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UNLOCKING THE ROLE OF MELATONIN TO ALLEVIATE NICKEL PHYTOTOXICITY IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

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Abstract

Nickel contamination in agriculture soils severely restricts sunflower growth and disrupts important physiological and biochemical process, making it necessary to identify effective approaches for improving plant tolerance. In this study, two sunflower cultivars, FH-701 (V1) and FH-741 (V2), were exposed to different nickel concentrations (0, 40, and 80 mg kg^{-1}) and treated with varying levels of exogenous melatonin (0, 25 and 50 µM) to evaluate its protective effects against nickel induced stress. The results showed that 50 µM melatonin application enhanced plant growth, increased chlorophyll content, improved antioxidant defense (SOD, POD, CAT), maintained better ion balance and reduced oxidative markers (MDA, H_2O_2) caused by nickel toxicity. According to this study, sunflower cultivar FH-741 had better growth as compared to FH-701. The results of present findings indicate that MEL is a plant growth regulator that has lessen Ni-induced oxidative damage in sunflower plants by altering secondary metabolites and antioxidant defense while maintaining ion homeostasis.

Key words: *Helianthus annuus*; Melatonin; Heavy metal stress; Antioxidant defense system; Ion homeostasis; Chlorophyll pigments

Introduction

In Pakistan, sunflower ranks fourth among oilseed crops after cotton, rapeseed, and mustard (Khan *et al.,* 2024). With approximately 70 species identified worldwide, sunflowers are plants that belong to *Helianthus annuus* and the Asteraceae family. (Nguyen *et al.,* 2021). As of 2017, about 16 million tons of sunflower oil were produced worldwide, with Russia and Ukraine being the largest producers. As a result, 51.9 million metric tons of sunflower were produced worldwide in 2024, with the following production rates for each country: Turkey with at least 0.65 million tons, Argentina with 1.65 million tons, Ukraine with 6.7 million tons, and Russia with 5.6 million tons (Bispo *et al.,* 2024).

Sunflowers (*Helianthus annuus* L.), a short-season plant native to North America, are now cultivated worldwide (Adeleke & Babalola, 2020). Sunflower seeds contain 40% oil and vitamins A, B, and K. Sunflower oil is considered beneficial for heart patients because it contains 90% unsaturated fatty acids. Sunflower seeds contain omega-3 fatty acid, 30 % of which are monounsaturated and 59% are polyunsaturated (Vasudha & Sarla, 2021). It contains mineral elements and phytochemicals such as dietary fiber, manganese, vitamins, tocopherols, phytosterols, triterpene glycosides, α-tocopherol, glutathione reductase, peptides, chlorogenic acid, caffeic acid, alkaloids, tannins, and saponins, which help to develop its functional and nutraceutical qualities (Aldalin *et al.,* 2024). However, sunflower production is increasingly challenged though several abiotic stresses, including heavy metal contamination, which can adversely affect plant growth, physiological processes and productivity (Chen *et al.,* 2021).

The water and land pollution by toxic heavy metals (HM) has become critical environmental concerns, deteriorating the growth and productivity of plants, biomass, grain yield, and crop quality (Rashid *et al.,* 2023). Nickel is an essential micronutrient required in trace amount for plant growth and metabolic functions. At high levels, nickel accumulation can become phytotoxic and prevent germination of seeds, inhibit photosynthesis, and disrupt the absorption of vital mineral nutrients (Hasanuzzaman *et al.,* 2021). Sunflower is one of the plants identified as a potential source in terms of absorption and removal of heavy metals in the environment (phytoremediation). Sunflowers have a high capacity to absorb and tolerate heavy metals as compared to other crops such as mustard, maize (Ullah *et al*., 2025), alfalfa, and *Ricinus* (Rizwan *et al.,* 2016).

Among these metals, Ni is of particular concern because its concentration in agriculture soil is increasing due to anthropogenic processes such as, application of phosphate-based fertilizers, smelting, wastewater sludge production, and electroplating. Elevated Ni become toxic and inhibit plant growth by impairing respiration, photosynthesis, uptake of mineral nutrients, and transport of assimilates and reduced production of dry mass (Ameen *et al.,* 2019).

Nickel toxicity disrupts the uptake of important soil nutrients like calcium, manganese, and iron and interferes with the activity of several metalloenzymes, leading to metabolic disorders (Sachan & Lal, 2017). The high amounts of Ni lead to oxidative stress through overproduction of toxic molecules by reactive nitrogen species (RNS), such as (NO) and ROS, such as hydrogen

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peroxide, ($OH^{\cdot}$), and (O^{-2}) (Espinosa-Vellarino *et al.,* 2020). This oxidative burst alters the cell membrane, causes lipid peroxidation, and damages biomolecules such as chloroplasts, proteins, pigments, enzymes, RNA, and DNA, resulting in impaired metabolic function (Garcia-Caparros *et al.,* 2021).

Plants respond defensively to metal stress by enhancing the activity of many antioxidant enzymes, including peroxidase (POD), ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT) to combat excessive ROS generation (Rajput *et al.,* 2021). However, under severe nickel stress, endogenous defense mechanism may not be sufficient to protect plant from oxidative damage (Abbas *et al.,* 2023). Consequently, the use of appropriate bio stimulants or signaling molecule is help for enhancing plant tolerance to heavy metal stress (Bartucca *et al.,* 2022). Melatonin (N-acetyl-5-methoxytryptamine) is a multifunctional plant growth regulator derived from tryptophan that has emerged as a powerful signaling molecule to increase tolerance to many abiotic stresses (Naqve *et al*., 2021). External application of plant growth regulators (PGR) is a useful strategy for improving plant growth and tolerance to various abiotic stressors. Melatonin, a tryptophan-derived multifunctional PGR, has recently expanded its role in modulating transcriptional feedback pathways to improve many abiotic stress tolerance processes in plants (Nawaz *et al.,* 2020). Exogenous MEL increase root formation, seed germination, osmotic adjustment, stimulation of photosynthesis, inhibition of leaf senescence and delayed fruit ripening by regulation of gene expression. (Sun *et al.,* 2021). Its protective effect has been reviewed under several abiotic stress including drought, salinity and heavy metal toxicity (Oyebamiji *et al.,* 2024). Recent reviews indicate that exogenous melatonin can alleviate metal toxicity by enhancing antioxidant enzyme activities, controlling nutrient uptake and prohibiting the accumulation and translocation of toxic metals within the plants (Chen *et al.,* 2026).

Melatonin foliar spray is quickly absorbed through leaves and eventually transported in plant thorough systematic signaling. This systematic movement allows melatonin to alter antioxidant metabolism, improve nutrient homeostasis and maintains the balance of essential elements including N, Mg, Mn, Ca, and Fe (Altaf *et al.,* 2021). Applying melatonin directly to the leaves, not only protect photosynthetic tissues from oxidative damage but also contribute to improved plant tolerance to heavy metal stress (Masoumi *et al.,* 2024). Exogenous melatonin has been reported to increase antioxidant defense and maintain photosynthetic activity under heavy metal stress, thereby reducing oxidative damage and improving plant growth (Khalid *et al.,* 2022). Recent evidence also showed that melatonin can adjust homeostasis of mineral nutrient and inhibit the harmful effects of heavy metal stress on photosynthetic structure and cellular metabolism (Altaf *et al.,* 2023). Therefore, the present study was conducted to evaluate the impact of melatonin as foliar application on morphophysiological and biochemical traits in sunflower cultivars FH-701, FH-741 under different level of nickel stress and to illustrate nickel phytotoxicity in sunflower.

Materials and Methods

An experiment was conducted to investigate the use of exogenous melatonin to measure physiological and biochemical changes in sunflowers under nickel stress. In January 2024, a pot trial was conducted in experimental area of the Botanical Garden of the University of Agriculture Faisalabad, Community College PARS. Sunflower seeds of two types, FH-701 and FH-741, were received from Oilseeds department at the Ayyub Agricultural Research Institute (AARI) in Faisalabad. Seeds were sown in plastic pots (30 cm deep and 24 cm diameter) with 8 kg of sandy-loam soil (7:3). The pots were placed in an open air to obtain the optimum temperature and amount of light. Soil moisture was calculated through the weight of 100 grams of fresh soil, then oven-dried at 105ºC for 24 h, and soil-dry weight was measured. Soil moisture content was calculated using following formula: soil moisture (%) = (soil fresh weight-soil dry weight / soil fresh weight) × 100. The physicochemical characterization of the soil indicated that the soil was non-saline/sodic (ECe = 2.04 dSm^{-1}) with pH 7.08. The soil contained about 0.86% organic matter, including nitrogen, phosphorus and potassium respectively. The initial background of nickel concentration in soil was 18.4 mg kg^{-1}. The experiment was arranged in a three-factor factorial completely Randomized Design (CRD) with three replications. The experimental factors included three nickel levels (0, 40, and 80 mg kg^{-1} soil), three melatonin concentrations (0, 25 and 50 µM), and two sunflower cultivars (FH-701 and FH-741) (Fig. 2). The experiment consisted of 3 × 3 × 2 =18 combinations of treatments × 3 replications resulting 54 experimental pots. Nickel was applied to the soil at 0 mg kg^{-1} (control), 40 mg kg^{-1}, and 80 mg kg^{-1} in the form of $NiCl_2.6H_2O$ to 40-day-old plants. After five days of Ni treatment, MEL was supplied as a foliar spray using hand sprayer, a fixed volume of 100 ml melatonin spray litter per pot was applied to ensure uniform foliage across treatments. Control plants received the same volume (100 ml/pot) of distilled water without melatonin, followed by two additional applications at five days after the plants reached 55 days of growth. Morphological, physiological, biochemical, ionic and enzymatic characteristics were determined after three to four weeks of melatonin treatment. Analysis of variance (ANOVA) was used to assess the impact of cultivars, melatonin concentrations, nickel levels and their interaction. All treatments maintained the same level of replication.

The graphical abstract and experimental setup were presented in Figs. 1, 2.

Morphological attributes: Plants were carefully uprooted, shoots and root length were measured using a measuring tape, and values are recorded in centimeters (cm). Samples of uprooted plants were measured for fresh weight of roots and shoots, using a digital weighing balance. Both samples were placed under sunlight for two-three days to measure the dry weight of shoot and roots. After that, samples were placed in an oven-dried at 80°C for two days. When the shoots and roots were completely dry, then dry weight was measured with an electronic weighing balance. Weight measurements were recorded in grams (g).

