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Changes in ultrastructure, photosynthetic abilities, and secondary metabolite due to individual and interactive effects of chromium and ultraviolet-B radiation in *Adhatoda vasica*

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Abstract

The study was executed to assess individual and interactive effects of elevated ultraviolet-B (eUV-B) radiation and chromium (Cr) on a medicinal plant *Adhatoda vasica* Nees. The experiment was conducted under field conditions involving control, Cr, eUV-B, and Cr+eUV-B treatments. The results showed that Cr content was the highest in roots as compared to other parts under Cr+eUV-B. Significant reductions in photosynthetic rate, intercellular CO_2 concentration, and stomatal conductance were observed under all treatments with maximum under Cr+eUV-B. Chlorophyll (Chl) fluorescence parameters showed variable responses under Cr and Cr+eUV-B. Chl content showed reductions under all treatments whereas Chl *a*/*b* ratio and carotenoids showed increment under eUV-B and reductions under Cr and Cr+eUV-B. The ultrastructure of leaves showed changes in chloroplasts under treatments. Vasicine (medicinally important secondary metabolite) increased under treatments. Our study revealed that *A. vasica* showed variable responses towards individual and interactive stress of Cr and eUV-B.

Keywords: bioconcentration factor; dry mass; physiology; translocation factor; UV-B absorbing compounds.

Introduction

Chromium (Cr) is contemplated as the 7th most copious element of the Earth's crust (Shanker *et al*. 2005). The extensive utilization and release of Cr from various anthropogenic and natural sources lead to global Cr pollution (Srivastava *et al*. 2021). Cr is widely utilized in several industrial processes, such as leather processing, dying of textiles, electroplating, refractory steel, cleaning agents, wood preservatives, and chemical production

Highlights

- The eUV-B and Cr individually and in combination showed variable responses
- Higher reductions in photosynthesis, chlorophylls, and dry mass were observed under Cr+eUV-B
- Vasicine (medicinally important compound) increased maximally under Cr+eUV-B

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Abbreviations: BCF – bioconcentration factor; C_i – intercellular CO_2 concentration; E – transpiration rate; eUV-B – elevated UV-B; F_0 – minimal fluorescence; F_m – maximum fluorescence; F_m/F_0 – electron transport rate through PSII; F_v – variable fluorescence; F_v/F_0 – the activity of PSII; F_v/F_m – maximum quantum yield of PSII; g_s – stomatal conductance; P_N – net photosynthetic rate; RDM – root dry mass; RSR – root-to-shoot ratio; SDM – shoot dry mass; TF – translocation factor.

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Conflict of interest: The authors declare that they have no conflict of interest.

(Shanker *et al*. 2005). The Agency for Toxic and Disease Registry ranked Cr as the 17th among the highly hazardous substances (Sharma *et al*. 2020). In the environment, Cr is present in various oxidation states, however, the stable forms of Cr are Cr^{3+} and Cr^{6+} and among these, Cr^{6+} is a highly toxic, soluble, and mobile form of Cr. The average content of Cr worldwide in soil ranges from 10 to 100 mg kg^{-1} (Srivastava *et al*. 2021). Cr does not have any known role in the plants, thereby Cr toxicity affects various processes in plants including photosynthesis, carbohydrate metabolism, cellular ultrastructure, enzyme activities, and secondary metabolism, which ultimately leads to hampered growth, physiology, yield, and the overall metabolism of plants (Shanker *et al*. 2005, Sharma *et al*. 2020, Srivastava *et al*. 2021).

Although being a minor component of the solar spectrum, ultraviolet-B (UV-B) radiation remarkably affects entire life on the Earth's surface. Researchers of the diverse groups revealed that independent of stratospheric ozone variations, climate change is extremely accountable for variations in the surface UV-B radiation (Barnes *et al*. 2019). UV-B radiation potentially affects several aspects of plants including morphology, growth, biochemistry, and secondary metabolism (Takshak and Agrawal 2015, Pandey *et al*. 2021). UV-B radiation can perturb the photosynthetic apparatus, such as the D1/D2 protein of the reaction center, other components of PSII, and the oxygen-evolving complex (Kataria *et al*. 2014). It also affects the stomatal functions and chloroplast ultrastructure, causes the destruction of chlorophylls (Chls) and carotenoids, and reduces the activity of Rubisco, which consequently leads to alteration in the physiology of plants (Kataria *et al*. 2014). In addition to its role as a stressor, UV-B radiation also plays an important regulatory role by controlling gene expressions, growth, development, and cellular and metabolic activities in plants (Hideg *et al*. 2013). At a low fluence rate, UV-B stimulates the genes that are responsible for phenolics and flavonoid production and protects against UV-B (Kataria *et al*. 2014).

Adhatoda vasica Nees. (commonly called adusa, vasak) is an important medicinal plant of the family Acanthaceae. It possesses an important pyrroquinazoline class of alkaloids, namely vasicine, vasicinone, vasicoline as well as sterols, flavonoids, fatty acids, vitamins, and terpenes. Its leaves are frequently used for various respiratory disorders, such as cold, asthma, and tuberculosis. *A. vasica* also possesses various biological activities including antidiabetic, antioxidant, anticestodal, chemo-preventive, and anti-inflammatory activities (Pandey *et al*. 2021). Despite its medicinal importance, studies regarding the abiotic stress responses of *A. vasica* are scarce in the literature.

Furthermore, in a natural environment, multiple stresses such as Cr and UV-B may likely coexist and the effect of a stress factor may intensify or be lessened by the simultaneous action of other stress. Gondor *et al*. (2014) reported that UV-B radiation may either have a negative or positive impact on wheat depending on other abiotic stress, *i.e*., Cd or drought. An aquatic plant *Elodea nuttallii* exposed to UV-B and Hg stress showed that increased UV-B radiation results in co-tolerance to Hg toxicity at the physiological and transcriptomic level (Regier *et al*. 2016). However, studies dealing with the interactive effects of Cr and UV-B on plants in natural environment are insufficient. A single study performed by Singh *et al*. (2016) is known till date; it deals with the interactive effect of Cr and UV-B on maize seedlings. Besides, nothing is renowned regarding the interaction of UV-B and Cr on the accumulation of Cr, physiology, and secondary metabolites in medicinal plants. Considering all the above details in mind, this study was executed with objectives: (*1*) to evaluate the individual and interactive effect of eUV-B and Cr on the accumulation of Cr in different plant parts, (*2*) to estimate the effect of eUV-B and Cr individually and in combination on gas exchange, chlorophyll fluorescence, pigments, and UV-B absorbing compounds, and (*3*) to assess the effect of eUV-B and Cr individually and in combination on ultrastructure, dry mass, and vasicine content (a medicinally important metabolite) of *A. vasica*.

