high light stress on specific energy fluxes, phenomenological energy fluxes, and quantum yield were studied, and results indicated that UVB radiation under high light conditions significantly altered the photosynthetic process of *Dracocephalum kotschyi* plants (Figure 3). The quantum yield of primary photochemistry (φP_o), which exhibits the overall photosynthetic potential of active PSII reaction centers, was not influenced by UVB radiation and high light stress in high-altitude plants (Figure 3). In contrast, in this study, a significant decrease of φP_o was detected in low-altitude plants under HL-alone or combined stress (UVB+HL) treatments. In the thylakoid membrane model, the specific energy fluxes, such as absorption flux per reaction center (ABS/RC), trapped energy flux per reaction center (TR_0/RC), electron transport flux per reaction center (ET_O/RC), and dissipated energy flux per reaction center (DI_O/RC), were determined to analyze the photosynthetic performance of active PSII reaction centers of *Dracocephalum kotschyi* subjected to UVB-alone, HL-alone, or combined stress (UVB+HL) treatments (Figure 3). In both low and high-altitude plants, a remarkable enhancement in absorption flux per reaction center (ABS/RC) was recorded under

UVB15 alone, HL alone, or combined UVB15+HL treatments. A similar trend in TR_0/RC and DI_O/RC was observed, as shown in Figure 3. In contrast, a significant decrease in electron transport flux per reaction center (ET_O/RC) was recorded in low-altitude plants. On the contrary, in the leaf model, phenomenological energy fluxes mean absorption flux per cross section (ABS/CS), trapped energy flux per cross-section (TR_O/CSM), electron transport flux per cross-section (ET_0/CS), and dissipated energy flux per cross-section (DI_O/CSM) significantly decreased by UVB15 alone, HL alone, or combined UVB15+HL treatments. It is of interest to investigate if UVB30 stress changes the specific energy fluxes, such as absorption flux per reaction center, as well as phenomenological energy fluxes, mean absorption flux per cross section in high-altitude plants under UVB30+HL treatment. While the phenomenological energy flux per cross section parameters increased, the specific energy flux per reaction center parameters decreased.

Ultrastructural analyses revealed an accumulation of plastoglobules only in high-altitude plant leaves under control conditions (Figsure 4, 5). In both low and high-altitude plants, UVB30 alone or combined UVB30+HL treatments caused a significant increase in starch granules in chloroplasts, and those chloroplasts tended to be round, especially in high-altitude plants (Figure 4, 5). In control plants, the chloroplast had a normal oval shape, and the boundaries of complete external envelopes were clear.

Discussion

Soluble sugar and free proline are two important osmotic adjustment substances that can enhance osmotic pressure and maintain the normal metabolism of plants (Cui *et al*., 2019). Many plants, when exposed to salinity stress (Spormann *et al*., 2023), accumulate proline in large quantities, as an ROS scavenger and osmotic regulator (Chun *et al*., 2018). In this study, we showed that priming with HL+UVB led to high levels of proline in high-altitude plants. These results further demonstrated a great positive correlation between altitude and proline accumulation (Ashraf and Harris, 2013; Cui *et al*., 2019), which may provide a notable protective function. Sugars, as important signaling molecules, are known to accumulate during stress in leaves (Zhang *et al*., 2018). To the contrary, in this study, a significant increase in soluble sugar concentration was not detected in high-altitude plants under UVB-alone, HL-alone, or combined stress (UVB+HL) treatments.