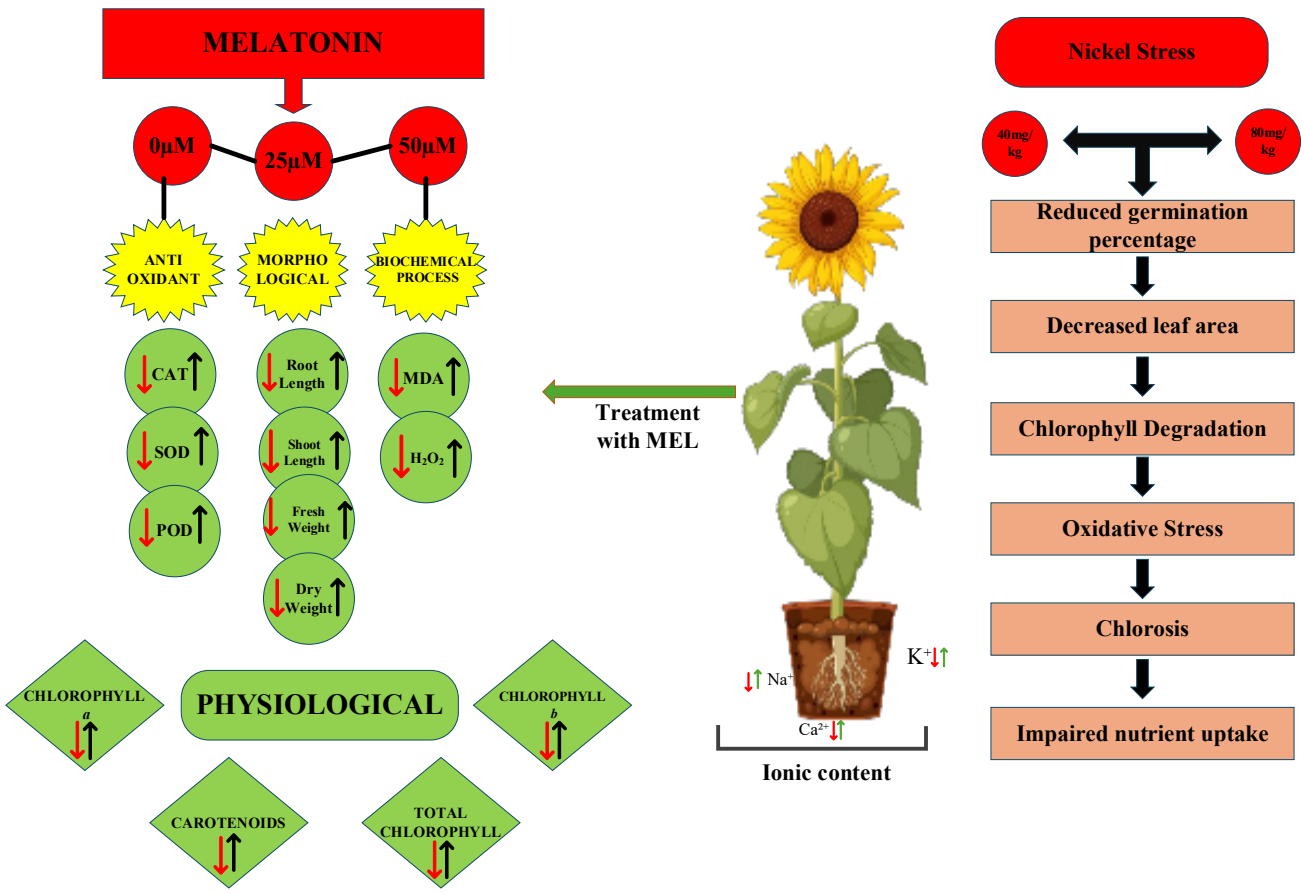


Fig. 1. Graphical abstract of foliar application of melatonin on sunflower for allevating nickel stress.



Fig. 2. Experimental setup.

Physiological attributes: Photosynthetic pigments were determined using a protocol defined by Arnon (1949) for the determination of chlorophylls a and b. First take a 0.1-gram fresh leaf from each sample. Put these leaves in plastic bottles and add 5 ml of 80% acetone solution. Keep these samples at 4ºC overnight. The extract was then centrifuged at 10000 rpm for 5 minutes. The absorbance of the extract was measured at three wavelengths 480 nm, 645 nm and 663 nm in carotenoids, chlorophyll b and chlorophyll a through spectrophotometer (Fig. 2)

Determination of ionic content: Nutrients were assessed using the Cresser & Parsons, (1979) methodology. 0.1 g of dried samples of roots and shoots were weighed and placed in digestion flasks containing 3 ml sulfuric acid. Flasks were covered with aluminum foil and left overnight. Flask-containing samples were heated on a hotplate, and hydrogen peroxide was added in drops using droplet until the samples were colorless. Next, chill the samples and filter them. Then distilled water was added to dilute solution up to 50 ml. The values of Ca, K, and Na were obtained using a flame photometer.

Estimation of secondary metabolites content

Anthocyanin (ANTs) content: The anthocyanin content is determined using Giusti & Wrolstad (2003). Pestle and mortar were used to ground 0.1g fresh leaf samples in 2 ml acidified methanol. The samples were then digested in H_2O bath at 50 ˚C for 60 mints. A spectrophotometer was used to assess the absorbance at 535 nm.

Quantification of proline: Proline content was measured according to (Bates, 1973). 5 ml of 3 % sulfosalicylic acid was homogenized with a 0.05 g of plant sample. Leave the homogenized mixture for 3 hours to finish the extraction. The extract was centrifuged across 1500 rpm for 10 minutes. In test tubes, 2 ml supernatant was mixed with 2 ml glacial acetic acid and 2 ml ninhydrin. Then, in a water bath at 100ºC for 60 minutes, place these test tubes containing the sample. The reaction was stopped by placing these test tubes in an ice bath. 4 ml toluene was poured into the test tubes and vortexed until a ring formed and two layers developed. A pipette was used to pick the upper pink layer, and a spectrophotometer was used to take a reading at 520 nm.

Biochemical parameters

Total soluble protein content: This protocol was used to determine the amount of TSP by using (Kruger, 2009). Take 0.1 g of commercial bright blue dye first. Add 100 ml of 85 percent phosphoric acid and 50 ml of 95 percent ethanol to the mixture. The volume of the solution was increased to 150 milliliters, and then 850 milliliters of distilled water was added to bring the total volume to one liter. Before usage, the mixture was filtered three times. In a prechilled pestle and mortar, 0.25 g of leaf samples was grinded in 5 ml phosphate buffer. This material was placed into an Eppendorf tube and centrifuged for 15 minutes at 15000 rpm. In a new Eppendorf, the supernatant was withdrawn and the pellet was discarded. The entire project was completed in ice. To determined total soluble protein, 100 µl enzyme extract and 5 ml Bradford reagent were added to test tubes from each sample. Then in test tubes, vortex the sample. A spectrophotometer was used to assess the absorbance at 595 nm.

Determination of enzymatic antioxidants

Peroxidase (POD): The procedure described by Durner & Klessig (1995) was used to determine peroxidase. The peroxidase concentration was determined using supernatant extracted with phosphate buffer at pH 7.8. In a cuvette, 100 µl supernatant, 100 µl guaiacol, 1 ml buffer, and 100 µl hydrogen peroxidase were introduced for this purpose. After time of interval of 0, 20, 40, 60, and 80 seconds, the absorbance was measured at 470 nm.

Catalase (CAT): Durner & Klessig (1995) described a method for determining catalase. The catalase concentration was determined using supernatant extracted with phosphate buffer at pH 7.8. 1 ml hydrogen peroxide, 1.9 ml phosphate buffer, and 100 µl enzyme extract was added in order to a cuvette, and absorbance was measured at 240nm after 0, 20, 40,60, and 80 seconds.

Superoxide dismutase (SOD): The procedure presented by Giannopolities & Ries (1977) was used to assess SOD. The Superoxide concentration was determined using extracted supernatant with phosphate buffer at pH 7.8. 50 µl of NBT (nitro-blue- tetrabenazine), 250 µl of phosphate buffer, 100 µl of Triton X, 100 µl of methionine, 400 µl of distilled water and 50 µl of supernatant was placed in a cuvette. After that, 50 µl of riboflavin was added to above solution and a cuvette was placed on top of it.

Determination of reactive oxygen species (ROS)

MDA (Malondialdehyde): Malondialdehyde was determined using Heath and Packer technique (1968). Firstly, 3 ml 5% trichloroacetic acid was used to crush a 0.5 g fresh leaf sample. Fill Eppendrops with the mixture and centrifuge for 15 minutes at 12000 rpm. 1ml of supernatant and 4 ml of Thio barbituric acid were added to test tubes. The samples should be placed in a water bath at 95˚C for 30 minutes. Centrifuge for another 10 minutes at 12000 rpm. Using a spectrophotometer, the absorbance for MDA content was determined at 532 nm and 600 nm.

Hydrogen peroxide standardization: The concentration of H_2O_2 was evaluated using Velikova *et al.,* (2000). A 0.5 g fresh leaf sample was obtained from each replicate and grinded with 0.1 percent TCA in a prechilled pestle and mortar. Fill an Eppendorf tube halfway with the extract and centrifuge for 15 minutes at 12000 rpm. 500 µl supernatant, 500 µl potassium phosphate buffer with pH 7, and 100 µl one molar potassium iodide was combined to make a sample solution. Mix the above-mentioned solution and use a spectrophotometer to take a measurement at 390 nm.

Determination of Ni contents: The samples of plants were dried in an oven to obtain average weight at 80ºC approximately two days. The concentration of Ni in root

and stem was determined by Avci *et al.,* (2013). The instrument used to determine the nickel content in plant is atomic absorption spectrophotometer (Model iCE 3400, Thermofisher, India).

Statistical analysis

The statistics 8.1 was used to analyze the data by performing 3 ways ANOVA (3 Ni levels × 3 MEL levels × 2 cultivars) with Tukey HSD test. Data presented are in the form of means (appropriately; n= 3 replicates) ± standard errors.

Results

Morphological parameters: The graphical data shows in-significant differences ($p \leq 0.05$) in growth parameters under nickel stress and melatonin application with a notable interaction effect ($p \leq 0.05$) (Fig. 3, Table 1).The increasing nickel stress reduced root and shoot length, while exogenous melatonin greatly improved length under unstressed condition Root and shoot length in V1 decreased at 40 and 80 mg kg^{-1} Ni, representing decrease of 27.22%, 17.98% and 39.05%, 33.70%, but in V2 reduced by 24. 0%, 22.63% and 40.44%, 42.43%. In V1, 25 µM MEL improved root and shoot length by 35.50%, 35.77%, 28.16% and 25.62%, 34.47%, 27.63% but 50 µM MEL increased with 46.67%, 52.12%, 64.76% and 52.66%, 66.67%, and 56.31% at 0, 40, and 80 mg kg^{-1} Ni. Similarly, in V2, 25 µM MEL increased the root and shoot length by 12.89%, 28.65%, 26.12% and 34.1%, 24.19%, and 30.32%, while at the same time, 50 µM MEL enhanced by 32.00%, 51.87%, 44.03% and 23.17%, 41.03%, and 65.01% at 0, 40, and 80 mg kg^{-1} Ni, respectively.