Materials and methods

Experimental area, soil, meteorological conditions, and plant material: The pot study was carried out in the field of Botanical garden, Department of Botany, Banaras Hindu University (25°16'14.1"N, 82°59'20.9"E), Varanasi, from the period August 2019–February 2020. The physicochemical properties of experimental soil were as follows.

Property	Values
Texture	Sandy loam
Sand [%]	45
Silt [%]	28
Clay [%]	27
pH	7.4
Electrical conductivity [mS cm^{-1}]	0.24
Total organic carbon [%]	0.85
Total nitrogen [%]	0.32
Available phosphorus [mg g^{-1}]	0.65
Cr [mg kg^{-1}]	41.9

The meteorological data were acquired from Indian Meteorological Division, Banaras Hindu University, Varanasi. During the experimental period, maximum and minimum mean temperature was 28.7 and 19.4°C, respectively. The total rainfall recorded was 1,162 mm. The mean cloud cover and relative humidity were 31.3 and 70.1%, respectively.

Stock plants of *Adhatoda vasica* Nees. were obtained from the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India as reported in Pandey *et al*. (2021). From the stock plants, stem cuttings of 10 cm having two nodes were planted in polybags filled with garden soil. Cuttings were placed in the shade and

watered regularly to provide moist conditions. After two months when the cuttings have four fully mature leaves, they were ready for transplantation and used for the experiment.

Experimental setup, design, and plant sampling: The experiment comprised four sets, such as (*1*) control (*i.e.*, ambient UV-B), (*2*) Cr [30 mg kg^{-1}(soil)], (*3*) eUV-B (ambient + 7.2 kJ m^{-2} d^{-1} UV-B), and (*4*) Cr+eUV-B [30 mg(Cr) kg^{-1} and the ambient + 7.2 kJ m^{-2} d^{-1} UV-B]. Each treatment had three replicates of experimental plots consisting of overall twelve experimental plots of 1 × 1 m size. The soil used in the experiment was taken at depth of 25 cm from the experimental site, *i.e.*, the botanical garden. Cr was added [30 mg kg^{-1}(soil)] as potassium dichromate ($K_2Cr_2O_7$; *Sigma-Aldrich*) and mixed gradually and evenly in the soil before filling in pots. The given dose of Cr was determined based on prior studies and fell within the range of Indian standards for soil (Pathak *et al*. 2011). A total of 144 earthen pots (height of 40 cm, diameter of 30 cm; in each experimental plot, 12 earthen pots were kept) were filled with 10 kg of soil (with and without Cr addition). For soil stabilization, pots were left as such for 10 d. After soil stabilization, one plant of *A. vasica* was transplanted into each pot. For eUV-B and Cr+eUV-B treatment, the elevated dose of UV-B (ambient + 7.2 kJ m^{-2} d^{-1} UV-B) was provided by UV-B lamps (*Q Panel UV-B 313 40 W*, *Q Panel Inc*., Cleveland, OH, USA) covered with cellulose diacetate filters (0.13 mm; *Cadillac Plastic Co.*, Baltimore, MD, USA) for 3 h (11:00–14:00 h) up to the culmination of the experiment. Criteria for UV-B dose selection and the details of UV-B treatment were as provided in Pandey *et al*. (2021). Tap water was added to each pot in the same amount at regular intervals by avoiding the leakage of water from the pots. The vegetative, pre-reproductive, and reproductive stages of *A. vasica* were represented by 60, 120, and 180 d (time after transplantation in days). The gas exchange, chlorophyll (Chl) fluorescence, pigments, and UV-B absorbing compounds were analyzed at three growth stages, whereas Cr content, dry mass, ultrastructural changes, and the vasicine content were analyzed at the reproductive stage (180 d).

Gas exchange and Chl fluorescence: Gas-exchange parameters, namely stomatal conductance (g_s), transpiration rate (E), intercellular CO_2 concentration (C_i), and net photosynthetic rate (P_N), were determined using *LI-6400XT* portable photosynthesis system (*LI-COR Inc.*, Lincoln, NE, USA) between 8:00–11:00 h on the third or fourth fully developed leaves, from control and treatment plots. The system was calibrated before measurements by using a known external source, *i.e.*, CO_2 cylinders (*9964-037* 12 g CO_2 cylinder, *LI-COR Inc.*, Lincoln, NE, USA) at a concentration of 509 ppm. For the measurement of gas-exchange parameters through *LI-6400XT* portable photosynthesis system, the average photosynthetically active radiation fixed was 1,200 µmol(photon) m^{-2} s^{-1}. Chl fluorescence was evaluated on the same leaves, which were used for gas-exchange measurements, by employing the plant efficiency analyzer (*PEA*, *MK2*, *9,414*, *Hansatech Instruments Ltd*., Norfolk, UK). By placing clips on the adaxial surfaces, leaves were adapted in dark for 30 min, and after that exposed to red light (650 nm) at saturating irradiance of 3,000 µmol(photon) m^{-2} s^{-1}. Minimal fluorescence (F_0) and maximum fluorescence (F_m) were measured and variable fluorescence ($F_v = F_m - F_0$), the maximum quantum yield of PSII (F_v/F_m), electron transport rate through PSII (F_m/F_0), and the activity of PSII (F_v/F_0) were calculated as reported by Singh *et al*. (2016).

Pigments and UV-B absorbing compounds: The content of pigments such as Chls and carotenoids was analyzed as given in Takshak and Agrawal (2015). Leaves samples were homogenized in 80% acetone and then centrifuged for 15 min at 5,000 × *g*. The absorbance of the extract was noted at 645 and 663 nm for Chl content and at 480 and 510 nm for carotenoids content using a double beam spectrophotometer (*Model 2203*, *Systronics*, India). Further, Chl *a*/*b* was calculated. The UV-B absorbing compounds (anthocyanins and flavonoids) were determined by the spectrophotometric assay using the methods of Gitelson *et al*. (2007) and Flint *et al*. (1985), respectively. For estimation of anthocyanins content, leaf tissue (0.1 g) was macerated in 10 mL of methanol (which contained 1% calcium carbonate and a drop of hydrochloric acid). The extract was centrifuged at 6,000 × *g* for 20 min and filtered. After that, the absorbance of the extract was measured at 535 and 650 nm on double beam spectrophotometer (*Model 2203*, *Systronics*, India). For analysis of flavonoid content, 0.1 g of leaf tissue was extracted in 10 mL of methanol (80%) and centrifuged at 6,000 × g for 15 min, and then filtered. The reaction mixture containing 1 mL of extract, 0.5 mL of potassium acetate (120 mM) and aluminium chloride (1.2%) was incubated at room temperature for 30 min and the absorbance of the reaction mixture was recorded at 450 nm on double beam spectrophotometer (*Model 2203*, *Systronics*, India). The standard of quercetin (*Sigma-Aldrich*) was used for the estimation of flavonoid content.

