Exogenous melatonin regulates chromium stress-induced feedback inhibition of photosynthesis and antioxidative protection in *Brassica napus* cultivars

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Abstract

**Key message** Melatonin is an early player in chromium stress response in canola plants; it promotes ROS scavenging and chlorophyll stability, modulates PSII stability and regulates feedback inhibition of photosynthesis conferring chromium tolerance.

**Abstract** The development of heavy metals, especially chromium (Cr)-tolerant cultivars is mainly constrained due to poor knowledge of the mechanism behind Cr stress tolerance. In the present study, two *Brassica napus* contrasting cultivars Ac-Excel and DGL were studied for Cr stress tolerance by using chlorophyll *a* fluorescence technique and biochemical attributes with and without melatonin (MT) treatments. Cr stress significantly reduced the PSII and PSI efficiency, biomass accumulation, proline content and antioxidant enzymes in both the cultivars. The application of MT minimized the oxidative stress, as revealed via a lower level of reactive oxygen species (ROS) synthesis (H$_2$O$_2$ and OH$^-$). Enhanced enzymatic activities of important antioxidants (*SOD*, *APX*, *CAT*, *POD*), proline and total soluble protein contents under MT application play an effective role in the regulation of multiple transcriptional pathways involved in oxidative stress responses. Higher NPQ and Y(NPQ) observed in Cr stress tolerant cv Ac-Excel, indicating that the MT-treated tolerant cultivar had better ability to protect PSII under stress by increasing heat dissipation as photo-protective component of NPQ. Reduced PSI efficiency along with increased donor end limitation of PSI in both canola cultivars further confirmed the lower PSII activity and electron transport from PSI. The Cr content was higher in cv. DGL as compared to (that in Ac-Excel). The application of MT significantly decreased the Cr content in leaves of both cultivars. Overall, MT-induced Cr stress tolerance in canola cultivars can be related to improved PSII activity, Y(NPQ), and antioxidant potential and these physiological attributes can effectively be used to select cultivars for Cr stress tolerance.

**Keywords** OJIP · Chlorophyll fluorescence · Melatonin · Antioxidants · Chromium stress · *Brassica napus*

**Abbreviations**

ANOVA Analysis of variance
APX Ascorbate peroxidase

CAT Catalase
DW Dry weight
EDTA Ethylenediaminetetraacetic acid
Fv/Fm Primary photochemistry of PSII

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**Introduction**

Rapid urbanization and anthropogenic activities have exacerbated environmental degradation and become a major constraint to crop production due to increased heavy metal accumulation in soil (Yang et al. 2019; Samanta et al. 2020; Islam et al. 2017). Chromium (Cr) is a non-essential toxic metal and its availability has aggravated pollution to agricultural soil and water, posing severe human health risks due to its involvement in the food chain (Ulhaßan et al. 2019; Islam et al. 2018). Hyper-accumulation of Cr in plant tissues causes severe phytotoxicity, with the consequences of reduced plant growth, biomass accumulation and chlorophyll degradation and increased reactive oxygen species (ROS) production. This increment in the production of ROS may damage the biological macromolecules and disrupt the antioxidant system in plants. Under oxidative stress, plants have antioxidant defense machinery (such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX), which helps in eliminating the toxic effects of ROS (Ahmad et al. 2020; Chen et al. 2016; Samanta et al. 2021). Metal-induced generation of ROS mainly occurs in chloroplast as superoxide around PSI via Mehler reaction, or singlet oxygen around PSII (Chaturvedi et al. 2020). Metal toxicity affects photosynthesis by inhibition of pigment biosynthesis, photosynthetic electron transport and Calvin–Benson cycle, by enhancing lipid peroxidation and disintegration of the thylakoid membrane (Ghori et al. 2019). Both light and dark reactions of photosynthesis are affected by poisoning of Cr metal (Banerjee and Roychoudhury 2021). In particular, this metal slows down the CO₂ fixation, mainly due to Rubisco inactivation, inhibits photosynthetic electron transport and causes damage to the photosynthetic apparatus (Rodriguez et al. 2012). In terms of PSII functional state, Cr minimizes turnover of the PSII reaction center D1 protein, alter 24 and 33 kDa proteins of the oxygen-evolving complex, disrupts electron flow from P680 to QA and affects PSII antenna variability (Rocchetta and Küpper 2009).

A large number of approaches are used to reduce metal toxicity damage in agricultural crops. One such approach is the foliar application of hormones to alleviate the metal stress environmental conditions (Bücker-Neto et al. 2017). Melatonin (MT) plays key physiological functions and protects plants against certain environmental stressors (Samanta et al. 2021). Previous studies have suggested that exogenously applied MT under Cr stress considerably enhanced plant growth, photosynthetic efficiency and antioxidants activity as well as reduced the chromium toxicity to crop plants (Altar et al. 2021). In another study, foliar application of MT in Brassica against chromium stress showed increased performance index (PI), indicating higher energy conservation of excited electron to reduce PSI (Ayyaz et al. 2020a).