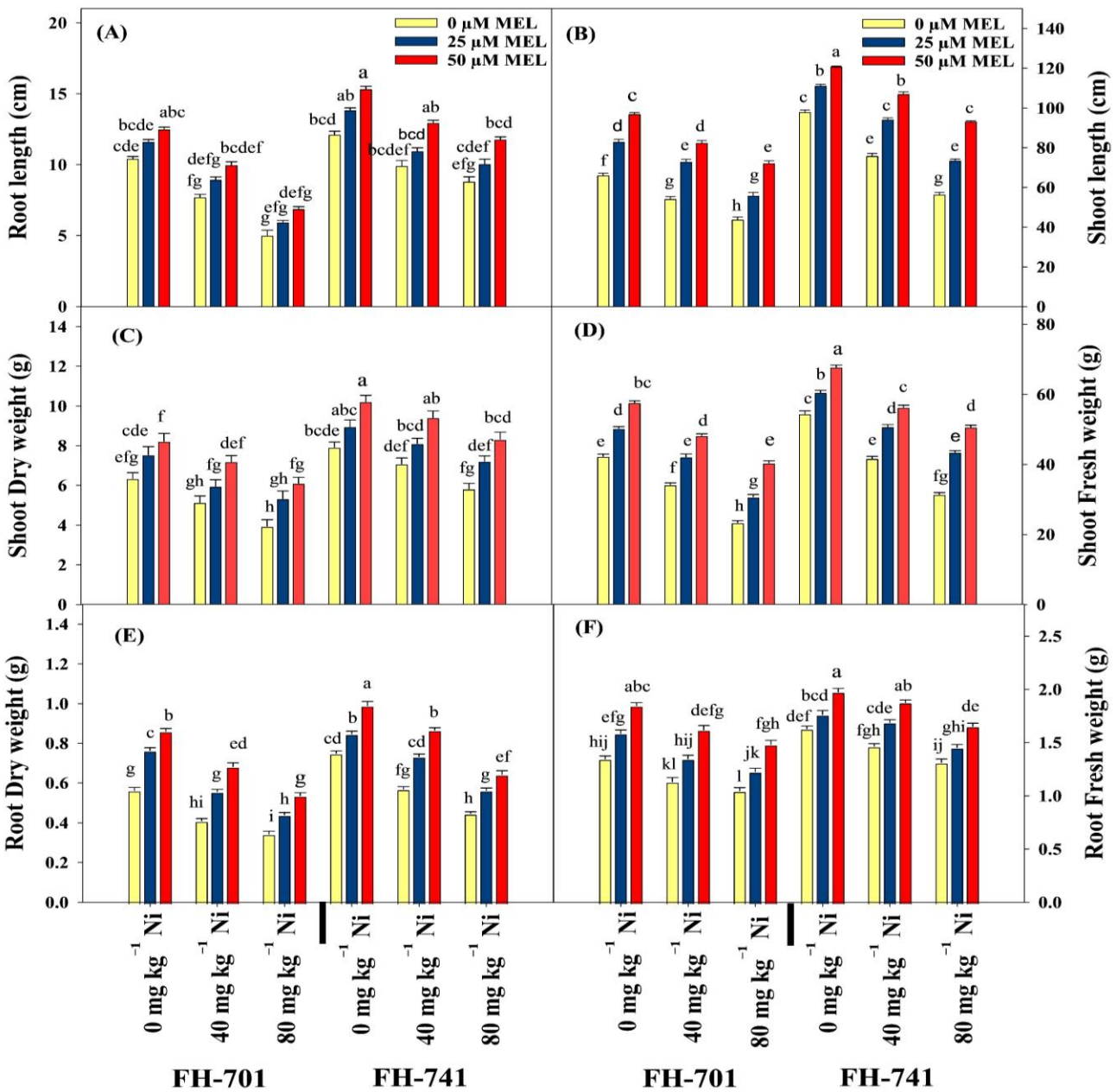


Fig. 3. Effect of melatonin and nickel stress on morphological parameters of two sunflower cultivars (FH-701, FH-741), where (A) Root length, (B) Shoot length, (C) Shoot dry weight, (D) Shoot fresh weight, (E) Root dry weight (F) Root fresh weight. The data shown are means of three replicates ± SE. Means within a variety followed by the same letter(s) are not significantly different ($p<0.05$).

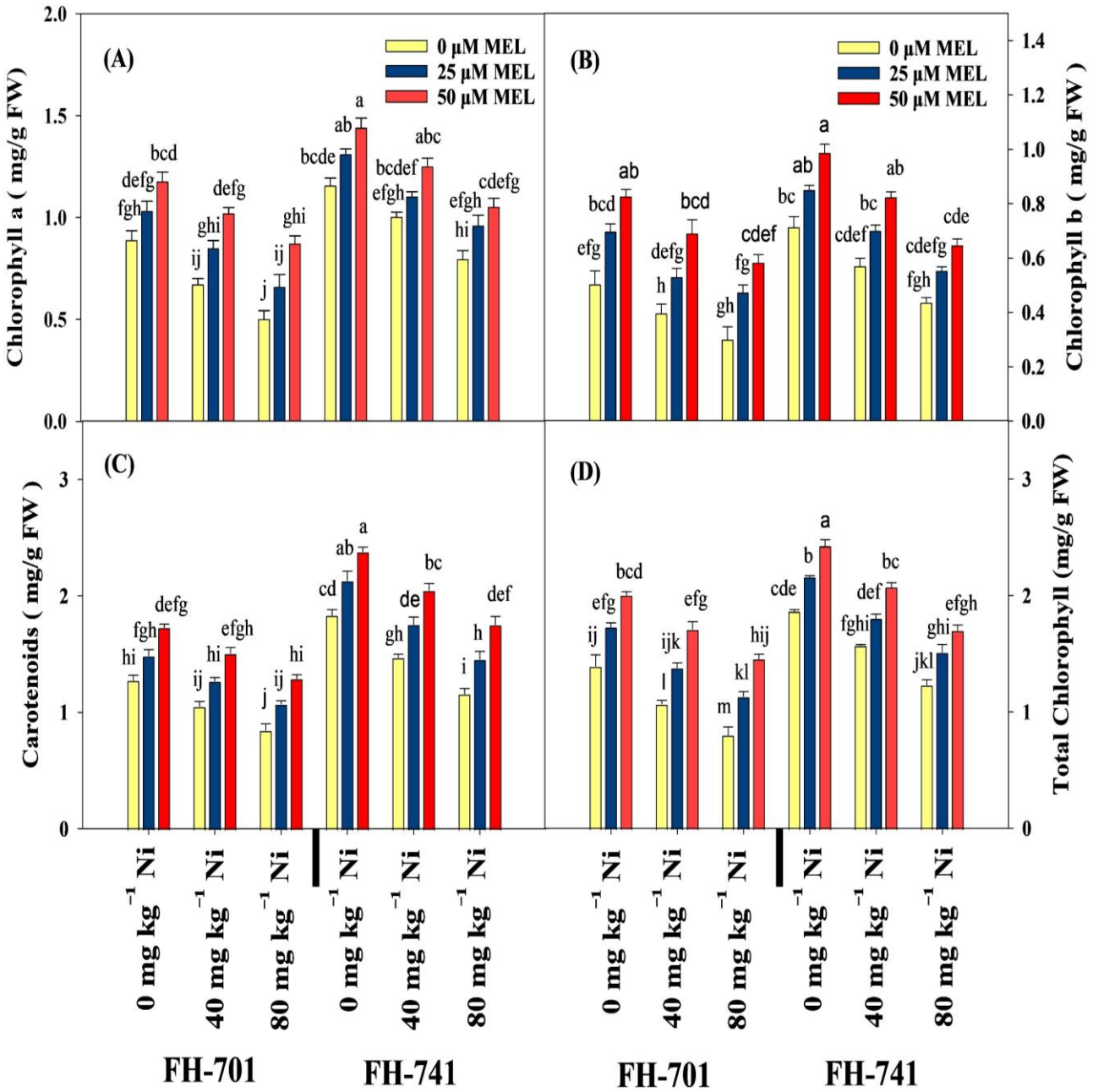


Fig. 4. Effect of melatonin and nickel stress on physiological parameters of two sunflower cultivars (FH-701, FH-741) where (A) Chlorophyll a, (B) Chlorophyll b, (C) carotenoids, (D) Total chlorophyll. The data shown are means of three replicates ± SE. Means within a variety followed by the same letter(s) are not significantly different ($p<0.05$).

Root-shoot fresh weight was not significantly ($p\leq0.05$) decreased with increasing nickel stress, as melatonin application enhanced weight in both cultivars. In V1, root and shoot fresh weight are not significantly ($p\leq0.05$) decreased under control conditions at 40 and 80 mg kg^{-1} Ni, representing reductions of 15.84%, 22.28% and 17.98%, 33.70% and whereas in V2, it was declined corresponding to reductions of 10.02%, 19.43%, and 23.39%, 42.26% respectively Application of 25 µM MEL insignificantly enhanced root and shoot fresh weight in V1 by 17.83%, 18.82%, 17.20% and 18.83%, 23.08%, and 31.48% at 0, 40, 80 mg kg^{-1} Ni, respectively, which is compared with the corresponding plants without MEL. The 50 µM MEL enhanced by 37.12%, 42.94%, 41.8 5% and 36.17%, 40.80%, 72.65% respectively. In V2, 25 µM MEL insignificantly increased the root and shoot fresh weight by 8.18%, 15.25%, 10.67%, and 11.14%, 21.66%, 38.28%, while 50 µM MEL enhanced the weights by 21.88%, 28.00%, 25.89% and 24.37%, 35.01%, 61.09% at 0, 40, 80 mg kg^{-1} Ni.

Nickel stress at 40, 80 mg kg^{-1} as compared to unstressed plant decreased the dry weight in V1 was of 27.22%, 39.05% and 24.00%, 40.44% in V2. But 25 µM MEL increased the plant dry weight by 35.50%, 35.77%, 28.16% at 0, 40, and 80 mg kg^{-1} Ni in V1 and 12.89%, 28.65%, and 26.12%, in V2 Whereas 50 µM MEL by 52.66%, 66.67%, 56.31% in V1 and 32.00%, 51.87%, 44.03% in V2 at 0, 40, and 80 mg kg^{-1} Ni. The highest growth parameters were recorded in V2 treated with 50 µM MEL under control conditions, while the lowest value occurred in V1 under 80 mg kg^{-1} Ni without MEL. Overall, 50 µM MEL was more effective than 25 µM MEL in maintaining plant growth under nickel stress.

Physiological parameters: Chl a, Chl b, carotenoid and total Chl levels were shown to be lower under nickel stress. As compared to unstressed plants, the reductions in Chl a, Chl b, Caro was noticed 43.45%, 40.40%, 33.63% with severe nickel exposure at 80 mg kg^{-1} in V1 cultivar but at 40 mg kg^{-1} there was less reduction in Chl a, b and carotenoids representing 24.34%, 21.19%, 17.42% However, as compared to untreated plants, the chlorophyll content also decreased in V2 was13.27%, 20.06% 19.75% at 40 mg kg^{-1} Ni, but there was more reduction at 80 mg kg^{-1} Ni by 31.12%, 38.74%, 36.80% respectively. In contrast, exogenous melatonin effectively reduced the adverse effect of Ni toxicity. In V1, 25 µM MEL improved the chlorophyll a content by 16.10%, 26.24%, 31.13%, Chl b 38.41%, 33.61%, 57.78% and caro by 16.56%, 20.37%, 26.79% under 0, 40, and 80 mg kg^{-1} Ni but, 50 µM MEL improved more Chl a by 32.21%, 51.49%, 73.51%, Chl b 64.24%, 68.07%, 94.44% and carotenoids by means of 35.56%, 42.99%, 52.36% at 0, 40, 80 mg kg^{-1} Ni respectively. Similarly, 50 µM MEL in V2 increased Chl a by 24.50%, 24.58%, 32.22%, Chl b by 38.39%, 44.44%, 48.47% and carotenoids by 29.72%, 39.13%, and 51.31%, respectively (Fig. 4). Overall, 50 µM MEL turned out to be more effective than 25 µM MEL, especially under excessive Ni stress. At 80 mg kg^{-1} Ni indicates that melatonin significantly reduced Ni-induced physiological damage and helped retain pigment-related processes under stress conditions. V1 showed a more relative response to MEL, while V2 maintained comparatively higher values under Ni stress indicates better tolerance of V2 to Ni toxicity.