Transmission electron microscopy (TEM) analysis: To visualize the ultrastructural changes in leaves of *A. vasica*, transmission electron microscopy (TEM) analysis was carried out. Leaves from control and treatments were cut into 1 × 1 mm (by avoiding midrib) and then fixed at 4°C in Karnovsky's fixative (made by 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4) using vacuum infiltration followed by washing with chilled 0.1 M sodium phosphate buffer, pH 7.4. The sections were subjected to secondary fixation using buffer and osmium tetroxide (1%). Then leaf sections were dehydrated in graded series of acetone and fixed in resin (epoxy resin). Furthermore, the sections (ultrathin) were cut using cryo-ultramicrotome (*UC7*, *Leica*) and then stained with lead citrate and uranyl acetate and photographed with a microscope (transmission electron microscope; *Talos L120C*, *FEI Company-Thermo Fisher Scientific Inc*., Massachusetts, USA).

Cr content and dry mass: The dry mass of *A. vasica* was determined in terms of root (RDM) and shoot dry mass (SDM). From each treatment, five plants were harvested randomly. After the removal of soil carefully, it was washed with water and separated into different parts such as roots and shoots (shoots comprised of the stem, leaf, and flower). Separated plant parts from each treatment were dried in an oven at 70°C until a fixed mass was achieved, and after that plant parts were weighed. The root-to-shoot ratio (RSR) was calculated by dividing the dry mass of roots and shoots. For estimation of Cr content, dried samples of leaf, stem, flower, and root were digested in a di-acid (HNO_3 and $HClO_4$ in a ratio of 9:4) mixture. The Cr content was determined in digested samples using atomic absorption spectrophotometer (*Analyst-800*, *Perkin Elmer Inc*., Norwalk, CT, USA) as detailed by Gautam *et al*. (2017). The translocation factor (TF; determined as a ratio of Cr in the shoots to Cr in roots) and the bioconcentration factor (BCF; as a ratio of Cr in roots to Cr in soil) were calculated as reported in Gajić *et al*. (2020).

Vasicine content was analyzed at the reproductive stage (180 d) because, in our earlier study, we found maximum alkaloids (vasicinone and vasicoline) at the reproductive stage of *A. vasica* (Pandey *et al*. 2021). Air-dried samples of leaf, stem, flower, and root from control and treatments were powdered using an electric grinder machine. Powdered samples of each part (1 g) from each treatment were extracted separately in 10 mL of HPLC-grade methanol for 72 h with continuous shaking at 25°C, followed by centrifugation at 5,000 × *g* for 20 min. Extracts were filtered through a 0.45-µm filter (*Millipore*, Billerica, MA, USA). Vasicine content in extracts was determined as reported in Bhambhani *et al*. (2012) using an HPLC system (*Waters*, Milford, MA, USA) with a photodiode array detector. The column used was the *C18-Nova Pak* column (250 × 4.6 mm, 5-µm particle size). The mobile phase used was methanol:water (40:60, V/V) and the separation was performed by the isocratic elution method. The flow rate was 0.7 mL min^{-1}, injection volume was 20 µL, and the wavelength of detection was 298 nm. The calibration curve was prepared from a standard of vasicine (*Sigma-Aldrich*). The stock solution was made by dissolving vasicine (1 mg) in 1 mL of HPLC-grade methanol (1 mg mL^{-1} stock solution). From the stock solution five additional calibrations from 20 to 100 µg mL^{-1} were prepared.

Statistical analysis: The data were represented as mean ± SE. To compare the mean of four sets (control, Cr, eUV-B, and Cr+eUV-B) one-way analysis of variance (*ANOVA*) was performed by applying *Tukey*'s *post hoc* test. The data of reproductive stage (180 d) of gas exchange, chlorophyll fluorescence, pigments, UV-B absorbing compounds, BCF, TF, dry mass, and total vasicine content (obtained by adding vasicine content in all plant parts) were subjected to bivariate *Pearson*'s correlation analysis to identify the relationship between various parameters. *GraphPad Prism version 8* was used for making graphs. The statistical analysis was carried out by *SPSS Inc. version 21.0* (*IBM Corp.*, Armonk, NY).

Results

Gas exchange and chlorophyll fluorescence: P_N was significantly reduced under treatments as compared to control at all of the growth stages with a maximum reduction under Cr+eUV-B treatment (25.5%) followed by Cr (19.1%) and eUV-B (10.4%) at 180 d (Fig. 1). Significant reduction in g_s was observed in comparison to control under Cr and Cr+eUV-B at all stages with a maximum reduction under Cr (25.9%) and Cr+eUV-B (33.2%) at 180 d. Further, C_i showed the same trend as P_N and g_s and was reduced by 23.8 and 29.8% under Cr and Cr+eUV-B treatments, respectively at 180 d. *E* was significantly reduced under treatments at all growth stages of *A. vasica* with a maximum reduction of 34.9% under Cr+eUV-B at 60 d (Fig. 1).

The Chl fluorescence parameters such as F_0 showed a significant increase under Cr and Cr+eUV-B treatment with the highest increase by 23 and 33.3% for Cr and Cr+eUV-B treatment, respectively, at 180 d. In contrast, F_v and F_m showed significant reduction under Cr and Cr+eUV-B treatment as compared to control at all stages (Fig. 1). F_v/F_m, which is an important indicator of stress and ranges from 0.84 to 0.72, showed significant reduction under Cr and Cr+eUV-B with a maximum reduction by 5.9 and 10.4% under Cr and Cr+eUV-B treatment, respectively, at 180 d. F_v/F_0 and F_m/F_0 also showed a significant reduction under Cr and Cr+eUV-B treatment with a maximum reduction of 40.2 and 32.5%, respectively, under Cr+eUV-B treatment at 180 d. All of the analyzed Chl fluorescence parameters showed nonsignificant variations under exposure to individual eUV-B treatment in comparison to their respective control at all of the growth stages (Fig. 1).