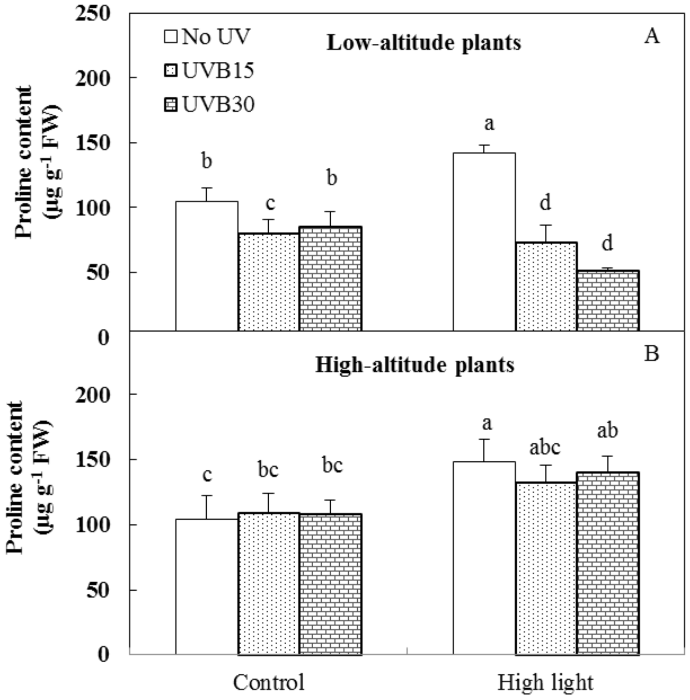

Figure 1. Effects of UVB radiation, high light intensity, and their combination on the proline contents in leaves of *Dracocephalum kotschyi* plants growing at low and high altitudes. Bars indicated with the *same letter* within each elevation site are not significantly different (P<0.05 Tukey test). Values are the mean ± SD (n=4).

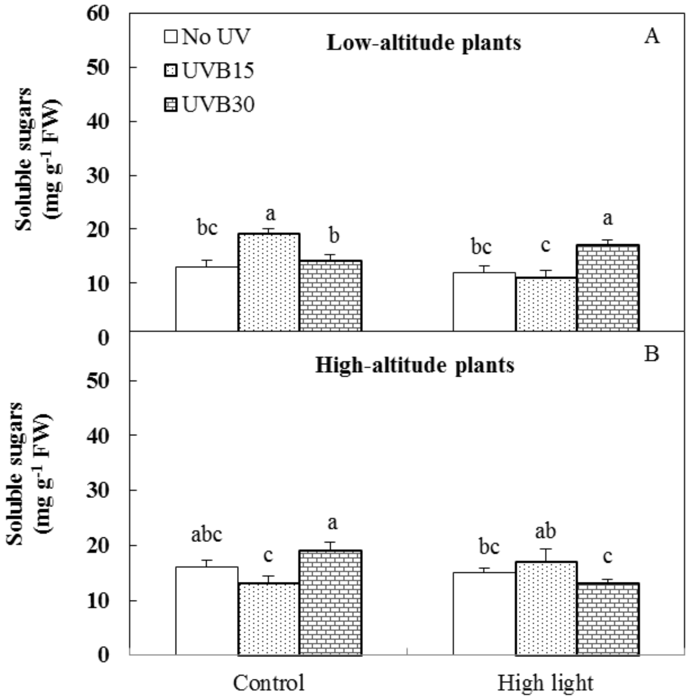

Figure. 2 Effects of UVB radiation or high light alone and their combination on the soluble sugars in leaves of *Dracocephalum kotschyi* plants growing at low and high altitudes. Bars indicated with the same letter within each elevation site are not significantly different (P<0.05 Tukey test). Values are the mean ± SD (n=4).