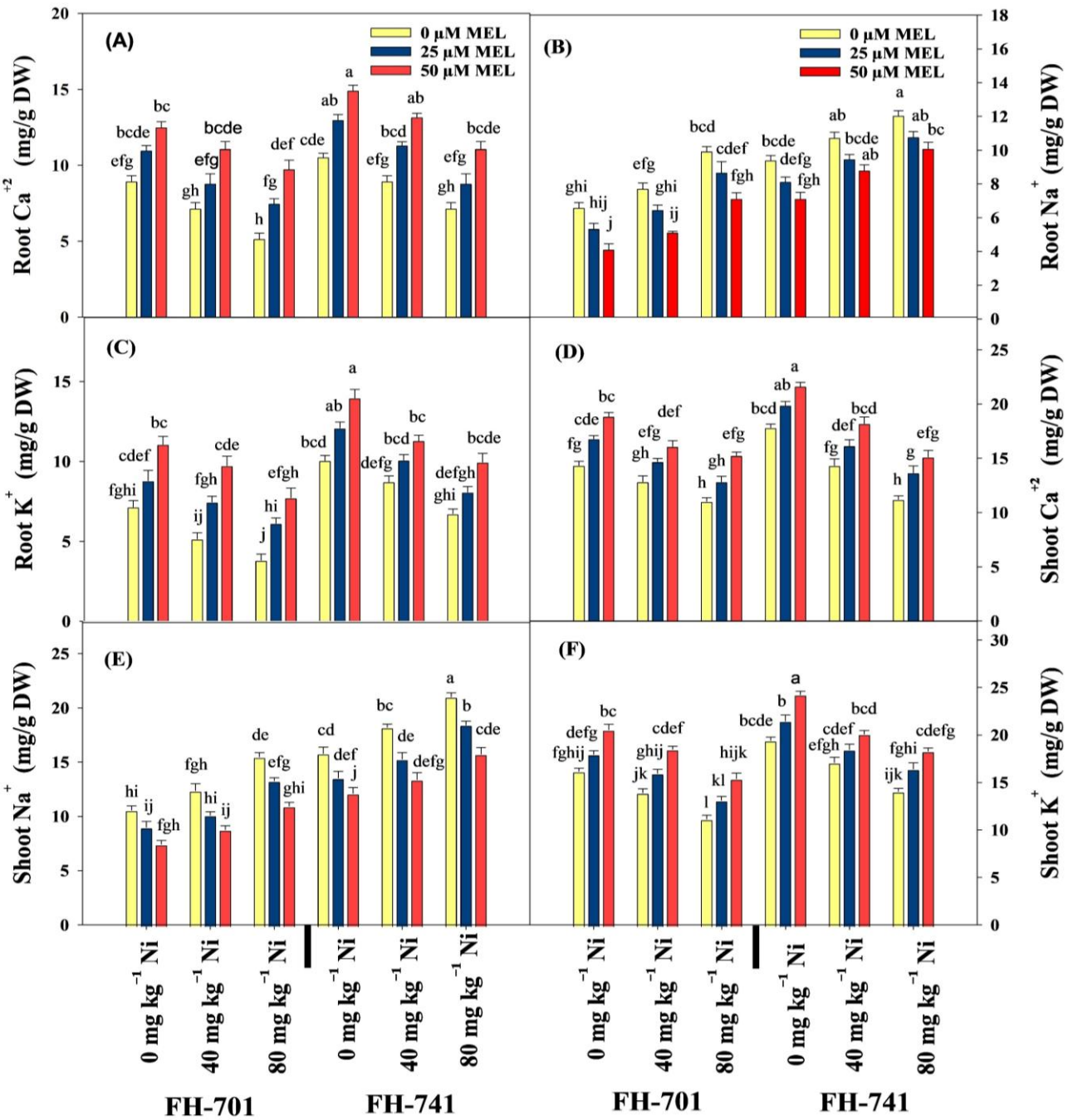


Fig. 5. Effect of melatonin on ion analysis of two sunflower cultivars under nickel stress. The data shown are means of three replicates ± SE. Means within a variety followed by the same letter(s) are not significantly different ($p<0.05$).

Table 1. Effect of melatonin on morphological attributes of sunflower under nickel stress.

Varieties	Treatments	RL (cm)	SL (cm)	RFW (g)	SFW (g)	RDW (g)	SDW (g)
	Control	10.40 ± 0.17 ce	66.06 ± 1.08 f	1.34 ± 0.02 h-j	42.3 ± 0.64 e	0.56 ± 0.01 g	6.34 ± 0.29 e-f
	25 µM MEL	11.61 ± 0.17 b-e	82.99 ± 1.23 d	1.58 ± 0.03e-g	50.26 ± 0.56 d	0.76 ± 0.01 c	7.54 ± 0.41 c-g
	50 µM MEL	12.46 ± 0.17 a-c	96.89 ± 0.89 c	1.84 ± 0.02a-c	57.6 ± 0.55 bc	0.86 ± 0.01 b	8.22 ± 0.39 f
	40 mg kg^{-1} + 0 µM MEL	7.70 ± 0.20 fg	54.19 ± 1.21 g	1.13 ± 0.03 kl	34.23 ± 0.49 f	0.41 ± 0.01 hi	5.14 ± 0.33 gh
FH-701	40 mg kg^{-1} + 25 µM MEL	8.93 ± 0.20 d-g	72.90 ± 1.28 e	1.34 ± 0.03 h-j	42.13 ± 0.84 e	0.55 ± 0.01 hi	5.96 ± 0.33 fg
	40 mg kg^{-1} + 50 µM MEL	9.96 ± 0.23 b-f	82.43 ± 1.21 d	1.62 ± 0.04 d-g	48.23 ± 0.55 d	0.68 ± 0.01 g	7.19 ± 0.30 d-f
	80 mg kg^{-1} + 0 µM MEL	5.01 ± 0.37 g	43.80 ± 1.34 h	1.04 ± 0.03 i	23.43 ± 0.55 h	0.34 ± 0.01 h	3.95 ± 0.32 h
	80mgkg^{-1} + 25 µM MEL	5.93 ± 0.14 e-g	55.90 ± 1.61 g	1.22 ± 0.03 jk	30.73 ± 0.63 g	0.44 ± 0.01 i	5.33 ± 0.38 gh
	80 mg kg^{-1} + 50 µM MEL	6.86 ± 0.17 d-g	72.16 ± 1.31 e	1.48 ± 0.03 f-h	40.40 ± 0.62 e	0.53 ± 0.01 h	6.11 ± 0.30 fg
	Control	12.10 ± 0.25 b-d	97.96 ± 0.99 c	1.63 ± 0.02 d-f	54.43 ± 0.84 c	0.75 ± 0.01 cd	7.92 ± 0.26 b-e
	25 µM MEL	13.38 ± 0.17 ab	111.1 ± 0.85 b	1.76 ± 0.03 b-d	60.53 ± 0.63 b	0.84 ± 0.01 b	8.96 ± 0.33 a-c
	50 µM MEL	15.3 ± 0.23 a	120.60 ± 0.44 a	1.97 ± 0.03 a	67.70 ± 0.64 a	0.99 ± 0.02 a	10.21 ± 0.32 a
	40 mg kg^{-1} + 0 µM MEL	9.93 ± 0.40 b-f	75.80 ± 1.31 e	1.46 ± 0.02 f-h	41.70 ± 0.60 e	0.57 ± 0.01 fg	7.08 ± 0.29 d-e
FH-741	40 mg kg^{-1} + 25 µM MEL	10.93 ± 0.20 b-d	94.13 ± 0.89 c	1.69 ± 0.02 c-e	50.70 ± 0.61 d	0.73 ± 0.01 cd	8.10 ± 0.25 b-d
	40 mg kg^{-1} + 50 µM MEL	12.93 ± 0.20 a-b	106.9 ± 1.25 b	1.87 ± 0.02 ab	56.30 ± 0.60 c	0.86 ± 0.01 b	9.41 ± 0.33 ab
	80 mg kg^{-1} + 0 µM MEL	8.80 ± 0.34 e-g	56.40 ± 1.07 g	1.31 ± 0.03 ij	31.4 ± 0.55 fg	0.44 ± 0.01 h	5.83 ± 0.27 fg
	80 mg kg^{-1} + 25 µM MEL	10.03 ± 0.35 c-f	73.50 ± 0.89 e	1.45 ± 0.02 g-i	43.4 ± 0.48 e	0.56 ± 0.01 g	7.21 ± 0.26 d-f
	80 mg kg^{-1} + 50 µM MEL	11.76 ± 0.20 b-d	93.06 ± 0.51 c	1.65 ± 0.02 de	50.6 ± 0.54 d	0.64 ± 0.01 ef	8.32 ± 0.36 b-d

RL= root length; SL= shoot length; RFW= root fresh weight; SFW= shoot fresh weight; RDW= root dry weight; SDW= shoot dry weight

Table 2. Effect of melatonin on antioxidant and biochemical parameters of sunflower under nickel stress.

Varieties	Treatment	SOD	POD	CAT	MDA	H_2O_2
	Control	4.69 ± 0.04 fg	3.46 ± 0.03 gh	1.90 ± 0.03 d-f	3.95 ± 0.07 jk	2.72 ± 0.06 gh
	25 µM MEL	5.52 ± 0.10 c	3.97 ± 0.08 d-f	2.25 ± 0.06 bc	3.55 ± 0.04 k	2.27 ± 0.07 i
	50 µM µM MEL	6.08 ± 0.06 b	4.47 ± 0.06 bc	2.47 ± 0.05 ab	3.17 ± 0.07 k	1.78 ± 0.06 j
	40 mg kg^{-1} + 0 µM MEL	3.76 ± 0.06 j-l	3.07 ± 0.08 i	1.64 ± 0.04 f-h	4.59 ± 0.05 g-i	3.05 ± 0.03 e-g
FH-701	40 mg kg^{-1} + 25 µM MEL	4.44 ± 0.07 gh	3.61 ± 0.03 fg	1.95 ± 0.05 c-f	4.01 ± 0.08 j	2.78 ± 0.05 f-h
	40 mg kg^{-1} + 50 µM MEL	4.98 ± 0.06 d-f	4.15 ± 0.04 c-e	2.23 ± 0.06 b-d	3.50 ± 0.06 k	2.45 ± 0.06 hi
	80 mg kg^{-1} + 0 µM MEL	2.88 ± 0.14 m	2.65 ± 0.04 j	1.38 ± 0.06 h	5.12 ± 0.09 d-f	3.25 ± 0.04 de
	80 mg kg^{-1} + 25 µM MEL	3.47 ± 0.06l	3.11 ± 0.04 hi	1.68 ± 0.05 f-h	5.48 ± 0.06 hi	2.99 ± 0.06 e-f
	80 mg kg^{-1} + 50 µM MEL	3.97 ± 0.09 ik	3.79 ± 0.05 e-g	1.97 ± 0.06 c-f	4.26 ± 0.07 ij	2.77 ± 0.04 f-h
	Control	5.33 ± 0.07 cd	4.15 ± 0.10 c-e	2.06 ± 0.06 c-e	5.32 ± 0.06 c-e	3.45 ± 0.03 cd
	25 µM MEL	5.99 ± 0.06 b	4.73 ± 0.11 b	2.45 ± 0.06 ab	4.89 ± 0.05 f-h	3.09 ± 0.09 ef
	50 µM MEL	6.54 ± 0.07 a	5.22 ± 0.09 a	2.75 ± 0.12 a	4.43 ± 0.08 i	2.74 ± 0.05 bc
	40 mg kg^{-1} + 0 µM MEL	4.28 ± 0.07 g-i	3.63 ± 0.07 fg	1.84 ± 0.07 e-g	5.97 ± 0.05 b	3.80 ± 0.10 ab
FH-741	40 mg kg^{-1} + 25 µM MEL	5.14 ± 0.05 c-e	4.18 ± 0.05 cd	2.14 ± 0.05 b-e	5.45 ± 0.09 cd	3.54 ± 0.06 b-d
	40 mg kg^{-1} + 50 µM MEL	5.96 ± 0.05 b	4.81 ± 0.06 b	2.45 ± 0.04 ab	4.97 ± 0.06 e-g	3.28 ± 0.05 c-e
	80 mg kg^{-1} + 0 µM MEL	3.67 ± 0.06 kl	3.16 ± 0.04 hi	1.54 ± 0.05gh	6.43 ± 0.07 a	4.10 ± 0.08 a
	80 mg kg^{-1} + 25 µM MEL	4.17 ± 0.10 h-j	3.76 ± 0.07 fg	1.94 ± 0.05 c-f	6.06 ± 0.08 b	3.83 ± 0.04 ab
	80 mg kg^{-1} + 50 µM MEL	4.86 ± 0.04 ef	4.23 ± 0.06 cd	2.24 ± 0.06 bc	5.67 ± 0.06 bc	3.62 ± 0.05 bc

SOD= superoxide dismutase; POD= peroxidase; CAT= catalase; MDA= malondialdehyde; H_2O_2= hydrogen peroxide

Table 3. Effect of melatonin on ion analysis of sunflower under Ni stress.