Pigments and UV-B absorbing compounds: Chl content was reduced under treatments as compared to control at all stages with a maximum reduction under Cr+eUV-B (18.3%) followed by Cr (15.8%) and eUV-B (6.9%) at the initial growth stage of *A. vasica* (Fig. 2). Chl *a*/*b* increased under eUV-B treatment at all stages with the highest increment at 180 d whereas this ratio showed reduction under Cr and Cr+eUV-B treatment with a maximum reduction of 12.5 and 12.7%, respectively, at 120 d. Carotenoids content showed the same trend of variations among treatments as the Chl *a*/*b*, however, stagewise it showed the opposite trend as the carotenoids content increased maximally under eUV-B at 120 d whereas it was reduced maximally under Cr and Cr+eUV-B treatment at 180 d (Fig. 2).

Flavonoids and anthocyanins significantly increased under eUV-B and Cr+eUV-B as compared to control at all of the growth stages with a maximum increment under Cr+eUV-B in anthocyanins (21.9%) at 60 d and in flavonoids (22%) at 180 d. However, under individual Cr treatment, both flavonoids and anthocyanins content showed nonsignificant variations (Fig. 2).

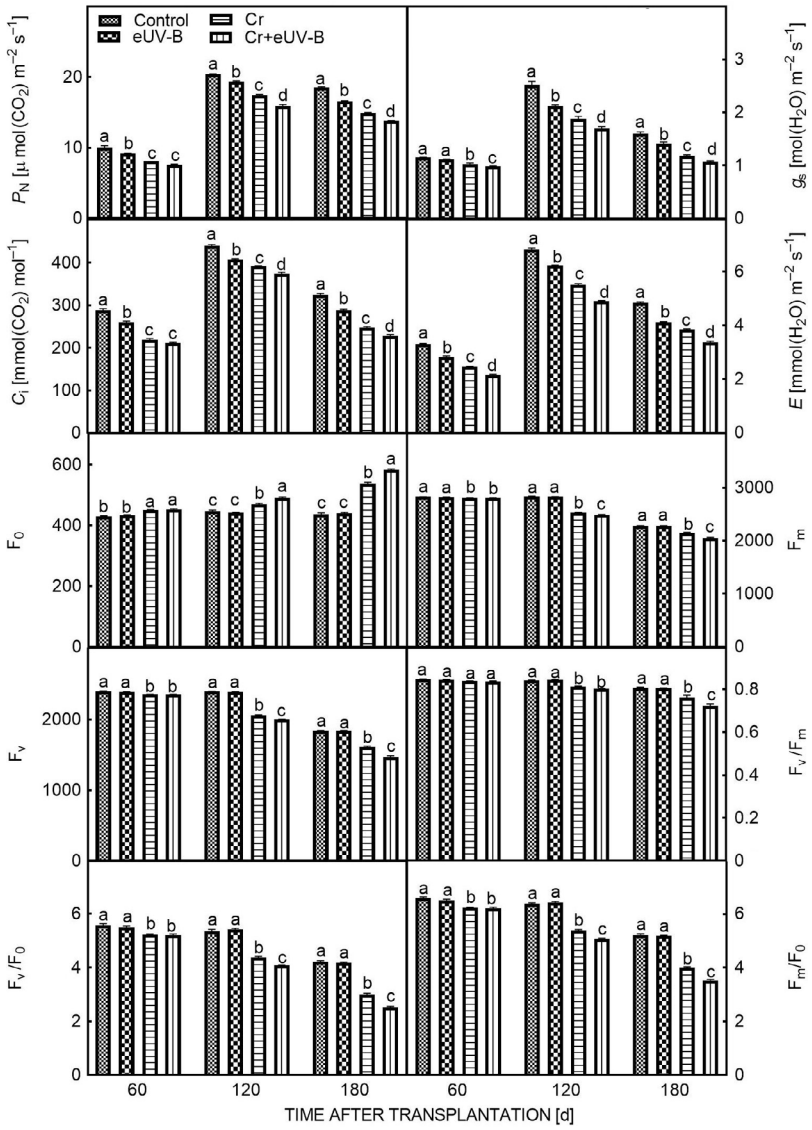

Fig. 1. Photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), transpiration rate (E), minimal fluorescence (F_0), maximum fluorescence (F_m), variable fluorescence (F_v), the maximum quantum yield of PSII (F_v/F_m), the activity of PSII (F_v/F_0), and electron transport rate through PSII (F_m/F_0) at three growth stages under control, eUV-B, Cr, and Cr+eUV-B treatments. Values are mean ± SE, n = 5. *Different letters* indicate significant differences between the treatments at each sampling stage according to *Tukey*'s test at p<0.05.

Ultrastructural changes: TEM analysis of *A. vasica* leaves showed well-organized chloroplast with dense thylakoids, grana, and one or two starch grains and plastoglobuli under control conditions. Most of the area of chloroplast was occupied by large-sized starch grain and several plastoglobuli under eUV and Cr+eUV-B treatment. Chloroplasts showed disorganized grana with several large-sized plastoglobuli under Cr treatment as compared to the control ones (Fig. 3).

Cr content and dry mass: The content of Cr in soil ranged from 42.5 to 78.4 mg kg^{-1} and was the highest under Cr+eUV-B treatment. In different parts of *A. vasica* analyzed, Cr was in the order of root > stem > leaf > flower (Fig. 4). The content of Cr significantly increased under individual Cr and Cr+eUV-B treatment with a maximum increase under Cr+eUV-B treatment in all parts of *A. vasica*. However, under eUV-B treatment, the content of Cr showed nonsignificant changes in soil and different parts of *A. vasica*. Both the BCF and TF ranges below 1 and significantly increased under Cr and Cr+eUV-B treatment as compared to the control (Fig. 4).

Both SDM and RDM showed significant reduction under treatments in comparison to control with the highest reduction under Cr+eUV-B followed by Cr and eUV-B treatment. Further, the RSR also showed a significant reduction under all treatments as compared to the control (Fig. 4).

Vasicine content: In different parts of *A. vasica* analyzed, vasicine content was the highest in leaf followed by stem, flower, and root. Vasicine content increased in all parts under all the treatments with the maximum in the root (27%) followed by the leaf (23.1%) under the Cr+eUV-B treatment (Fig. 5).

Fig. 6 represents correlation among different parameters of *A . vasica*. Further, an overview of the individual and interactive effects of Cr and eUV-B (at 180 d) on test plant is illustrated in the Fig. 7.