*Brassica napus* is the main cash crop widely cultivated in both the North and South Hemispheres during annual or biennial seasons and has an annual value of USD 41 billion (Lohani et al. 2020; Zhou and Leul 1998). *B. napus* is considered a very interesting crop plant; because of its higher stress tolerance response, it is known for high metal accumulation and phytoremediation at very large scale. Brassica plants have developed higher stress tolerance mechanism, which enables them to grow well in metal-contaminated soil. The study's main goal is to understand (1) up to what extent

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**Abbreviations**

| Acronym | Meaning |
|---------|---------|
| FW      | Fresh weight |
| H₂O₂    | Hydrogen peroxide |
| MDA     | Malonaldehyde |
| Na₂HPO₄ | Disodium phosphate |
| NBT     | Nitro blue tetrazolium chloride |
| PBS     | Phosphate buffer solution |
| POD     | Peroxidase |
| PSI, PSII | Photosystem I, photosystem II |
| ROS     | Reactive oxygen species |
| RWC     | Relative water content |
| SOD     | Superoxide dismutase |
| TBA     | Thiobarbituric acid |
| TCA     | Trichloroacetic acid |
| TW      | Turgid weight |
| MT      | Melatonin |
| Cr      | Chromium |
| ETR     | Electron transport rate |
| PI      | Performance index |
| KI      | Potassium iodide |
| QA      | Quinone A |
| ASA     | Ascorbic acid |
| Fo      | Minimum fluorescence |
| Fm      | Maximum fluorescence |
| Mo      | Maximum rate of accumulation of closed reaction centers |
| N       | Maximum turnovers of QA reduction until Fm was reached |
| Fv/Fm   | Maximum quantum yield of PSII |
| Fv/Fo   | Efficiency of electron donation to PSII; absorption per reaction center at PSII/ratio of active reaction centers in PSII (Clark et al. 2000) |
| TRo/RC | Trapped energy flux per reaction center |
| ETo/RC | Electron transport flux per reaction center |
| DIo/RC | Dissipation energy flux per reaction center |
| PI_ABS | Performance index on absorption basis |
| NPQ    | Non-photochemical quenching |
| Y(NPQ) | Protective photochemical quenching |
| Y(1)   | Quantum yield of PS(I) |
| Y(II)  | Quantum yield of PS(II) |
| Y(ND)  | Donor site limitation of PS(I) |
MT application alleviate the adverse effects of chromium on growth of canola cultivars; (2) Whether ameliorative effect of MT application occurs by reducing oxidative stress or enhancing the antioxidative protection to canola cultivars; and (3) Up to what extent MT application modulate PSII and PSI activities, and electron transport using chlorophyll fluorescence measurement (4) to assess contribution of other physiological attributes in Cr stress tolerance in canola cultivars.

**Materials and methods**

**Plant material and growth conditions**

Seeds of canola cultivars Ac-Excel and DGL were obtained from Ayyub Agriculture Research Institute (AARI), Faisalabad. In this study, 40 plastic containers of diameter 28 cm and with 7 kg sand had ten seeds sown in each. Thinning was carried out leaving four healthy plants in each pot. Plants were sprayed twice a day with foliar application of melatonin MT (10 µM) on the lower and upper surface of the leaves until runoff before 1 week of Cr treatment. Based on our previous study (Ayyaz et al. 2020b), 28 days old canola plants were applied with chromium (K₂Cr₂O₇) salt solution (50 µM) with Hoagland’s nutrients for 14 days. Two cultivars, two Cr levels (0 and 50 µM) and two melatonin levels (0 and 10 µM) having five biological replicates (one replicate = one pot) with four technical replicates (one replicate = one leaf) were selected at the third leaf stage of the plants in the pot. The experiment was based on CRBD (completely randomized block design). After harvesting plants separated into root and shoot, data regarding fresh and dry biomass were measured.

Table 1 Morpho-physiological and biochemical attributes of *Brassica napus* cultivars when subjected to melatonin application grown under chromium stress conditions