Variety	Treatments	Root Na	Root K	Root Ca	Shoot Na	Shoot K	Shoot Ca
	Control	6.58 ± 0.30 g-i	7.15 ± 0.38 f-i	8.93 ± 0.38 e-g	10.58 ± 0.37 hi	16.18 ± 0.32 f-j	14.35 ± 0.38 fg
	25 µM MEL	5.33 ± 0.32 h-j	9.13 ± 0.39 c-f	10.96 ± 0.33b-e	8.9 ± 0.53 ij	17.96 ± 0.37 d-g	16.79 ± 0.29 c-e
	50 µM MEL	4.11 ± 0.34 j	11.06 ± 0.50 bc	12.49 ± 0.39 bc	7.44 ± 0.33 f-h	20.56 ± 0.56 bc	18.87 ± 0.33 bc
	40 mg kg^{-1} + 0 µM MEL	7.71 ± 0.34 e-g	5.15 ± 0.38 il	7.15 ± 0.38 gh	12.38 ± 0.62 f-h	13.93 ± 0.38 jk	12.85 ± 0.53 gh
FH-701	40 mg kg^{-1}+25 µuM MEL	6.45 ± 0.29 g-i	7.46 ± 0.34 f-h	8.7 ± 0.64 e-g	10.12 ± 0.28 hi	15.96 ± 0.41g-j	14.69 ± 0.29 e-f
	40 mg kg^{-1}mgkg−1+50 uM MEL	5.11 ± 0.07 ij	9.73 ± 0.60 c-e	11.06 ± 0.50 b-e	8.78 ± 0.34 ij	18.49 ± 0.32 c-f	16.09 ± 0.53 d-f
	80 mg kg^{-1}+0 µM MEL	9.92 ± 0.28 b-d	3.82 ± 0.38 j	5.15 ± 0.38 h	15.48 ± 0.38 de	11.15 ± 0.38 l	11.03 ± 0.32 h
	80 mg kg^{-1}+25 µM MEL	8.66 ± 0.65 c-f	6.13 ± 0.33 h	7.46 ± 0.34 fg	13.27 ± 0.28 e-f	13.13 ± 0.39 kl	12.84 ± 0.49 gh
	80 mg kg^{-1}+50 µM MEL	7.11 ± 0.37 f-h	7.73 ± 0.60 e-h	9.73 ± 0.60 d-f	10.94 ± 0.35 g-i	15.39 ± 0.60 h-k	15.26 ± 0.30 e-g
	Control	9.38 ± 0.30 b-e	10.06 ± 0.30 b-d	10.52 ± 0.28 c-e	15.80 ± 0.58 cd	19.42 ± 0.35 b-e	17.82 ± 0.33b-d
	25 µM MEL	8.12 ± 0.28 d-g	12.08 ± 0.37 ab	12.96 ± 0.37 ab	13.53 ± 0.62 d-f	21.47 ± 0.62 b	19.88 ± 0.35 ab
	50 µM MEL	7.11 ± 0.39 f-h	13.96 ± 0.54 a	14.89 ± 0.38 a	12.12 ± 0.54 j	24.24 ± 0.36 a	21.62 ± 0.36 a
	40 mg kg^{-1}+0 µM MEL	10.71 ± 0.34 ab	8.72 ± 0.36 d-g	8.93 ± 0.38 e-g	18.20 ± 0.30 bc	17.10 ± 0.55 e-h	14.33 ± 0.61 fg
FH-741	40 mg kg^{-1}+25 µM MEL	9.45 ± 0.29 b-e	10.08 ± 0.33 b-d	11.20 ± 0.25 b-d	15.28 ± 0.60 de	18.46 ± 0.56 c-f	16.15 ± 0.56 d-f
	40 mg kg^{-1}+50 µM MEL	8.78 ± 0.34 ac	11.29 ± 0.34 bc	13.15 ± 0.27 ab	13.38 ± 0.64 d-g	20.10 ± 0.37 b-d	18.20 ± 0.60 b-d
	80 mg kg^{-1}+0 µM MEL	12.01 ± 0.32 a	6.72 ± 0.30 g-i	7.15 ± 0.38 gh	21.01 ± 0.36 a	14.06 ±0.33 i-k	11.21 ± 0.31 h
	80 mg kg^{-1}+25 µM MEL	10.75 ± 0.33 ab	8.08 ± 0.33 d-h	8.79 ± 0.64 e-g	18.42 ± 0.35 b	16.41 ± 0.66 f-i	13.66 ± 0.64 g
	80 mg kg^{-1}+50 µM MEL	10.07 ± 0.38 bc	9.96 ± 0.54 b-e	11.06 ± 0.50 b-e	15.74 ± 0.60 c-e	18.29 ± 0.34 c-g	15.12 ± 0.60 e-g

Root Na= root sodium ion; Root k= root potassium; Root Ca= root calcium

Ion analysis: Plants coming in contact with high nickel concentrations have a tendency to make too much sodium within their cells. The general balance of essential ions is disturbed by this unbalanced sodium accumulation, which can also disrupt metabolic processes regularly and can cause harmful consequences. The sodium content of the plant increases with nickel concentration. Nickel treatments (40 mg kg^{-1} and 80 mg kg^{-1}) increased root Na^+ concentration was 17.15%, 50.60% in V1 and 14.21%, 28.08% in V2 respectively, while reducing root potassium ion was 27.94%, 48.57% in V1 and 13.25%, 33.13% in V2. Exogenous MEL at a concentration of 50 µM increased the K^+ content was 54.56%, 88.65%, 102.11% and Na^+ concentration 37.61%, 33.69%, 28.39% of the roots decreased at 0, 40, 80 mg kg^{-1} in V1 and K^+ enhanced by 24.15%, 18.98%, 16.15% and Na^+ reduced by 38.84%, 29.41%, 48.04% in V2 (Fig. 5b & c). Furthermore, the data showed that at 40, 80 mg kg^{-1}, the Ca^{+2} content was considerably reduced in roots representing 19.91% and 42.28%. However, when measured with un-stressed plants, the Ca^{+2} level increased in root after applying MEL at a rate of 50 µM was 39.77%, 54.56%, 88.64% in V1 and 41.48%, 47.23%, 54.56% in V2 (Fig. 5a). When heavy metal stress (40 mg kg^{-1} and 80 mg kg^{-1}) was applied, the shoot Na^+ concentration was increased by 16.97%, 46.30% in V1 and 15.21%, 33.02% in V2. In comparison to the plants under stress, exogenous MEL supplementation at rates of 25 µM by 18.28%, 14.29% in V1 and 16.05%, 12.34% in V2 and 50 µM reduced the amount of Na^+ in the shoots by 29.07%, 29.31% in V1, 26.46%, 25.09% in V2 at 40, 80 mg kg^{-1} (Fig. 5e). The K^+ and Ca^{+2} contents of shoots were raised by exogenous MEL (50 µM) at 80 mg kg^{-1} by 37.99%, 38.39% in V1, 30.11%, 34.80% in V2 but it was decreased by 31.07%, 23.11% in V1 and 27.59%, 37.07% in V2 due to nickel stress (80 mg kg^{-1}) (Fig. 5 d & f) (Table 3).

Activity of enzymatic antioxidant: Enzymatic antioxidant was strongly influenced through the use of nickel stress and melatonin. Increasing nickel stress generally decreased antioxidant activity in each variety, while exogenous MEL was increased this activity under control and stress conditions. In V1, SOD, POD, Catalase activity decreased under control conditions at 40 and 80 mg kg^{-1} Ni, to 19.83%, 38.59% and 11.28%, 23.22% and 13.49%, 27.07% respectively but in V2 similar decreased was observed for SOD by 19.75%, 31.25% POD by 12.56%, 23.82% and catalase by 10.51% 25.07% (Fig. 6, Table 2).

Application of 25 µM MEL enhanced SOD activity in V1 through 17.70%, 18.17%, and 20.49% POD 14.72%, 17.58%, and 17.70% catalase 18.49%, 19.10%, 21.36%, respectively at 0, 40, 80 mg kg^{-1} Ni, compared with the corresponding untreated plants. The response was stronger by 50 µM MEL, with 29.67%, 32.54%, 37.96%, POD 29.38%, 35.39%, 42.72% and catalase 30.47%, 35.97%, and 42.16% but with 25 µM MEL application in V2 enhanced antioxidant activity by 12.30%, 20.07%, 13.73%, POD 13.96%, 15.29%, 18.97% and catalase 18.96%, 16.25%, 26.07%, while 50 µM MEL improved more activity by 22.62%, 39.30%, and 32.60% POD 25.72%, 32.45%, 33.78% CATALASE 33.82%, 33.04%, and 45.33% at 0, 40, and 80 mg kg^{-1} Ni, respectively. Overall, 50 µM MEL is more effective than 25 µM MEL in maintaining antioxidant activity under nickel stress. V2 exhibited higher increased than V1 in all activities, indicating more potent antioxidant protection and comparatively increasing tolerance to Ni-induced oxidative stress.

Lipid peroxidation (MDA) and H_2O_2: Graphical data showed a significantly increase in lipid peroxidation with increasing nickel stress, whereas foliar application of melatonin reduced MDA and H_2O_2 accumulation in each sunflower cultivar. In V1 both MDA and H_2O_2 was significantly enhanced by 16.24%, 29.48% and 12.05% and 19.54% at 40, 80 mg kg^{-1} as compared to control level but melatonin gradually decreased MDA and H_2O_2 accumulation under each Ni stress. Application of 25 and 50 µM MEL reduced MDA, H_2O_2 under 40 mg kg^{-1} Ni was 12.63%, 23.82% and 8.63%, 19.67% with corresponding 0 µM MEL treatment. At 80 mg kg^{-1}, significantly reduction was observed as 10.48%, 16.72% and 7.90% to 14.66% respectively. But in V2, nickel stress significantly enhanced MDA, H_2O_2 was 12.03%, 10.21% at 40 mg kg^{-1} and 20.77%, 18.82% at 80 mg kg^{-1} under 0 µM MEL Whereas, 25 and 50 µM MEL treatment, significantly decreased both MDA was 8.72%, 16.63% and H_2O_2 6.72%, 13.58% at 40 mg kg^{-1} but at 80 mg kg^{-1}, there was observed a significantly decreased 6.68%, 11.90% and H_2O_2 6.58% and 11.72% respectively (Fig. 7). The reduction in MDA and H_2O_2 concentrations was observed with 50 µM MEL under Ni stress, indicating that higher MEL treatment was more effective in preventing oxidative stress. Overall, FH-701 showed greater results in MDA, H_2O_2 under Ni stress was than FH-741 at 80 mg kg^{-1}, suggesting more oxidative stress sensitivity in FH-701. But FH-741 reduced MDA, H_2O_2 accumulation under Ni stress but melatonin application enhanced the plant antioxidant defense systems and indicating higher tolerance. The protective effect of 50 µM MEL was generally producing greater reductions in MDA and H_2O_2.

Secondary metabolites: The soluble protein, anthocyanin and proline contents enhanced with increasing nickel stress. At 40, 80 mg kg^{-1} Ni in V1 significantly enhanced the total soluble protein by 12.7%, 34.4%, anthocyanin by 19.5%, 32.9% and proline by 7.2%, 16.2% but in V2 TSP increased by 21.0%, 37.4%, anthocyanin by 12.9%, 24.5% and proline by 8.6%, 17.1% as compared to untreated plants respectively. The exogenous melatonin under both stress and unstressed conditions enhanced the accumulation of these biochemical constituents. The 50 µM MEL turned out to be more potent than 25 µM MEL, in V1 25 µM increased the TSP by 27.0%, 22.1%,19.2%, anthocyanin by 23.6, 16.3, 12.3% and proline content by 16.9%, 15.5%, 14.6% at 0, 40, 80 mg kg^{-1}. But 50 µM enhanced the TSP by 41.1%, 45.4%, 36.8%, anthocyanin by 38.7%, 26.7%, 22.7% and proline by 37.3%, 37.6%, 32.6% However in V2, 25 µM increased the total soluble protein by 16.7%, 13.1%, 11.0% anthocyanin by 14.5%, 11.3%, 9.5% and proline by 10.8%, 8.9%, 10.4% but 50 µM increased the total soluble protein by 39.8%, 33.7%, 25.9% and anthocyanin by 30.0%, 20.5%, 17.6% and proline by 19.2%, 17.5%, 18.7%. The overall findings showed that 50 µM enhanced the plant biochemical defense against nickel stress (Fig. 8).