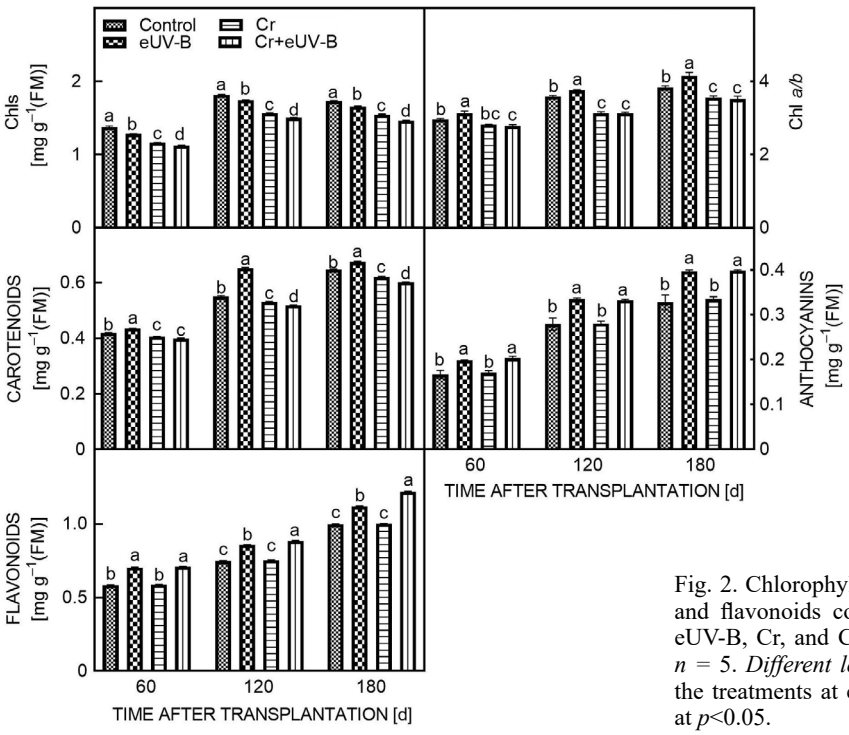

Fig. 2. Chlorophylls (Chls), Chl *a*/*b*, carotenoids, anthocyanins, and flavonoids content at three growth stages under control, eUV-B, Cr, and Cr+eUV-B treatments. Values are mean ± SE, n = 5. *Different letters* indicate significant differences between the treatments at each sampling stage according to *Tukey*'s test at $p<0.05$.

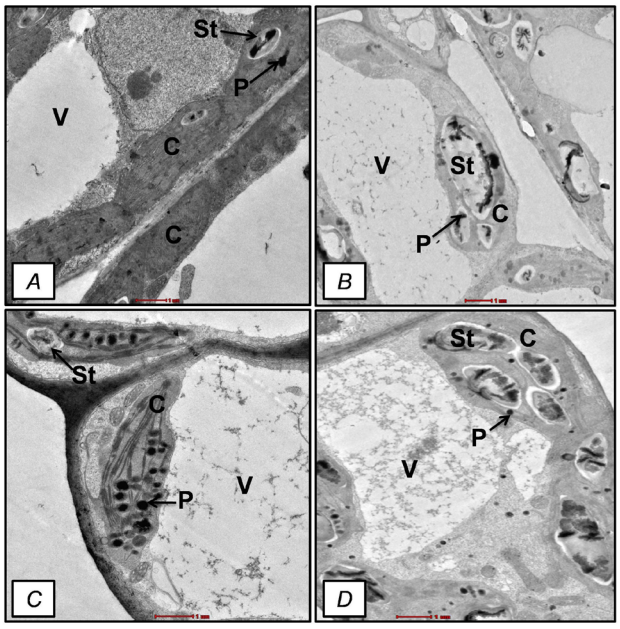

Fig. 3. Transmission electron microscopy (TEM) images of *Adhatoda vasica* under (*A*) control, (*B*) eUV-B, (*C*) Cr, and (*D*) Cr+eUV-B treatments at 180 d (time after transplantation). C – chloroplast; St – starch; V – vacuole; P – plastoglobuli. Scale bar – 1 µm.

Discussion

The present study depicted that *A. vasica* showed the variable responses under individual and combined stress of eUV-B and Cr. Under abiotic stresses such as Cr and UV-B, the photosynthetic rate of plants get altered by several factors including stomatal and nonstomatal factors, such as stomatal conductance, intercellular CO_2 concentration, loss of Rubisco activity, carboxylation efficiency, reduction in Chl content, damage to chloroplast ultrastructure and the photosynthetic electron transport (Tripathi *et al*. 2012, Sharma *et al*. 2020). However, the responses of plants vary depending on cultivars, developmental stage of the plant, genetic setup, and other environmental variables (Zhao *et al*. 2015). In our study, a reduction in P_N was observed under individual and combined stress, however, the reduction was more severe under combined Cr+eUV-B stress (Fig. 7) at a later growth stage which might be explained as the effect of eUV-B was aggravated by the concurrent action of Cr stress. Reduction in g_s was found to be associated with reduced C_i which in turn might regulate the P_N (stomatal limitation) as showed by the positive correlation between P_N, g_s, and C_i (Fig. 6). In line with the present study, reduction in P_N, g_s, and C_i under eUV-B and Cr were also reported by other studies (Subrahmanyam 2008, Jaiswal and Agrawal 2021). Reduction in E under treatments with maximum under combined Cr+eUV-B treatment might be seen as an adaptive strategy of *A. vasica* to cope with the excess of Cr and reduced water content (Vernay *et al*. 2008).

The Chl fluorescence parameter F_0 denotes the minimal fluorescence yield, and it occurs when all reaction centers are in an active 'open' state, whereas F_m denotes the maximum fluorescence yield, and occurs when all of the reaction centers are in an inactive 'close' state. In the present study, an increment in F_0 was observed under Cr and Cr+eUV-B treatment at all growth stages which