| Parameters                  | AC-Excel     | MT 10 µM | Cr 50 µM | MT 10 µM+ Cr 50 µM | DGL          | MT 10 µM | Cr 50 µM | MT 10 µM+ Cr 50 µM |
|-----------------------------|--------------|----------|----------|-------------------|--------------|----------|----------|-------------------|
| Shoot fresh weight          | 17.13±1.51a  | 29.13±2.18b | 10.02±1.52c | 14.14±1.75bc      | 19.55±1.84a  | 21.19±1.79ab | 4.51±1.14c | 9.25±1.31d       |
| Shoot dry weight            | 2.26±0.21b   | 2.44±0.18a | 0.95±0.16ac | 1.65±0.15cd       | 1.8±0.22a    | 2.63±0.39b  | 0.64±0.04bc      | 1.20±0.08cd      |
| Root fresh weight           | 8.37±0.58a   | 9.07±0.53c | 4.73±1.12bc | 7.9±1.53b         | 7.25±0.35a   | 8.17±0.71b  | 3.87±0.45c       | 4.18±0.61bc      |
| Root dry weight             | 1.15±0.057a  | 1.18±0.062ab | 0.46±0.06b  | 0.62±0.15b        | 0.85±0.07a   | 0.96±0.073bc | 0.31±0.05ab      | 0.42±0.047cd     |
| Leaf number                 | 11.25±0.47a  | 12.50±0.70b | 5±0.85c    | 10.25±0.85bc      | 8±0.64a      | 12±0.75a    | 3±1.08bc         | 7.25±1.10c       |
| RWC                         | 80.10±6.54b  | 85.75±2.46c | 52.75±4.31cd | 64.75±9.97b      | 77±7.38ab    | 81.75±10.53b | 38.25±6.60bc     | 58±6.22cd       |
| Chlorophyll a               | 1.02±0.03ab  | 1.17±0.05b | 0.37±0.08bc | 0.6±0.07cd        | 1.1±0.09a    | 0.7±0.22b   | 0.49±0.12b       | 0.81±0.08c       |
| Chlorophyll b               | 0.24±0.01a   | 0.26±0.01ab | 0.07±0.03b  | 0.13±0.02bc       | 0.25±0.02a   | 0.24±0.02bc | 0.12±0.01c        | 0.15±0.02ac      |
| Total chlorophyll           | 1.7±0.19a    | 1.95±0.07b | 0.45±0.12cd | 0.6±0.13bc        | 0.8±0.17a    | 0.95±0.06ab | 0.34±0.15cd       | 0.45±0.11bc      |
| Carotenoids                 | 11.62±1.36a  | 8.9±0.54a  | 6±0.87bc   | 7.5±0.17ab        | 11.25±0.41a  | 8.45±0.93b  | 4.27±0.69c        | 6.02±0.18cd      |
| Xanthophyll                 | 0.14±0.006c  | 0.15±0.005ab | 0.14±0.001b | 0.15±0.001cd      | 0.15±0.002a  | 0.15±0.002b  | 0.13±0.006c       | 0.16±0.005c      |
| MDA                         | 15.3±4.2b    | 11.9±1.28a | 29.4±4.08ab | 20.2±3.26cd       | 9.99±3.48b   | 9.27±1.54ab | 51.11±1.62c       | 28.61±5.52       |
| H₂O₂                        | 18.20±4.78a  | 19.38±16.05b | 65.38±27.36ac | 40.64±4.68b      | 15.67±2.40b  | 12.02±5.38b | 82.98±9.04c       | 60.66±16.67      |
| SOD                         | 27.52±6.32cd | 46.71±6.17a | 85.00±13.17b | 125.32±13.15d     | 19.15±5.35bc | 22.12±5.94b | 78.36±8.35ab      | 108.56±17.91c    |
| POD                         | 0.018±0.005a | 0.016±0.004b | 0.014±0.002bc | 0.0031±0.004cd   | 0.015±0.003ab | 0.017±0.001b | 0.013±0.003c      | 0.016±0.002ac     |
| CAT                         | 0.64±0.007b  | 1.05±0.017ab | 0.32±0.220c | 1.72±0.281ac      | 0.18±0.148a  | 0.64±0.077b | 0.87±0.191cd       | 1.57±0.281c      |
| APX                         | 0.21±0.091a  | 0.58±0.088b | 1.44±0.088ab | 1.32±0.042c       | 0.57±0.042a  | 0.62±0.113a | 0.91±0.042bc       | 1.47±0.310c      |
| TSP                         | 4.55±1.21a   | 5.82±0.40a  | 11.77±0.12c | 12.40±1.11bc      | 7.53±0.87b   | 8.37±0.12ab | 12.11±0.14c       | 10.20±0.22bc     |
| Proline                     | 13.29±1.07b  | 62.02±7.16ab | 34.52±11.44a | 78.02±5.94c       | 7.53±1.36a   | 28.11±3.54bc | 18.88±1.90bc       | 41.11±4.10c      |
| Cr                          | 0.17±0.43a   | 0.002±0.02a | 18.02±6.05c | 13.72±3.29bc      | 0.2±0.06a    | 0.007±0.05b | 29.31±5.14bc       | 16.29±1.46cd     |

*ns non significance

*, **, ***Significant at 0.05, 0.01, 0.001 probability levels, respectively.
Quantum yield (Fv/Fm) measurements

For Fv/Fm measurement, mature leaves of canola plants were selected and kept in dark for about 30 min by using a handheld device Fluor Pen FT100 (Bano et al. 2021).

Leaf relative water content (RWC) measurement

For RWC evaluation, full mature leaves were randomly harvested and weighed for fresh weight measurement. After the leaves were thoroughly submerged in distilled water for 6 h, the leaf TW (turgid weight) was calculated. Then, after 24 h at 70 °C, leaf DW (dry weight) measured (Islam et al. 2016). Following the given formula, RWC was determined:

\[
\text{RWC} = \left( \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \right) \times 100
\]

Chlorophyll content

Chlorophyll pigments were extracted from 0.2 g of frozen mature leaves (pooled from one plant) in 80% acetone at room temperature. The extract was centrifuged at 2800g for 15 min, and the supernatant collected for absorbance measurement at 750, 663, 652, 645 and 470 nm by using a fluorescence spectrophotometer (Hitachi F-4600). (Zheng et al. 2020; Najeeb et al. 2011).