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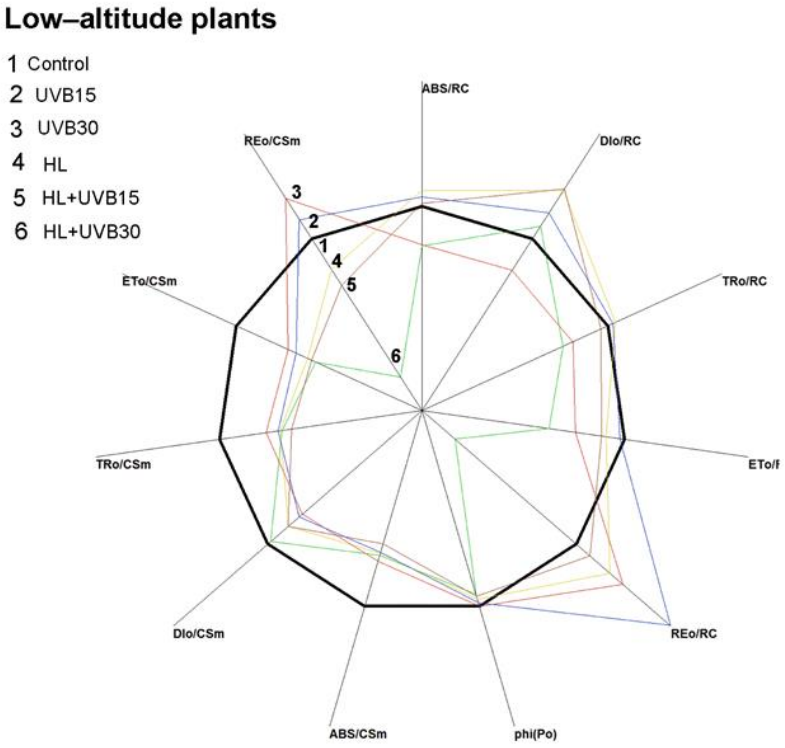

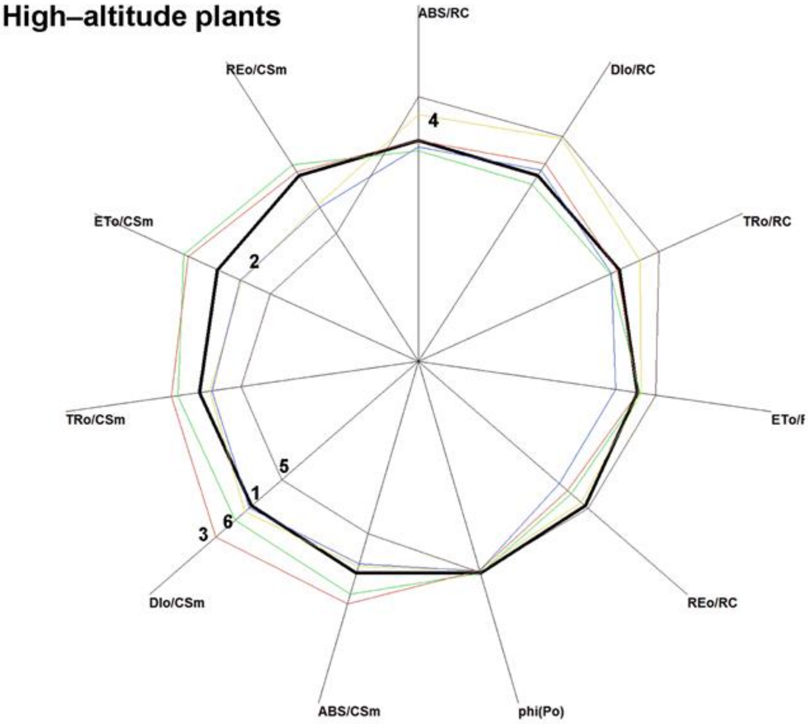

Figure 3. Radar plots showing various technical fluorescence parameters in both low and high-altitude *Dracocephalum kotschyi* plants exposed to UVB30 alone or UVB30+HL combination. TRo/RC: Trapped energy flux per RC. ETo/RC: Electron transport flux per RC. DIo/RC: Dissipated energy flux per RC. ABS/CS: Absorption flux per CS; represents the amount of photon energy absorbed by the antenna associated with active and inactive reaction centers of PSII. TRo/CSm: Trapped energy flux per CS. ETo/CSm: Electron transport flux per CS. DIo/CSm: Dissipated energy flux per CS. RC/CSm: Amount of active PSII RCs per CS. φPo: Maximum quantum yield of primary photochemistry.