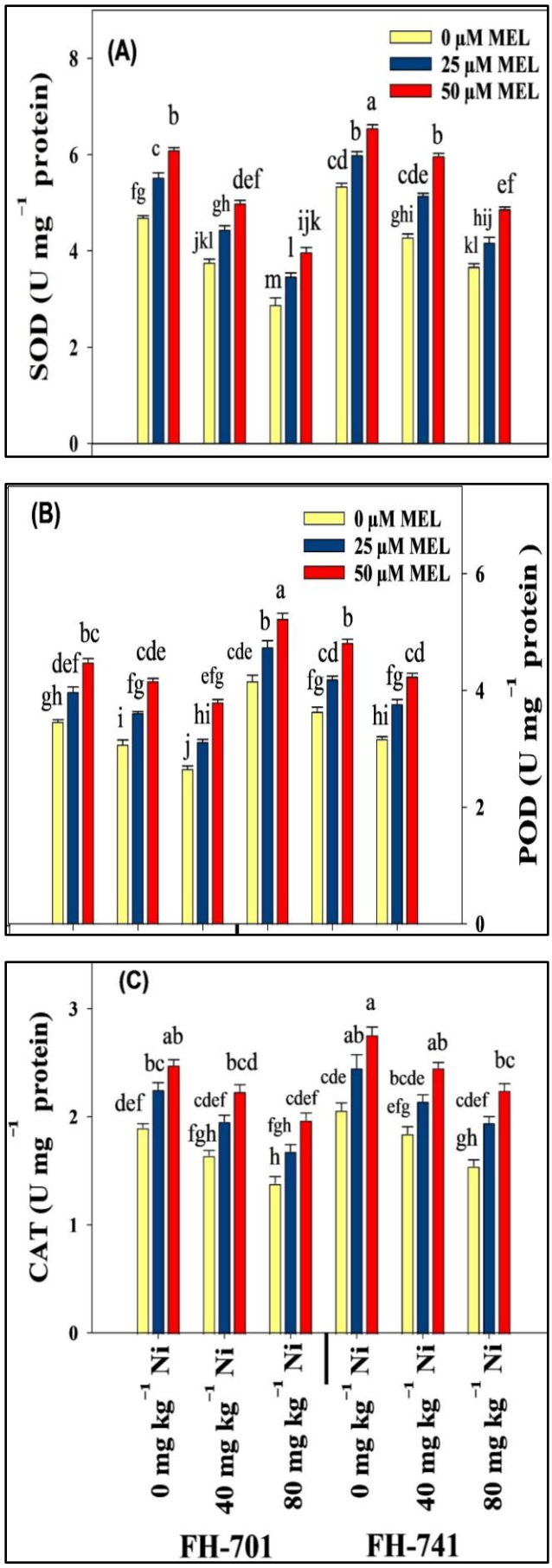


Fig. 6. Exogenous MEL showed effect on SOD (A), POD (B), Catalase (C) of two sunflower cultivars (FH-701, FH-741) under Ni stress.

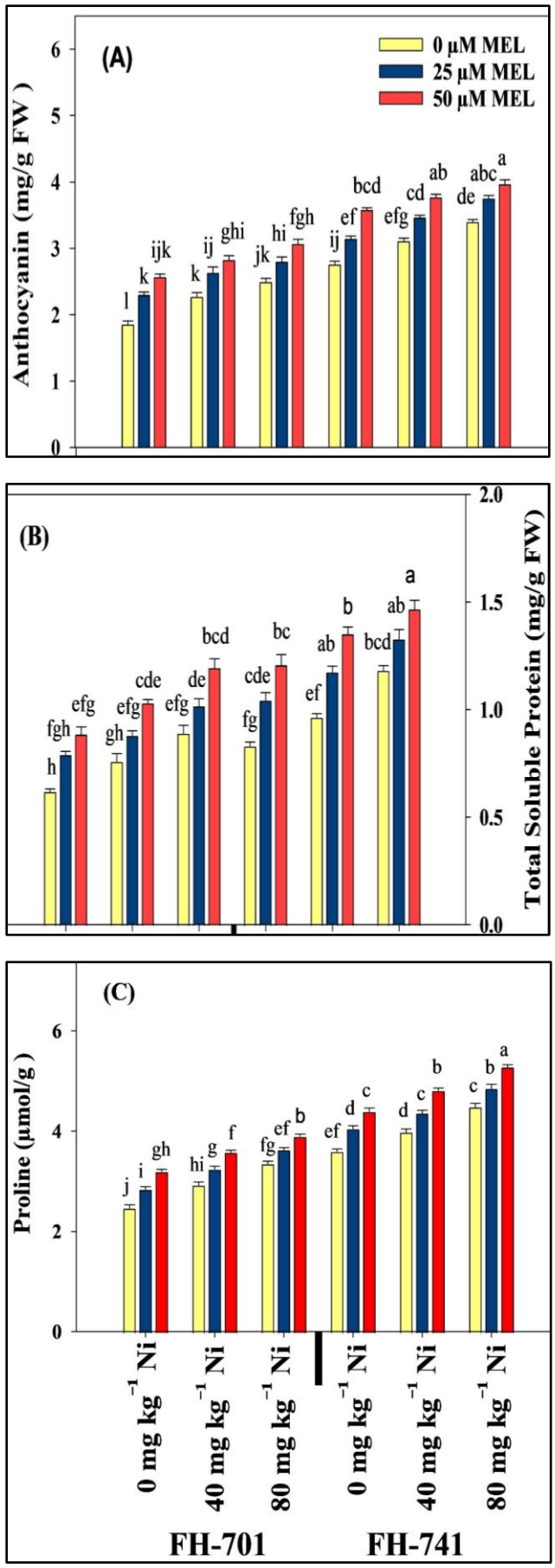


Fig. 8. Exogenous MEL showed effect on anthocyanin (A) Total soluble protein (B) and Proline (C) two sunflower cultivars (FH-701, FH-741) under Ni stress.

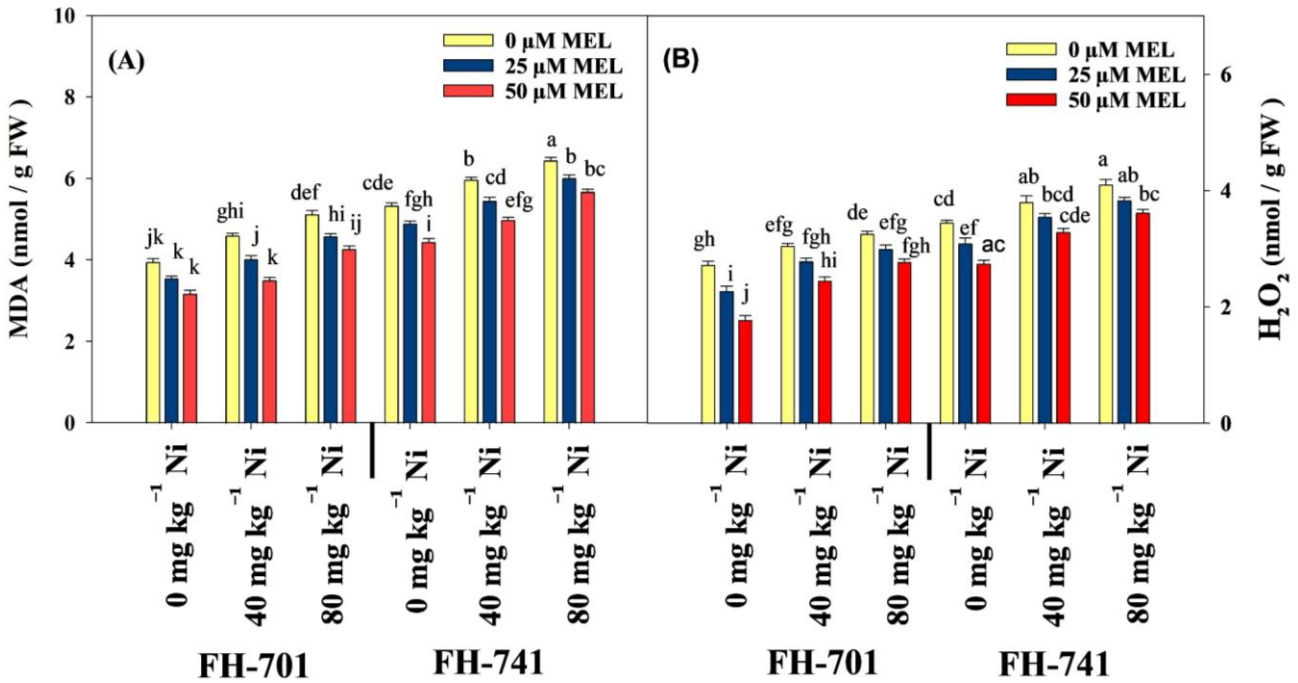


Fig. 7. The effect of melatonin and nickel stress on MDA and hydrogen peroxide of two sunflower cultivars.

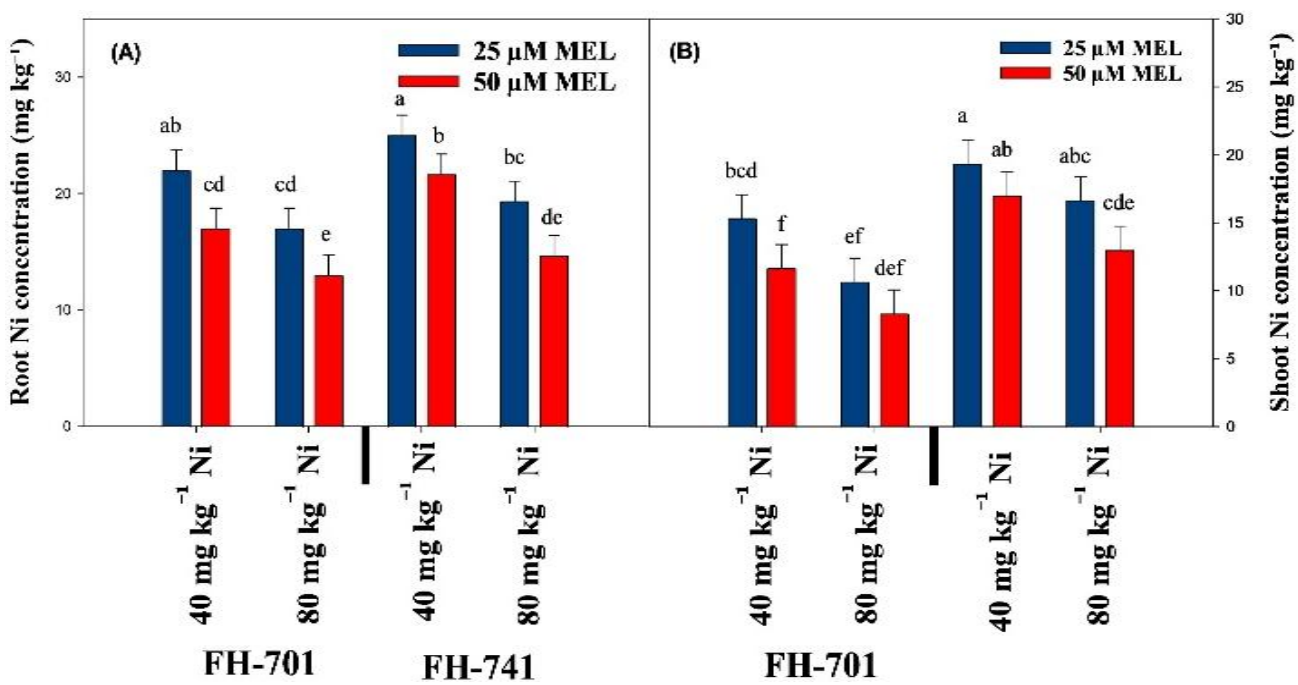


Fig. 9. Effect of melatonin application on nickel concentration in the roots and shoots of FH-701 and FH-741 sunflower cultivars.