Proline, hydrogen peroxide (H₂O₂), malonaldehyde (MDA) and total soluble protein (TSP) analysis

Proline content was determined by following Bates et al (1973). For reagent toluene-containing chromophores aspirated from the aqueous process, spectrophotometric readings at 520 nm were taken. Using Zhou and Lin (1995) methods, the quality of H₂O₂ was determined. The H₂O₂ content was determined using a normal curve and the absorbance estimated at 390 nm. Lipid peroxidation was observed in terms of content of MDA, measured by using TCA and TBA as reagents, at 532 and 600 nm according to Zhou and Leul (1999). The total soluble protein (TSP) contents were measured using the Bradford (1976) procedure and bovine serum albumin as a reference.
Fig. 2  A Chlorophyll a; B chlorophyll b; C chlorophyll a/b; D total chlorophyll; E carotenoids. F Xanthophyll of *Brassica napus* L. cultivars under melatonin and chromium stress.
Biochemical analysis of enzymatic activities

Leaf tissue (0.5 g) was ground in a pre-cooled mortar and pestle in 50 mM PBS (pH 7.8) and centrifuged for 20 min at 16,128 g. The supernatant was collected in another tube and used for further study of the enzymes activity as “enzyme extract”. SOD activity assessed following Farooq et al. (2016), which is based on the ability of SOD to inhibit NBT photochemical reduction. The reaction mixture consists of 50 mM PBS (pH 7.8), 2 μM riboflavin, 13 mM methionine, 75 μM NBT and 0.1 mM EDTA and 100 μl enzyme extract in a total volume of 3 ml. The same unilluminated solution was used as a blank. One unit of SOD activity is the amount of enzyme required to cause 50% inhibition of the NBT reduction was measured at 560 nm. CAT activity was assessed according to the method of Aebi (1984) and POD activity assayed according to Li et al. (2019) with some modifications. The reaction mixture comprised 50 mM potassium phosphate buffer (pH 7.0), 1% guaiacol, 0.4% H₂O₂ and 100 μl enzyme extract. The oxidation of guaiacol was measured at 470 nm. APX activity was assessed by the H₂O₂-dependent oxidation rate of ascorbic acid (AsA) following the method of Gill et al. (2014).

OJIP fast chlorophyll transient test

Mature leaves of canola were kept in the dark for about 20 min covered with aluminum foil around them with saturating light pulse of 3000 mol m⁻² s⁻¹ light intensity for 0.8 s length exposed over a 4 mm leaf area by using hand-held chlorophyll fluorometer FluorPen FP-100 (Photon System Instruments, Czech Republic). Raw curves were plotted as XY scatter points with line plot against log x-axis. The data obtained were subjected to JIP-test and computed values were plotted as radar plot.

Rapid light curve analysis using PAM-fluorometer

At the third leaf stage, mature and healthy leaves from each plant were used for the assessment of PSII and PSI efficiency. Before taking measurements, the plants were dark adapted for 10 min by using aluminum foil. After 10 min of
dark adaptation, the aluminum foil was carefully removed and immediately the leaves were mounted to the measuring panel of the DUAL-PAM100 (Walz). Leaf absorbance for PSI at 830 and 870 nm was balanced first. A weak red light was used to measure Fo and then a saturated pulse of 8000 μmol m⁻² s⁻¹ with 0.8 s width was applied to measure Fm and maximum QY (Fv/Fm) of PSII photochemistry. Various intensities of actinic red light were used which increased gradually from 0, 11, 18, 27, 58, 100, 131, 221, 344, 536 and 830 in 20 s intervals over 5 min. The quantum efficiency of PSII, electron transport through PSII, non-photochemical quenching (NPQ), photoprotective component of NPQ as Y(NPQ) and nonregulated heat dissipation as Y(NO) were calculated (Hanelt et al. 2018; Maxwell and Johnson 2000).

**Estimation of chromium (Cr)**

Take 0.1 g of dried soil plant material in vials (digestion flask) and add 2 ml of digestion mixture for 10 h. Heat vials using hot plate at 50–200°C. Add an appropriate amount of HClO₃ (0.5 ml) into each vial having the samples using a plastic dropper. Vials cool down when the color of the mixture becomes clear (transparent). Dilute up to 10 ml to each sample with deionized water.

**Statistical analysis**

Data of rapeseed oil varieties of various parameters were subjected to complete randomization (three-way ANOVA) and LSD 0.05 level of confidence by using Co-STAT (COHORT Software, California, USA). All the data contain mean values with standard error.

![Fig. 4](image-url) A Malonaldehyde (MDA) content. B Hydrogen peroxide H₂O₂ content. C Proline estimation. D Total soluble protein contents of *Brassica napus* L. cultivars under melatonin and chromium stress.
Results

Genotypic variation of physiological responses to chromium stress

Plant growth attributes

Chromium (Cr) stress considerably ($P < 0.001$) declined the plants' growth attributes including fresh and dry biomass of plants (Table 1). The control and melatonin (MT) treatment against (0 and 50 µM) Cr stress effectively increased the plants' biomass (shoot dry weight) accumulation with maximum increase of 16%, 63% and 33%, 104% in tolerant Ac-Excel cultivar relative to 8%, 73% and 45%, 83% in sensitive DGL cultivar, respectively (Fig. 1). However, our results suggested that under Cr stress, cultivar Ac-Excel exhibited higher dry weight accumulation than that of DGL as compared to the control plants. Overall, Ac-Excel was found to be tolerant relative to DGL in terms of plant biomass accumulation under Cr stress (Fig. 1A–D).