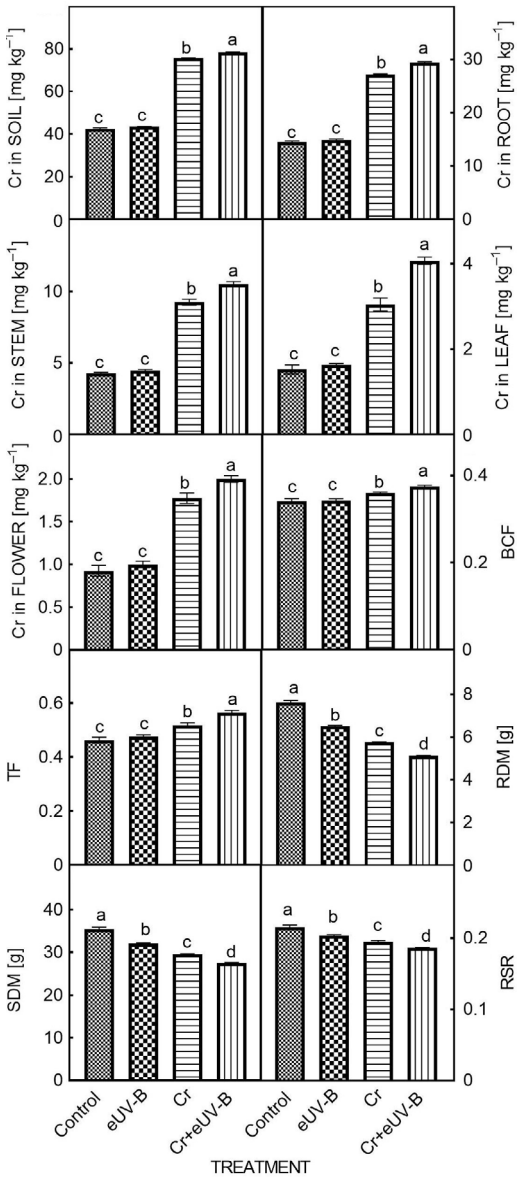

Fig. 4. Chromium (Cr) content in soil, root, stem, leaf, and flower, and the bioconcentration factor (BCF), translocation factor (TF), root dry mass (RDM), shoot dry mass (SDM), and root-to-shoot ratio (RSR) at 180 d (time after transplantation) of *Adhatoda vasica*. Values are mean ± SE, n = 5. *Different letters* indicate significant differences between the treatments at each sampling stage according to *Tukey*'s test at p<0.05.

indicated the impairment in PSII reaction centers' energy trapping efficiency or disconnection of antennae from the reaction centers (Li *et al*. 2015). Reduction in F_m under Cr and Cr+eUV-B revealed a change in the thylakoid membrane ultrastructure that affects the electron transport rate (Vernay *et al*. 2008). F_v/F_m is an important and frequently used indicator of photoinhibition or any other stress caused to PSII (Vernay *et al*. 2008), furthermore, F_v/F_0 and F_m/F_0 are also used to analyze the photosynthetic efficiency of a plant under stress conditions. In the present study, reduction in F_v/F_m, F_v/F_0, and F_m/F_0 under Cr and Cr+eUV-B showed that the maximum quantum efficiency of PSII, the activity of the PSII, and the electron transport rate through PSII were affected leading to decrease in these ratios and the structural and functional alteration in photosynthetic processes (Singh *et al*. 2016). Nonsignificant variations in Chl fluorescence parameters at all of the growth stages under eUV-B showed the absence of any photoinhibition or photodamage to PSII which was in accordance with the study of Martínez-Lüscher *et al*. (2013) on *Vitis vinifera* and Jaiswal and Agrawal (2021) on *Curcuma caesia*. In contrast, Singh *et al*. (2016) found a reduction in Chl fluorescence parameters under all treatments.

A marked reduction in Chl content observed under eUV-B, Cr, and Cr+eUV-B treatment at all growth stages in our study complied with earlier studies made on UV-B and Cr (Vajpayee *et al*. 2000, Vernay *et al*. 2008, Takshak and Agrawal 2015, Pandey *et al*. 2021). Vajpayee *et al*. (2000) reported that Cr impaired the biosynthesis of Chl which resulted in a reduction of Chl content in *Nymphaea alba*. Cr can degenerate δ-aminolevulinic acid dehydratase (which is an important enzyme of the Chl biosynthesis pathway) which in turn affects the utilization of δ-aminolevulinic acid (ALA) resulting in the buildup of ALA and reduction of Chl content. Kataria *et al*. (2014) also revealed that reduction in Chl occurs due to the destruction of Chl or its reduced synthesis under UV-B exposure. In addition to stomatal limitation, the reduction in Chl content might be also one of the reasons for a reduction in P_N in the present study (Fig. 6; as P_N and Chl showed a positive correlation). Chl *a*/*b* increased under eUV-B whereas decreased under Cr and Cr+eUV-B treatments which denotes that the content of Chl *b* was affected more than Chl *a* under eUV-B. Under Cr and Cr+eUV-B stresses, Chl *a* was impacted more than Chl *b*. In accordance with our study, Chl *a*/*b* increased under UV-B stress in bean, barley, radish, and corn whereas

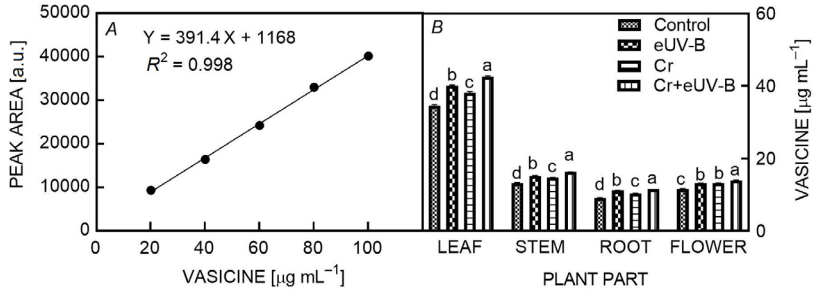

Fig. 5. High-performance liquid chromatography (HPLC) analysis of *Adhatoda vasica*. (*A*) Calibration graph of vasicine, (*B*) vasicine content in leaf, stem, root, and flower under control, eUV-B, Cr, and Cr+eUV-B treatments at 180 d (time after transplantation). Values are mean ± SE, n = 5. *Different letters* indicate significant differences between the treatments according to *Tukey*'s test at p<0.05.