Nickel concentration: Nickel accumulation in roots and shoots was altered by melatonin foliar spray. Increasing MEL concentration from 25 to 50 µM caused a significantly steady decrease in Ni accumulation in each sunflower cultivar under 40, 80 mg kg^{-1} Ni stress. In roots of V1, 50 µM MEL reduced Ni accumulation through 22.7% and 23.5% at 40 and 80 mg kg^{-1} Ni respectively as compared to 25 µM MEL. The corresponding decreased in V2 were 13.3% and 24.1%. Thus, the most potent reduction in Ni accumulation was observed in V2 under 80 mg kg^{-1} Ni. The similar results were found in shoots. In V1, 50 µM MEL reduced shoot Ni accumulation using 23.9% and 21.9% at 40, 80 mg kg^{-1} Ni, respectively. Shoot Ni accumulation in V2 is reduced via 12.1% at 40 mg kg^{-1} and 22.0% at 80 mg kg^{-1} Ni (Fig. 9). These findings demonstrate that increasing MEL concentration effectively limited Ni accumulation and translocation to underground tissues. Overall, 50 µM MEL was more effective than 25 µM MEL in reducing Ni accumulation, with generally decreases from 13.3% to 24.1% in roots and 12.1% to 23.9% in shoots (Fig. 9). The higher reductions were obtained at 80 mg kg^{-1} which indicates that melatonin play role in inhibiting Ni uptake and translocation.

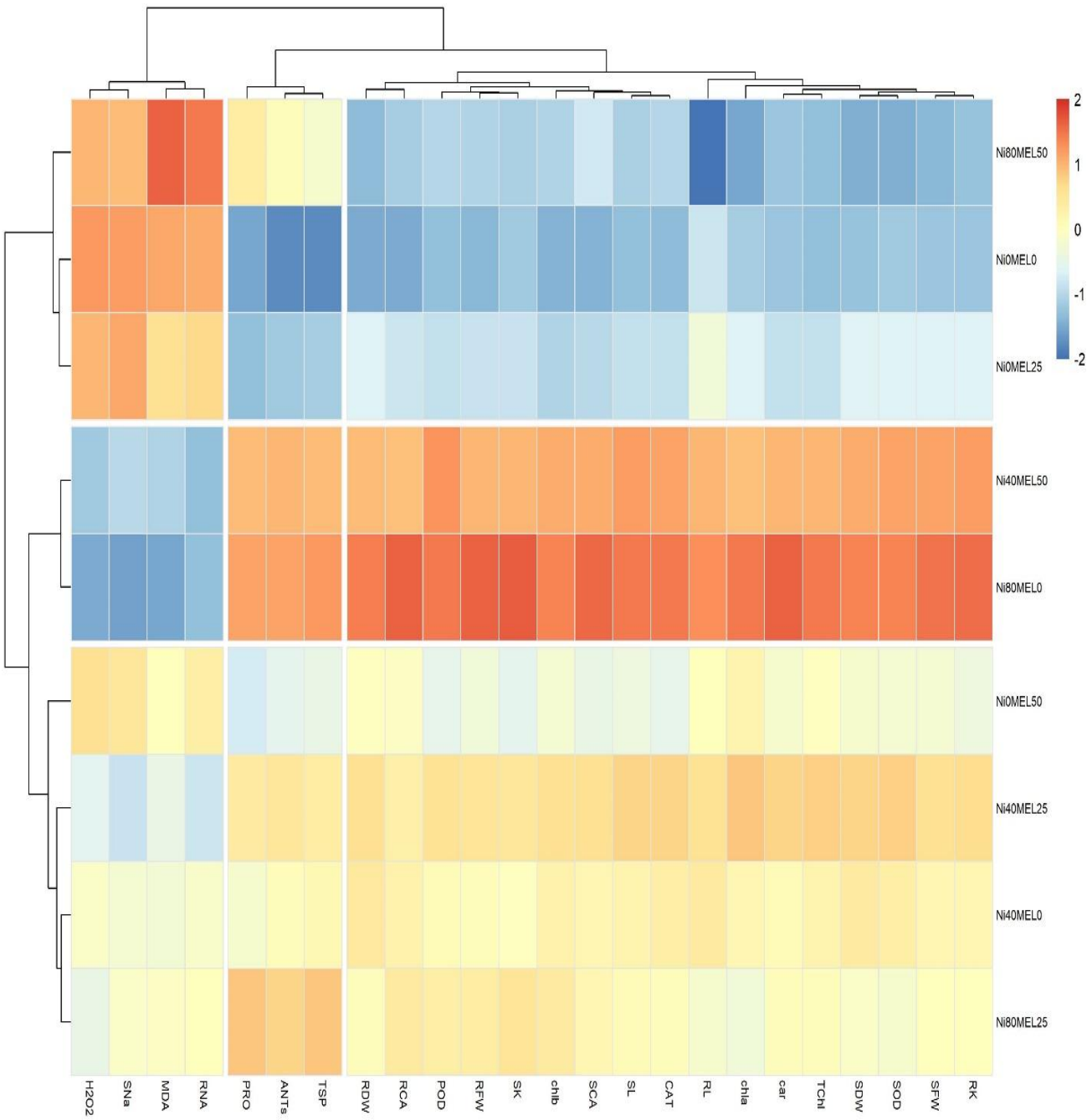


Fig. 10. Hierarchical clustering showing heatmap relative responses of physiological, biochemical, antioxidant and growth-related parameters under different Ni-MEL treatments of sunflower cultivar (FH-701). The color scale represents standardized relative values from - 2 (blue, low values) to + 2 (red, higher values).

Heatmap: The FH-701 heatmap was created to show associations between sunflower morpho-physiological and biochemical parameters at varying nickel stress levels (0, 40, and 80 mg kg^{-1}) and melatonin levels (0, 25, and 50 µM) (Fig. 10). Three sub-clusters were visible within the main cluster. Root length, shoot length, shoot fresh weight, shoot dry weight, Chl b, carotenoids, and Chl a, as well as root and shoot calcium and potassium connected at Ni 80 (0 µM MEL), Ni 40 (50 µM MEL) and Ni 80 (25 µM MEL), were all strongly positively associated with the first sub-cluster. However, at 80 Ni (50 µM MEL) 0 Ni (0 µM MEL), all of these factors showed a strong negative association. Total soluble protein, anthocyanin, and proline exhibited a strong positive association with Ni 80 (0 µM MEL) and Ni 40 (50 µM MEL) in the second sub-cluster, whereas Ni 0 (0 µM MEL) and Ni 0 (25 µM MEL) demonstrated a negative association. Biochemical attributes including hydrogen peroxide and malondialdehyde were strongly positively associated with the third sub-cluster at 80 Ni (50 µM MEL) and Ni 0 (0 µM MEL). In contrast, the third sub-cluster showed negative associations at different levels, with the highest association occurring at 40 Ni (25 µM MEL) and 80 Ni (0 µM MEL). Sodium ions from roots and shoots likewise exhibited a high positive association in this sub-cluster at 80 Ni (50 µM MEL) and Ni 0 (0 µM MEL). Overall, there was a negative association between plant secondary metabolites (anthocyanin, total soluble protein, and proline concentration).

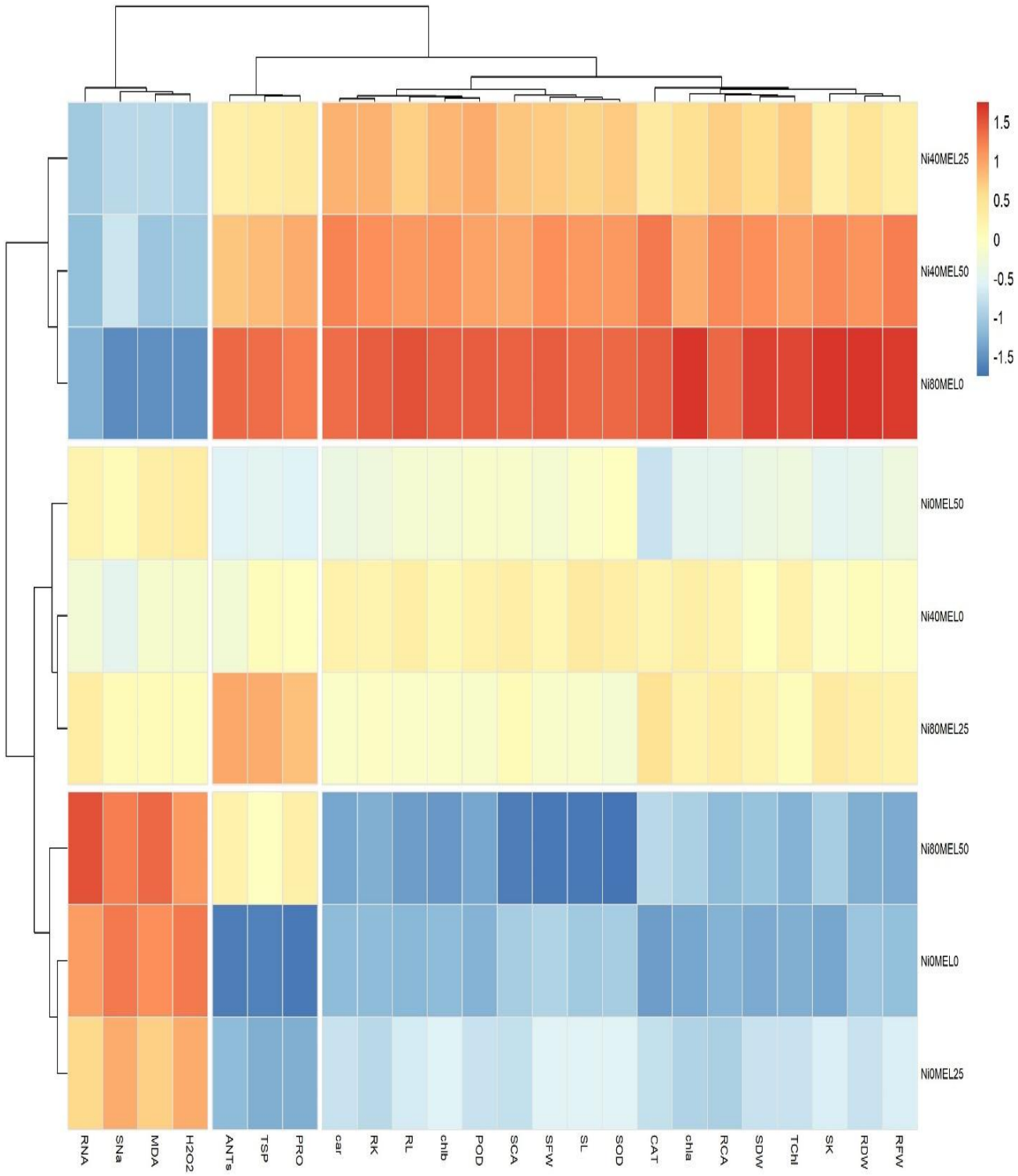


Fig. 11. Hierarchical clustering showing heatmap relative responses of physiological, biochemical, antioxidant and growth-related parameters under different Ni-MEL treatments of sunflower cultivar (FH-741). The color scale represents standardized relative values from – 1.5 (blue, low values) to + 1.5 (red, higher values).