Chlorophyll content, leaf number, relative water content (RWC) and quantum yield (Fv/Fm)

Photosynthetic pigments dramatically ($P < 0.001$) declined in both B. napus cultivars under Cr stress (Table 1). However, MT-treated plants under Cr stress showed higher photosynthetic pigments in Ac-Excel relative to sensitive DGL cultivar with different range of chlorophyll a (20%, 89%) and (48%, 92%), chlorophyll b (28%, 60% and 11%, 78%) and total chlorophyll (14%, 64% and 33%, 79%) in Ac-Excel. In DGL, maximum increase of chlorophyll a (15%, 53% and 7%, 59%), chlorophyll b (15%, 57% and 18%, 64%) and total chlorophyll (4%, 56% and 37%, 63%) was observed respectively, (Fig. 2A–D). In addition, carotenoids content in Ac-Excel showed different range, (8%, 53%) and (40%, 91%), compared to DGL, (14%, 49%) and (36%, 58%), whereas under Cr stress MT-treated and non-treated plants did not show significant effect on xanthophyll content as in Ac-Excel (7%, 9%) and (3%, 12%), but increased somehow in DGL with maximum increase of (2%, 8%) and (2%,19%) (Fig. 2E, 2F).
According to (Table 1), Cr stress considerably reduced the number of leaves of both Brassica cultivars. However, MT-treated plants of Ac-Excel showed significant increase in the number of leaves (7%, 45%, and 18%, 66%) relative to DGL with maximum increase of 4%, 34% and 20%, 62%, respectively (Fig. 3A). Table 1 shows that Cr caused significant reduction in RWC and was higher under MT treatment in Ac-Excel (17%, 56%, and 18%, 62%), relative to DGL (23%, 42% and 10%, 32%), respectively (Fig. 3B). Cr stress considerably reduced the yield of photochemistry of PS(II) in terms of quantum yield (Fig. 3C). However, under MT treatment (Fv/Fm), the values were significantly ($P < 0.001$) increased under control and chromium stress (7%, 63% and 35%, 104%) in Ac-Excel relative to (20%, 59% and 11%, 78%) in DGL, respectively (Table 1). Higher Fv/Fm values in Ac-Excel showed higher PSII efficacy including primary photochemistry that was found to be lowest in the DGL cultivar under chromium stress.

**Chromium (Cr) concentration**

The chromium content in leaves was found to be increased in Cr stress treatments. Increased Cr uptake significantly reduced the plant growth (root, shoot) by directly damaging the roots’ structure and function and as a consequence reduced nutrients’ uptake in Cr-treated plants (Table 1). There was higher Cr content accumulation in sensitive cultivar DGL compared to tolerant cultivar Ac-Excel leaves (0.12%, 1.20% and 51%, 19%) and (0.7%, 1.07% and 28%, 12%) under control and MT treatments, respectively, causing more damage to the root structure. However, MT-treated plants showed significant increase in plant growth along with reduced Cr ions uptake and translocation resulting in reduced Cr toxicity damage in *Brassica* plants (Fig. 3D).
MDA, H₂O₂ and proline contents

Chromium stress considerably affected the membrane lipid peroxidation and increased MDA level ($P < 0.001$), while MT-treated plants considerably reduced MDA content formations (Table 1) with maximum decrease (25%, 20% and 53%, 33%) in Ac-Excel compared to that in (7%, 4% and 74%, 41%) in DGL (Fig. 4A). Under Cr stress, H₂O₂ content was considerably ($P < 0.001$) increased in both canola plants (Table 1), but reduced under MT treatments (36%, 26% and 56%, 41%) in Ac-Excel and (12%, 9% and 83%, 38%) in DGL (Fig. 4B). Proline content significantly ($P < 0.001$) enhanced under Cr and MT treatments in *B. napus* cultivars compared to that of control plants (Table 1). Proline contents were observed to be higher (14%, 74% and 51%, 130%) in Ac-Excel, but decreased (39%, 74% and 56%, 88%) in DGL (Fig. 4C).

Total soluble protein and antioxidative enzymes assay

Cr stress induced changes in total soluble protein and antioxidative enzymes activities between *B. napus* cultivars including SOD, POD, CAT and APX. Generally, it was observed that under Cr stress, total soluble protein and antioxidant activates increased in Ac-Excel compared to that of DGL (Table 1). Application of MT increased the total
soluble proteins in both canola cultivars under chromium stress or control conditions. Moreover, MT-induced increase in total soluble proteins was higher in cv. Ac-Excel (27%, 35%, and 43%, 60%) than in cv. DGL (11%, 24% and 27, 41%) (Fig. 4D). However, SOD activity enhanced in a range of 51%, 66% and 77%, 89% in Ac-Excel and25%, 20% and 35%, 55% in DGL as compared to their respective control plants (Fig. 5A). Results (Table 1) indicated that POD activity was increased (21%, 65% and 75%, 91%) in Ac-Excel and (31%, 45% and 58%, 71%) in DGL as compared to the respective control plants (Fig. 5B). Similarly, CAT activity was increased (6%, 38% and(87%, 132%) in Ac-Excel and (40%, 58% and 58%, 71%) in DGL plants, respectively (Fig. 5C) (Table 1). APX activity showed a maximum increase (10%, 72% and 98%, 140%) in Ac-Excel compared to that (32%, 49% and 58%, 96%) in DGL plants, respectively (Fig. 5D). However, data suggested that Ac-Excel possesses greater antioxidative activity, indicating higher Cr stress tolerance than DGL.