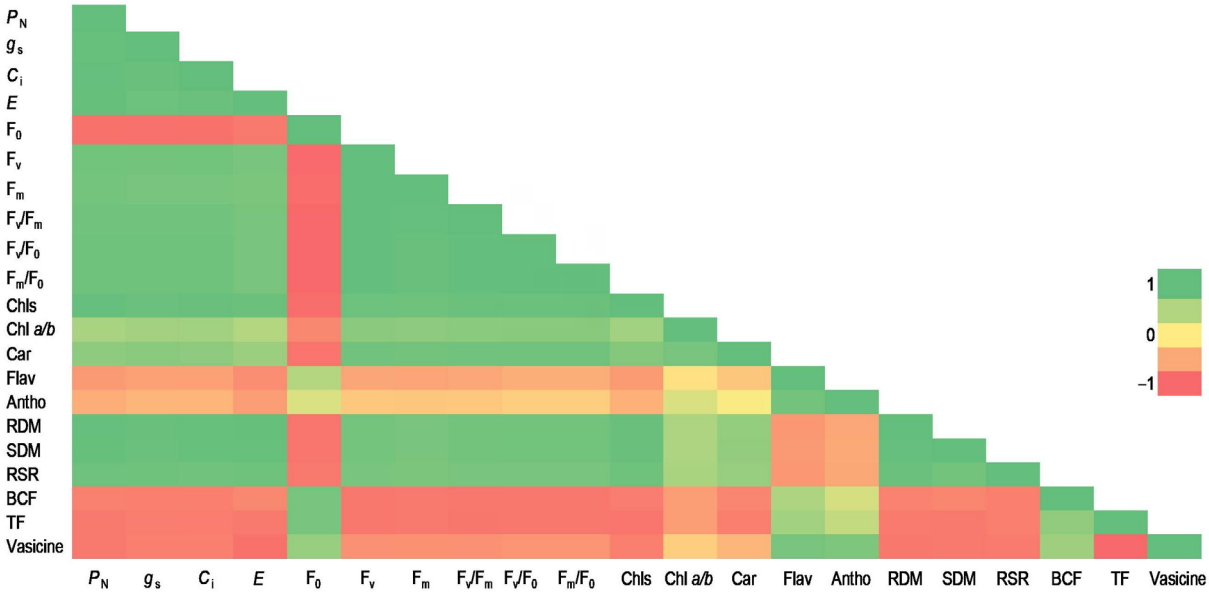

Fig. 6. Correlation matrices based on *Pearson*'s correlation coefficient between physiological parameters (gas exchange, chlorophyll fluorescence, pigments, and UV-B-absorbing compounds), dry mass, bioconcentration factor, translocation factor, and the total vasicine content at 180 d (time after transplantation) of *Adhatoda vasica*. A *Pearson*'s coefficient closer to 1 indicates a positive correlation, closer to 0 means no correlation, and closer to –1 shows a negative correlation. P_N – photosynthetic rate; g_s – stomatal conductance; C_i – intercellular CO_2 concentration; E – transpiration rate; F_0 – minimal fluorescence; F_m – maximum fluorescence; F_v – variable fluorescence; F_v/F_m – maximum quantum yield of PSII; F_v/F_0 – the activity of PSII; F_m/F_0 – electron transport rate through PSII; Chls – chlorophylls; Chl *a*/*b* – chlorophyll *a*/*b* ratio; Car – carotenoids; Flav – flavonoids; Antho – anthocyanins; RDM – root dry mass; SDM – shoot dry mass; RSR – root-to-shoot ratio; BCF – bioconcentration factor; TF – translocation factor; Vasicine – total vasicine content.

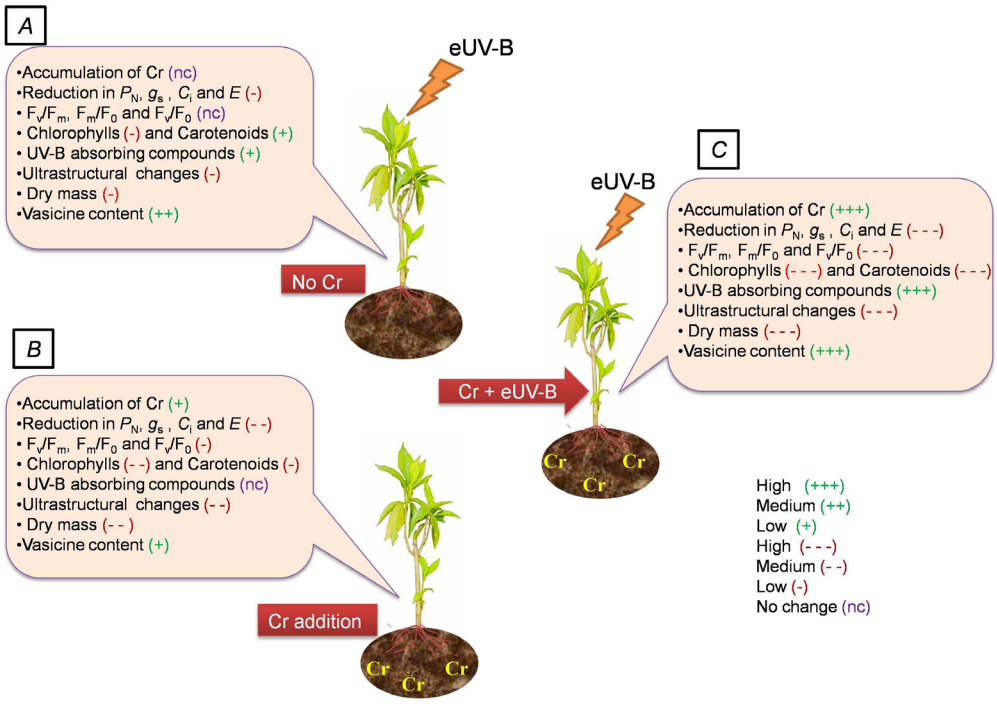

Fig. 7. An overview of the effects of (*A*) eUV-B, (*B*) Cr, and (*C*) Cr+eUV-B treatments on *Adhatoda vasica* at 180 d (time after transplantation). Cr – chromium; eUV-B – elevated UV-B; Cr+eUV-B – the combined effect of Cr and eUV-B; P_N – photosynthetic rate; g_s – stomatal conductance; C_i – intercellular CO_2 concentration; E – transpiration rate; F_v/F_m – maximum quantum yield of PSII; F_v/F_0 – the activity of PSII; F_m/F_0 – electron transport rate through PSII.

a reduction in Chl *a*/*b* under Cr stress in *Nymphaea alba* was observed (Tevini *et al*. 1981, Vajpayee *et al*. 2000). However, contrasting results were also reported in some other studies under Cr and UV-B stresses (Rai and Mehrotra 2008, Vernay *et al*. 2008). Carotenoids protect Chls from photooxidative destruction by dissipating excess light and heat energy. An increment in carotenoids content with a subsequent increase in Chl *a*/*b* under eUV-B treatment might have provided some protection to *A. vasica* under eUV-B treatment (as observed by a lesser reduction in P_N under eUV-B exposure as compared to Cr and Cr+eUV-B). In contrast, a reduction in carotenoids content was observed under Cr and Cr+eUV-B treatment in the present study. Li *et al*. (2014) reported a reduction in carotenoids content under all treatments, *i.e.*, UV-B, Cd, and Cd+UV-B with maximum under Cd+UV-B in soybean seedlings. Anthocyanins and flavonoids are considered UV-B absorbing compounds that help in the reduction of UV-B penetration deep inside the layer of leaves and also act as free radicals scavengers (Pandey *et al*. 2021). In the present study, anthocyanins and flavonoids increased under eUV-B and Cr+eUV-B whereas showed nonsignificant variations under Cr at all growth stages which were in support of the results obtained by Mishra and Agrawal (2006) under Cd/Ni and UV-B stress on *Spinacia oleracea*.