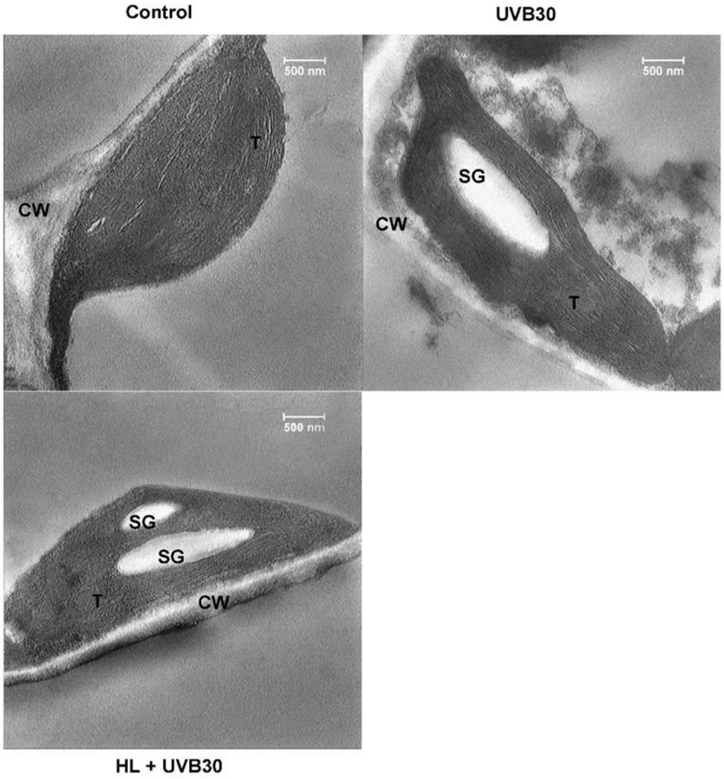

Figure 4. TEM micrograph of cross ultrathin sections through chlorenchyma cells of low-altitude *Dracocephalum kotschyi* plants exposed to UVB30 alone or UVB30+HL combination (T, thylakoids; SG, starch grain; CW, cell wall).

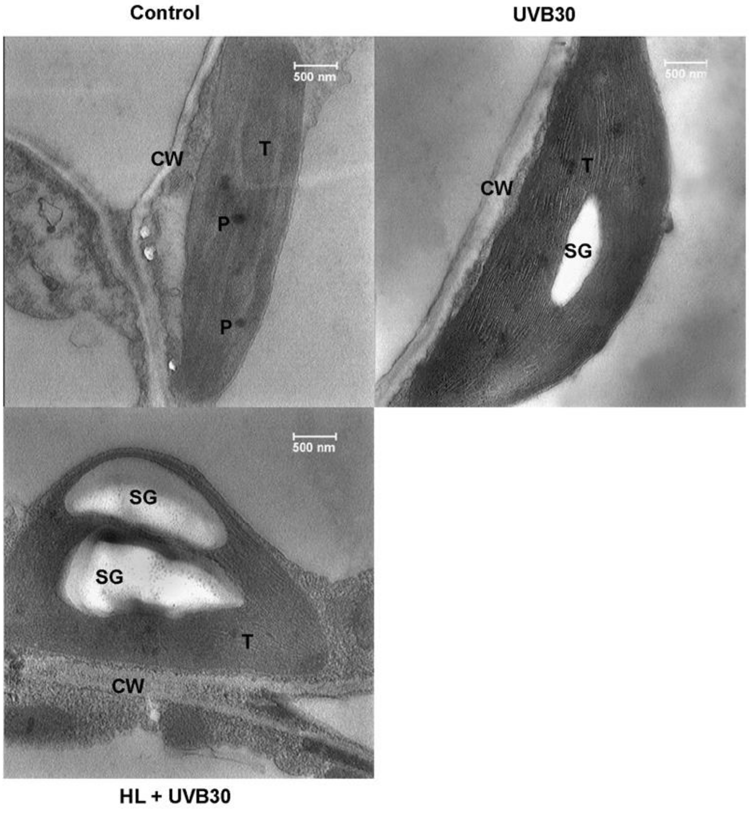

Figure 5. TEM micrograph of cross ultrathin sections through chlorenchyma cells of high-altitude *Dracocephalum kotschyi* plants exposed to UVB30 alone or UVB30+HL combination. T, thylakoids; SG, starch grain; P, plastoglobule; CW, cell wall