**Fast chlorophyll fluorescence kinetics (OJIP)**

Cr stress remarkably reduced the PSII efficiency in terms of quantum yield observed as measured ratio of chlorophyll fluorescence parameters in two Brassica cultivars. Exogenously applied MT treatment under Cr stress clearly showed considerable difference in chlorophyll fluorescence kinetics as compared to their respective control condition. However, Cr stress-induced reduction in fluorescence kinetics was more obvious in sensitive cultivar DGL relative to tolerant cultivar Ac-Excel. Meanwhile, MT and Cr treatments showed significant increase in amplitude of the OP band (FT-Fo/Fm-Fo) as compared to the respective control plants in both tolerant Ac-Excel and sensitive DGL cultivars (Fig. 6A–D), respectively. According to our results under Cr stress reduced maximum number of turns over for QA reduction until Fm reached (N), rate of QA reduction (Mo) and maximum turnover of QA reduction until Fm reached (N). In addition, MT application against Cr stress substantially improved energy flux for absorption (ABS/RC), trapping energy (TRo/RC) and electron transport (ETo/RC) in both Brassica napus cultivars (Fig. 7). However, in contrast dissipation energy flux per reaction center (D1o/RC) remarkably enhanced in both genotypes under Cr stress, although decrease of energy flux per reaction center was more obvious in sensitive cultivar DGL relative to tolerant cultivar Ac-Excel. Moreover, both cultivars showed significant decrease in efficiency of electron donation to PSI (Fv/ Fo) and maximum quantum yield of PSII (Fv/Fm) under Cr stress. Cr stress considerably decreased the area between ‘Fo’ and ‘Fm’, which indicated the electron transport inhibition from the reaction center (RC) to plastoquinone (PQ) and performance index on absorption basis (PIABS) in both cultivars. However under MT and Cr treatments, tolerant cultivar “Ac-Excel” showed greater photosynthetic characteristics than sensitive cultivar “DGL” (Fig. 7A, B).

**Rapid light curve analysis**

Chromium stress-induced excessive energy loss or photoprotective heat dissipation of PSII and PSI was assessed by using DUAL-PAM-100. Cr stress remarkably reduced the PS(I), PS(II) quantum yield expressed in terms of [Y(I)], [Y(II)] (Fig. 8A–D) and electron transport rate ETR(I), ETR(II) (Fig. 8E–H) in both Brassica cultivars, respectively. However, tolerant cultivar Ac-Excel showed maximum increase in Y(II) and ETR(II) under Cr and MT treatments, relative to sensitive cultivar DGL as compared to their respective control plants, respectively. Similarly, under Cr and MT treatment, increase in NPQ was more obvious in cultivar DGL relative to cultivar Ac-Excel (Fig. 9A, B). The quantum yield of photoprotective heat dissipation [Y(NPQ)] was increased under Cr and MT stress in both Brassica cultivars, although cultivar DGL showed higher [Y(NPQ)] values than Ac-excel (Fig. 9C, D). Moreover, non-photoprotective heat dissipation [Y(NO)] was increased because of Cr toxicity damage to PSII, which was clearly decreased by MT application in Ac-Excel than in DGL relative to their control plants (Fig. 9E, F). To assess the exact damage site of PSI, limitations of donor or acceptor sites of PSI were observed. According to our results, Cr stress considerably decreased Y(ND) (the donor side limitation of PSI), while MT application effectively increased Y(ND), maximum increase was observed in tolerant cultivar Ac-Excel relative to sensitive cultivar DGL (Fig. 9G, H).

**Discussion**

In the present work, variation in Cr stress tolerance observed in canola cultivars. However, based on our previous study (Ayyaz et al. 2020a), two canola cultivars, Ac-Excel and DGL, were further evaluated. It is presumed that such variability in Cr stress tolerance in canola cultivars might have been because of variation in physio-biochemical attributes (Farooq et al. 2013; Li et al. 2018a, b). Based on some physiological parameters, such as chlorophyll content, TSP and Fv/Fm, and PSII and PSI efficiency, Ac-Excel is considered as Cr stress tolerant and DGL as Cr stress sensitive. In our study, foliar application of MT was considered an effective strategy for the alleviation of metal stress (Farouk and Al-Amri et al. 2019). Our results suggested that Cr stress reduced plant growth including fresh and dry weight in both canola cultivars. Against MT and Cr treatments, reduction in biomass was more obvious in sensitive cultivar “DGL” relative to tolerant cultivar “Ac-Excel”. The present findings
were in correspondence with Hasan et al. (2015), which suggested that Cr stress induced reduction in canola plants growth attributes associated with inhibition of plant cell turgidity, cell division, biosynthesis of cell wall, cell elongation and reduced relative water content. Similarly, data suggested that Cr stress significantly declined chlorophyll pigments in all canola cultivars (Table 1). It may happen because of higher chlorophyllase enzyme activity, oxidative pigment degradation, distortion of thylakoid membrane and improper uptake of nutrients (Ul hassan et al. 2019; Gill et al. 2015). Protochlorophyllid reductase decreased activity further lowers the chlorophyll synthesis under Cr stress that is considered the main reasons of reduction in photosynthetic pigments (Zhao et al. 2017). Meanwhile, Zhao et al. (2019) reported that exogenous application of MT prevents chlorophyll degradation and increases photosynthesis, antioxidant’s ability and Cr stress tolerance in cucumber seedlings. It is assumed that MT induced photosynthetic ability in plants is because of some unusual biostimulating pathway through regulation of photosystem II efficiency in certain light and dark conditions (Zhang et al. 2014; Wang et al. 2013). Cr stress has significant influence on photosynthetic efficiency of plant and Fv/Fm supposed as key indication of photosynthetic efficacy. Our results indicated that Cr stress considerably reduced the plants’ quantum yield (Fv/Fm) ratios and reduction in QY more pronounced in tolerant cultivar “Ac-Excel” (Fig. 2F). Generally, Fv/Fm is often confirmed by chlorophyll contents and cell shape, which may be affected by many factors, influencing the PSII activity and nutrient status.