Our study emphasized that eUV-B, Cr, and Cr+eUV-B caused variable changes in the ultrastructure of chloroplast (Fig. 7). Increased number and size of starch grains as observed in our study under eUV-B and Cr+eUV-B might be due to impairment in biochemical processes of starch under stress conditions (Eleftheriou *et al*. 2015). It was reported by Steinmüller and Tevini (1985) that in normal conditions, chloroplast has few plastoglobuli (as observed under control in the present study) however, their number and size increased as thylakoid degraded, so it might be a plausible reason for increased number and size of plastoglobuli under treatments in the present study. Similarly, Gill *et al*. (2016) reported that damage to thylakoids increased the number of plastoglobuli and starch under Cr stress in *Brassica napus*. Changes in chloroplast ultrastructure were another potential reason for the reduction in Chl content that consequently leads to depression in photosynthesis as observed in the present study under stress conditions (Singh *et al*. 2013).

In the present study, higher content of Cr was accumulated in the roots as compared to other parts of *A. vasica*, which was corroborated well with several other findings (Shanker *et al*. 2005, Diwan *et al*. 2010, Srivastava *et al*. 2021). Higher accumulation of Cr in roots might be taken as one of the natural antitoxicity responses of *A. vasica* by sequestering the higher amount of Cr in the roots which lead to its lower translocation to aerial parts. It could also be plausible that the root serves as an interface between plant and soil, so being in direct contact with Cr, the root accumulates most of it and thus restricts its further mobility to aerial parts (Shanker *et al*. 2005, Diwan *et al*. 2010). In all parts, the highest Cr content was observed under combined (Cr+eUV-B) treatment which might have occurred due to altered permeability of the membrane, leading to increased uptake of Cr (Mishra and Agrawal 2006, Singh *et al*. 2016). In contrast, Regier *et al*. (2016) reported 30% reduction in Hg accumulation under combined treatment of Hg and UV-B as compared to Hg alone treatment. Further exposure of *A. vasica* to eUV-B alone treatment caused nonsignificant changes in the accumulation of Cr as compared to control which was following the study of Singh *et al*. (2016). BCF and TF in our study ranged below 1, which showed that *A. vasica* acts as phytostabilizer and accumulated most of Cr in its roots to avoid toxicity of aerial parts (Gajić *et al*. 2020).

The biomass or dry mass of a plant manifests the long-term integration of all of the physiological and biochemical processes. Teramura (1983) reported that even a minute effect of UV-B on physiological processes could result in a significant effect on the biomass of a plant. In addition, Cr toxicity also affects various physiological processes including the photosynthetic rate of plants, thereby resulting in reduced dry mass of plants (Singh *et al*. 2013). In the present study, reductions in SDM and RDM were found to be associated with a reduction in P_N (Fig. 6). The reduction induced by Cr+eUV-B was greater than that induced by Cr and eUV-B alone (Fig. 7) which was corroborated with the findings of Li *et al*. (2014), who noticed a maximum reduction in shoot and root dry mass under the interactive effect of UV-B and Cd on soybean seedlings. The reduction in RSR represents that the roots were affected more as compared to the shoots under all treatments in the present study. Similarly, Vernay *et al*. (2008) also noticed a higher reduction in roots as compared to shoots under Cr stress in *Datura innoxia*. The negative correlations of P_N and dry mass with that of BCF and TF (Fig. 6) as observed in the present study reflected that the accumulation of Cr (under Cr and Cr+eUV-B treatment) in plant parts might reduce P_N, which consequently led to the reduced dry mass of *A. vasica* (Fig. 7).

To grow and survive in adverse environments, plants possess a range of secondary metabolites. It has been revealed by various researchers that the accumulation of such metabolites often increases under stress conditions including Cr and UV-B (Vernay *et al*. 2008, Takshak and Agrawal 2015). Vasicine (important secondary metabolite belonging to pyrroquinazoline class of alkaloids) content was the highest in the leaf as compared to other parts and was found to be increased under all treatments in the present study. Furthermore, increment in vasicine content accompanied by reduced dry mass (as shown by the negative correlation between dry mass and total vasicine content; Fig. 6) might be explained as the reallocation of photoassimilates towards the increased synthesis of vasicine leading to reduced dry mass under stress conditions (Fig. 7). Takshak and Agrawal (2015) reported that increment in secondary metabolites as a defensive strategy of a plant may come at the expense of reallocation of assimilates and may cause the plant to compromise on its photosynthetic efficiency, dry mass, and yield depending on the efficacy and extent of defensive strategies of a plant.

Conclusions: The present study exhibited that individually and in combination, Cr and eUV-B caused differential

responses on *A. vasica* in terms of the accumulation of Cr, photosynthetic abilities, dry mass, and production of medicinally important secondary metabolite, with the impact being more prominent under Cr+eUV-B followed by Cr and eUV-B stress. Under the exposure of eUV-B, nonsignificant variations in chlorophyll fluorescence parameters and the lesser reduction in P_N, g_s, C_i, and Chl content with a concomitant increase in the carotenoids and UV-B absorbing compounds might offer some protection to *A. vasica* as evidenced from a lowered reduction in its dry mass. However, under combined stress (Cr+eUV-B), integrated and coordinated action of several factors (such as accumulation of Cr, higher reduction in most of the chlorophyll fluorescence parameters, Chl and carotenoids contents as well as ultrastructural changes and stomatal limitation) might have ultimately caused a higher reduction in P_N, that in turn leads to higher impact on dry mass of *A. vasica*. BCF and TF ranged below 1 which showed that *A. vasica* can act as phytostabilizer and accumulate most of Cr in its roots. Furthermore, an increment in the content of secondary metabolite vasicine under all treatments might be seen as a defensive strategy of *A. vasica.* Overall, considering the importance of *A. vasica* in pharmaceutical purposes and the higher accumulation of Cr in its roots, we can suggest promoting its cultivation in the area contaminated with Cr and having a high influx of UV-B. However, it is necessary to take care while consuming its plant parts especially leaves as such, because in that case, Cr content may be higher than the permissible limit. More research works are required on medicinal plants in this direction by incorporating different doses of UV-B and Cr in the natural environment.

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