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In both low and high-altitude plants, UVB15 alone, HL alone, or combined UVB15+HL treatments, changes in the specific energy fluxes, such as absorption flux per reaction center as well as phenomenological energy fluxes, mean absorption flux per cross section presented that various sites in PSII are sensitive to UVB radiation and high light stress. A similar trend was observed in many studies on the plant's physiological changes under UV radiation and high light stress (Zushi *et al*., 2012; Gautam *et al*., 2014). Based on present results, TR_O/CSm and ET_O/CSm decreased with UVB15+HL because active RCs are converted into inactive or closed RCs, consequently decreasing the trapping efficiency and electron transport from PSII (Tsimilli-Michael, 2020). TR_O/RC is the maximum rate at which an exciton is captured by the RC, resulting in a decrease in QA (Singh *et al*., 2022). In the present studies, UVB15+HL caused an increase in the TR_O/RC ratio, which indicates that all the QA has been reduced (Kalaji *et al*., 2014). In low-altitude plants, reduction in ET_O/RC showed that the re-oxidation of reduced QA through electron transport in an active RC was decreased because a greater number of the active RCs are available; hence, it only reflects the activity of active RCs, which, similarly, was previously reported by Zhao *et al*. (2023). The ratio of total dissipation of untrapped excitation energy from all RCs to the number of active RCs is described as DI_O/RC. Dissipation arises as heat, fluorescence, and energy transfer to other systems, and the ratios of active/inactive RCs also have an impact. Interestingly, in high-altitude plants exposed to UVB30+HL, the ratio of total dissipation to the number of active RCs (DI_O/RC) is not influenced significantly, due to the effective utilization of energy by the active RCs (Grieco *et al*., 2015).

To study the changes in the size and shape of chloroplasts in the mesophyll cells, the chloroplast membranes were analyzed by a transmission electron microscope (TEM). In high-altitude plants' leaves under control conditions, plastoglobule content was higher in chloroplasts, which was consistent with the finding of Lichtenthaler (2014), who reported that plastoglobules contain traces of xanthophylls. Owing to the protective effects of carotenoids (xanthophylls) in the dissipation of absorbed light energy as thermal energy as well as its function as a non-enzymatic antioxidant during exposure to stress (Lingwan *et al*., 2023), this higher accumulation of xanthophylls located in plastoglobules plays a crucial role in plant photoprotection under HL and UVB30 stress, especially in high-altitude plants.

We showed that plants exposed to UVB30-alone or combined UVB30+HL exhibited ultrastructural changes in chloroplasts, which more starch granules were observed during UVB30 treatment. These results were consistent with previous studies in *Oryza sativa* and *Aloe vera* leaves (Wang *et al*., 2019; Habibi, 2021) under drought stress, and with studies in *Mangifera indica* under enhanced ultraviolete-B radiation (Chen *et al*., 2023). In high-altitude plants, we proposed that chloroplasts preserve more starch granules in order to maintain the highest levels of photochemical activity, corresponding with a proline accumulation under UVB30 stress.

Conclusion

In the present study, significant variations in specific energy fluxes, phenomenological energy fluxes, and quantum yield were detected between low and high-altitude plants under UVB radiation and high light stress. While after exposure of low-altitude plants to stress combination, proline content decreased significantly, stress combination (HL+UVB) caused an increase in proline content in high-altitude plants that showed the significance of proline accumulation in high-altitude plants adaptation. High-altitude plants leave under control conditions exhibited higher accumulation of xanthophylls located in plastoglobules, which play a crucial role in plant photoprotection under HL and UVB30 stress.

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