Previous studies suggest the destructive effect of Cr on the reaction center and ETC, which consequently reduced net PSII quantum yield (Oukarroum et al. 2015). According to Ayyaz et al. (2020b) foliar application of MT under Cr stress prevents photosynthetic pigment degradation along with enhanced overall photosynthetic process in B. napus. The formation of ROS can also play a potential role in protein synthesis and gene expression regulation accompanied by plant stress defense. On the other hand, tremendous ROS generation is supposed to be cytotoxic and can affect the cell macromolecules such as phospholipids, protein, DNA, as well as other cell bodies (Hasan et al. 2015). The present study suggests that higher H2O2 contents in the sensitive cultivar leads to more lipid peroxidation, ultimately causing increased MDA content with consequent more cellular toxicity, causing cell death. A similar cellular effect was observed by Ding et al. (2017) where higher H2O2 content altered cellular structure, production and elimination of active oxygen radicals which cause cell death (Meng et al. 2014). Increased production of free radicals against Cr stress was also reported in B. napus species, which can cause severe membrane damage to Cr-sensitive crop plants. The generation of malondialdehyde (MDA) is the potential indicator of free radical formation in plant tissues under stress conditions. The present study indicated that elevated levels of MDA under Cr stress might lead to oxidative stress in B. napus plants, causing membrane leakage and loss of DNA, which results in severe damage to plant cell membrane. An increased lipid peroxidation against Cr stress was reported in maize cultivars (Ding et al. 2017). This study also showed higher MDA content formation especially in DGL relative to Ac-Excel, suggesting membrane damage of B. napus plants in response to Cr stress (Table 1). Cr stress affects the integrity of plasma membranes that are damaged by alteration in lipoxy gene activity in different crop plants, which ultimately causes cell death in plant tissues (Gill et al. 2015). However, previous studies suggested that MT-pretreated cucumber seedlings showed reduced ROS formation with enhanced antioxidants activity under Cu (Zhang et al. 2013).

Melatonin-induced regulation of photosynthetic attributes in canola cultivars was observed specifically in tolerant cultivar “Ac-Excel” relative to sensitive cultivar “DGL” that might involve its genetic potential, although its effects on the exact site of the photosynthetic apparatus is still unclear. Data of the present study suggested that both Brassica cultivars of the present study explained that primary photochemistry and photochemical quenching at the O–J and J–I step were reduced under Cr stress in both canola cultivars. MT application enhanced compensation reduction rate of PS(I) at the I–P phase, which consequently improved PSII quantum yield in both brassica cultivars. Decreased fluorescence values suggest Cr stress-induced reduction of energetic connectivity in the L-band. Increased chlorophyll fluorescence curve at the I–P phase suggested that MT application against Cr stress remarkably increased electron transport rate from PQH2 to secondary electron acceptor site of PS(I) and maintained plastoquinone redox state by transferring electrons to PS(I). These findings were in correspondence to Li et al. (2018a, b), who suggested that MT considerably enhanced PS(I) efficiency in wheat plants under cold stress, while Cr-treated plants showed decreased fluorescence curve at the I–P phase suggesting reduced PQ pool size, resulting in decreased redox potential, which may be because of sharp decline in plant water status as compared to control plants. However, this increase in I–P band was more pronounced in “Ac-Excel” which suggests the higher redox potential and quantum yield of PS(I) than of “DGL”. Similarly, Oukarroum et al. (2015) observed that under salt stress, higher
ROS accumulation causes reduction in quantum yield of PS(I) with enhanced photochemistry in *Limna gibba* plant.

Plants’ photosynthetic efficiency is considered very crucial as it directly contributes to plant growth and productivity against certain abiotic stressors. The photosynthetic capacity of plant chlorophyll fluorescence measurement under control and stressed conditions is considered as the best method for the evaluation of PSII efficiency (Maxwell and Johnson 2000). Cr stress severely affected the PSII functional and structural constancy, which resulted in reduced plant photosynthetic attributes in both *B. napus* cultivars. Our results were in correspondence with a previous study, which suggests that increased metal toxicity adversely affects the photosynthetic efficiency of crop plants (Goussi et al. 2018). Against Cr stress decreased *Fm* values indicate the denaturation of the antenna complex of PSII and reduced PSII efficiency, which result in maximum energy dissipation. This energy dissipation may occur due to reduced electron transport or poor quantum yield of PSII and PSI in both *Brassica* cultivars under Cr stress. However, MT application against Cr stress remarkably improved the electron transport rate ETR(I), ETR (II) and quantum yield *Y(I)*, *Y(II)* of PS(I), PS(II) in both *Brassica* cultivars respectively (Fig. 9).

This increase was more pronounced in tolerant cultivar “Ac-Excel” relative to “DGL” indicating MT induced structural and functional modulation of photosystem under Cr stress, while in contrast poor electron transport rate and quantum yield result in excessive energy dissipation in the sensitive cultivar “DGL” with increased NPQ and *Y(NO)* values, depicting higher energy loss and reduced PS(II) and PS(I) efficiency. This might have happened when the photosynthetic system was damaged at the end of Calvin cycle and all ferredoxins were reduced. Then, *Y(NO)* of Cr-stressed plants decreased and *Y(NA)* was enhanced. It caused photodamage by the production of ROS, which damaged the D1 protein of PSII, PSI and oxygen-evolving complex (Ruban et al. 2012). Cr increased NPQ and *Y(NPQ)*, and a greater increase in NPQ, *Y(NPQ)*, *Y(NO)* and *Y(NO)* was found in the tolerant cv Ac-Excel, indicating that the Cr stress-tolerant cultivar managed over-excitation of PSII by safe heat dissipation via the photoprotective component of NPQ. A decrease in PSI efficiency with an increase in donor end limitation of PSI in both *Brassica* cultivars further confirmed that the electron transport through PSII became downregulated. However, the Cr-sensitive cv. DGL had poor ability to manage overexcitation of PSII through buildup of *Y(NPQ)*, thereby causing greater oxidative stress with and without melatonin treatment (Ayyaz et al. 2021). Generally, non-photochemical quenching responds quickly and obstruct ROS formation by dissipating the light energy as heat from the antenna complex (Lambrechts et al. 2012). However, excessive light energy if not dissipated can be used in photosynthesis to cause photodamage in PSII. Hence, photoprotection is required for the efficient regulation of light harvesting to maintain optimum photosynthesis. So, in the antenna complex of PSII, non-photochemical quenching (NPQ) carefully dissipates the excessive light energy, whereas PSI is also protected from extra heat by non-photoprotective heat dissipation *Y(NO)* (Niyogi and Truong 2013). In the current study, *Y(NO)* enhanced in “DGL” under Cr stress to protect the plants from photodamage.

The present study indicated that Cr stress remarkably increased the plant antioxidative defense system of two *B. napus* cultivars. Cr stress stimulated the potential antioxidative defense enzymes such as SOD, POD, CAT and APX activities to reduce the oxidative stress in *B. napus* cultivars. CAT and APX, considered as potential antioxidative enzymes, are accompanied by *H2O2* removal in leaves (Jahan et al. 2019). Specifically, CAT splits *H2O2* into water and oxygen (Tang et al. 2018) and counteracts *H2O2* generation in peroxisomes by GOX activity during photorespiration, while APX protects chloroplast membrane integrity by reducing *H2O2* to water conversion through ascorbate oxidation (Li et al. 2018a, b). Reduced ascorbate regeneration is necessarily required for *H2O2* quenching and enzymes like APX participates in the ascorbate glutathione pathway, the central redox site in plants (Jahan et al. 2019). These findings are also supported by previous studies (Chen et al. 2018) which suggested that *B. napus* species exhibits higher CAT activities when exposed to salt stress. The free radical’s formation under Cr stress decreased in tolerant cultivar Ac-Excel, showing higher CAT activity compared to the sensitive cultivar DGL, while, surprisingly, SOD activity significantly increased in canola plants under Cr stress (Table 1). In plants, most of SOD activity occurs in chloroplast, mitochondria and cytosol and is responsible for the catalysis of highly oxidative anions to less harmful *H2O2* (Ni et al. 2018) Apparently, against Cr stress, increased SOD activity is independent of genotypes, though representing common shared ROS generating mechanism and in turn can be scavenged by genotype response and effective antioxidative responses. Previous studies suggested that MT application improved SOD activity in pepper seedlings when exposed to severe Cr stress. Enhanced SOD activity reveals significant protection against excessive ROS production under metal stress. Similarly, increased POD activity suggests enzymatic antioxidative protection against Cr stress in *B. napus* cultivars. However, the present study indicated higher POD activity against Cr stress. Similar results were experienced by Tang et al. (2018) in *Brassica* species against Cr stress.
Conclusion

The melatonin-induced observed differences suggest that metal tolerance capability of *Brassica* plants under Cr stress. Due to Cr toxicity, the plant growth, chlorophyll content and photosynthetic activity were reduced in canola plants. To overcome these stressful conditions, exogenous MT application can effectively increase the plant growth, development and PSII efficiency, reduce ROS generation and regulate stress tolerance by increasing antioxidant enzymes against Cr stress. The application of MT significantly reduced the uptake of Cr and promoted the different plant defense systems so that plants were able to cope with the excesses ROS and reduce the effects of oxidative stress. There is a need to focus on the exogenous application of growth-enhancing agents that enable plants especially agricultural crops to increase their yield and tolerance against toxic elements. Given that melatonin showed positive effect on plants, it is supposed that in future melatonin could have a potential role in developing photosynthetically efficient stress-tolerant transgenic crops.

Author contribution statement

HAR and MAH conceptualized, wrote and edited the manuscript and supervised the work. AA performed experiments. ZUZ helped in analyzing physiological parameters and provided technical and helpful discussions. All authors read and approved the manuscript.

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Declarations

Conflicts of interest The authors have no conflict of interest to declare.

Ethics approval This work complies with ethical standards.

Consent for publication All authors have read and approved the final manuscript.

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