To show associations between sunflower morpho-physiological and biochemical characteristics at varying nickel stress levels (0, 40, and 80 mg kg^{-1} and melatonin levels (0, 25, and 50 µM), the FH-741 heatmap was created (Fig. 11). There were three sub-clusters seen in the main cluster. Strong positive associations were found between the first sub-cluster and root length, shoot length, shoot fresh weight, shoot dry weight, Chl b, carotenoids, and Chl a, as well as between root and shoot calcium, root and shoot potassium linked at Ni 80 (0 µM MEL), and Ni 40 (50 µM MEL). All of these parameters, however, showed a significantly negative associations at 80 Ni (50 µM MEL) 0 Ni (0 µM MEL). The total soluble protein, anthocyanin, and proline in the second sub-cluster exhibited a substantial positive correlation at Ni 80 (0 µM MEL) and Ni 40 (50 µM MEL), however at two distinct levels, N i0 (0 µM MEL) and Ni 0 (25 µM MEL), there was a negative association.

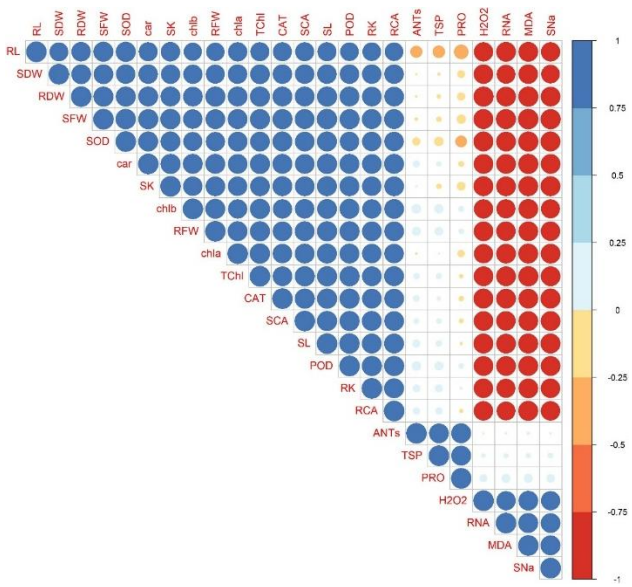


Fig. 12. Correlation plots of morphological, physiological, biochemical, enzymatic and ionic content of sunflower cultivar (FH-701) under nickel stress including SNa (shoot sodium ion), RNa (root sodium ion, SK (Shoot potassium ion) RK (Root potassium ion), SCA (Shoot calcium ion), RCA (Root calcium ion), MDA (Malondialdehyde), H_2O_2 (Hydrogen peroxide), TSP (Total soluble protein), ANTS (Anthocyanin) PRO (Proline), Tchl (Total chlorophyll), Car (Carotenoids), RL (Root length), SL (shoot length), RFW (Root fresh weight), RDW (Root dry weight), SOD (Superoxide dismutase), POD (peroxidase), CAT (Catalase).

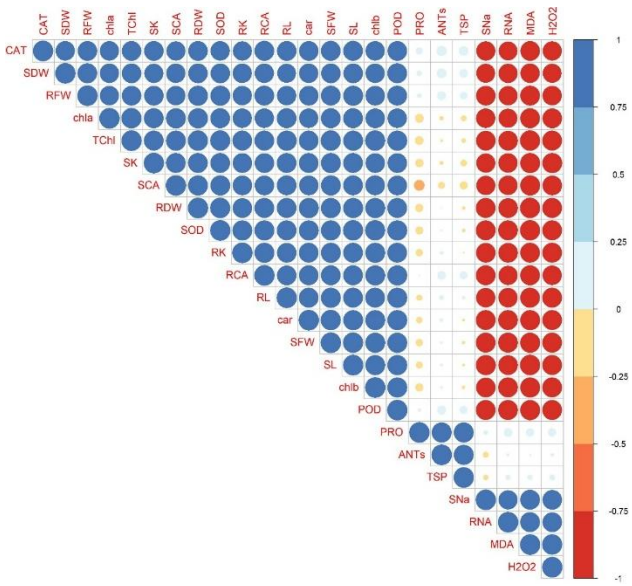


Fig. 13. Correlation plots of morphological, physiological, biochemical, enzymatic and ionic content of sunflower cultivar (FH-741) under nickel stress including SNa (shoot sodium ion), RNa (root sodium ion, SK (Shoot potassium ion) RK (Root potassium ion), SCA (Shoot calcium ion), RCA (Root calcium ion), MDA (Malondialdehyde), H_2O_2 (Hydrogen peroxide), TSP (Total soluble protein), ANTS (Anthocyanin) PRO (Proline), Tchl (Total chlorophyll), Car (Carotenoids), RL (Root length), SL (shoot length), RFW (Root fresh weight), RDW (Root dry weight), SOD (Superoxide dismutase), POD (peroxidase), CAT (Catalase).

Biochemical traits including hydrogen peroxide and malondialdehyde were strongly positively associated with the third sub-cluster at 80 Ni (50 µM MEL) and Ni 0 (0 µM MEL). In contrast, the third sub-cluster showed negative associations at different levels, with the highest association occurring at 40 Ni (50 µM MEL) and 80 Ni (0 µM MEL). Sodium ions from roots and shoots likewise exhibited a high positive correlation in this sub-cluster at 80 Ni (50 µM MEL) and Ni 0 (0 µM MEL). Overall, there was a negative correlation between plant secondary metabolites (anthocyanin, total soluble protein, and proline concentration).

Correlations: Correlations between the biochemical and morpho-physiological attributes of FH-701are presented in Fig. 12. Morphological parameters and superoxide dismutase, peroxidase, and catalase were positively correlated with photosynthetic contents of plants (chlorophyll a, chlorophyll b, Caro, and T Chl) and ion analysis (root and shoot potassium and calcium ions), while hydrogen peroxide, MDA, and root and shoot sodium ions showed a negative correlation. The stress indicators MDA and H_2O_2 exhibit a positive correlation with proline and TSP. The correlations between the biochemical and morpho-physiological attributes of FH-741 are shown in Fig. 13. MDA and H_2O_2 levels were positively correlated with sodium ions found in plant roots and shoots. Plant photosynthetic pigments (Chl a, Chl b, total Chl, and carotenoids), ionic parameters (calcium, K^+ ion), plant growth-related traits, and SOD, POD, catalase contents were strongly negatively correlated with sodium ions in the roots and shoots. There is a positive correlation between shoot sodium ions and PRO and TSP

Principal component analysis (PCA): Four distinct clusters were found in the PCA biplot (FH-701) between biochemical and morpho-physiological traits are presented in Fig. 14. MDA and root sodium ion levels were highly correlated with 80 mg kg^{-1} Ni (50 µM MEL) in the first group. Superoxide dismutase, CAT, and root Ca ions linked at 80 mg kg^{-1} Ni (0 µM MEL) and photosynthetic contents (chlorophyll b, chlorophyll a, and total chlorophyll) were weakly associated with total soluble protein, anthocyanin, and total proline contents, which were strongly related to each other at 80 mg kg^{-1} (25 µM MEL). Shoot sodium ions connected at 0 mg kg^{-1} (50 µM MEL) were highly correlated with plant hydrogen peroxide concentrations. SOD was poorly connected at 40 mg kg^{-1} Ni (0 µM MEL) and 40 mg kg^{-1} Ni (25 µM MEL), and plant photosynthetic contents (carotenoids, chlorophyll a) shown a high correlation with ionic contents (root K, Ca ions) as well as shoot dry weight, root fresh weight, and root dry weights. Four distinct clusters were shown by principal component analysis of FH-741 between morpho-physiological and biochemical characteristics are in Fig. 15. Catalase, shoot and root calcium and potassium ions, shoot dry weights, and root dry and fresh weight connected at 80 mg kg^{-1} Ni (0 µM MEL) are all positively correlated with chlorophyll in the first cluster. MDA and H_2O_2 concentrations linked at 80 mg kg^{-1} Ni (50 µM) were substantially correlated with sodium ions in roots and shoots. Super oxide dismutase, peroxidase, carotenoids, total chlorophyll, and chlorophyll b root potassium ions connected at 40 mg kg^{-1} Ni (50 µM MEL) shown a substantial correlation with plant growth-related parameters (root length, shoot length, and shoot fresh weights). Strong correlations were observed between root fresh weight, root calcium, and shoot potassium ions linked at 80 mg kg^{-1} Ni (0 µM MEL), as well as plant proline content, total soluble protein, and anthocyanin significantly associated at 80 mg kg^{-1} Ni (25 µM MEL).

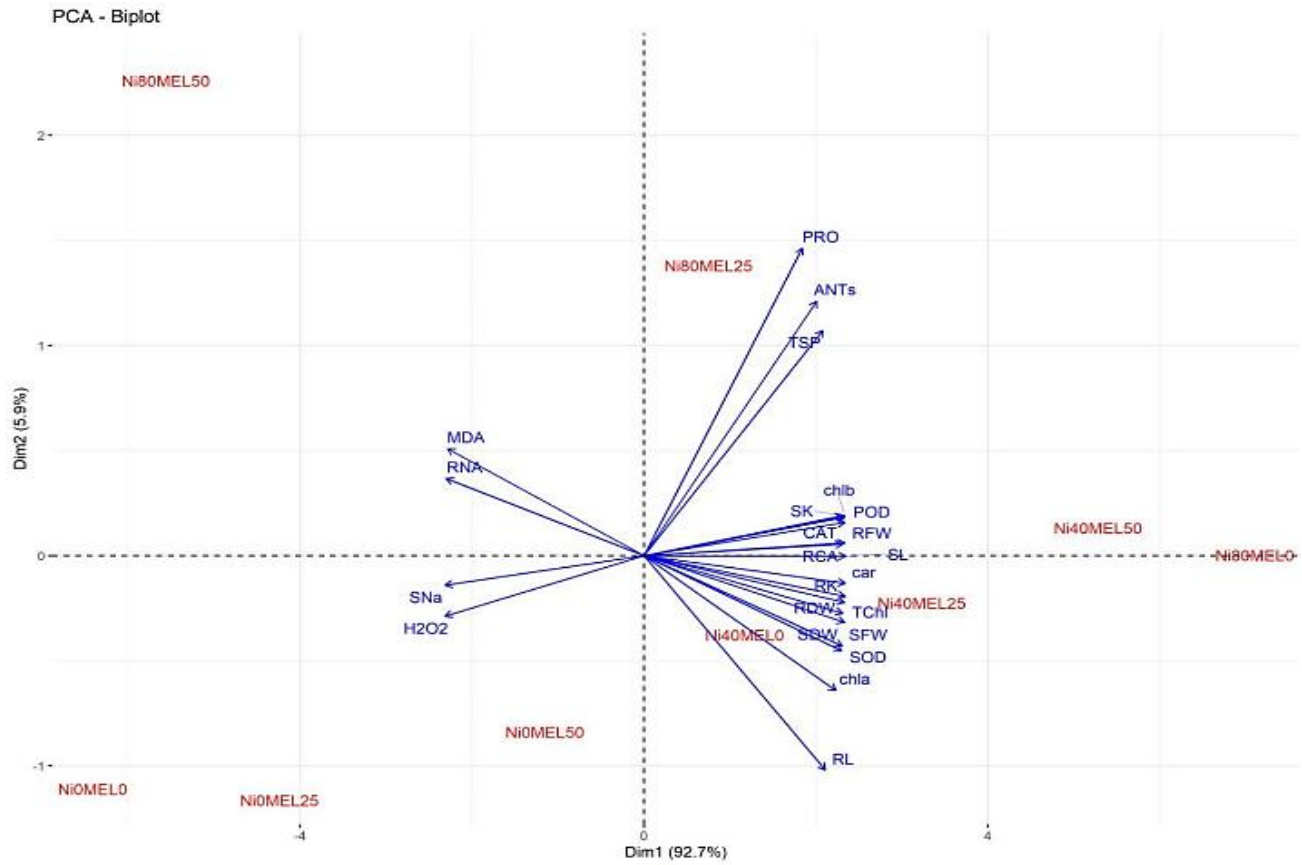


Fig. 14. Principal component analysis (PCA) biplot of sunflower (FH-701) between morpho-physiological, biochemical, and ionic characteristics under nickel stress.

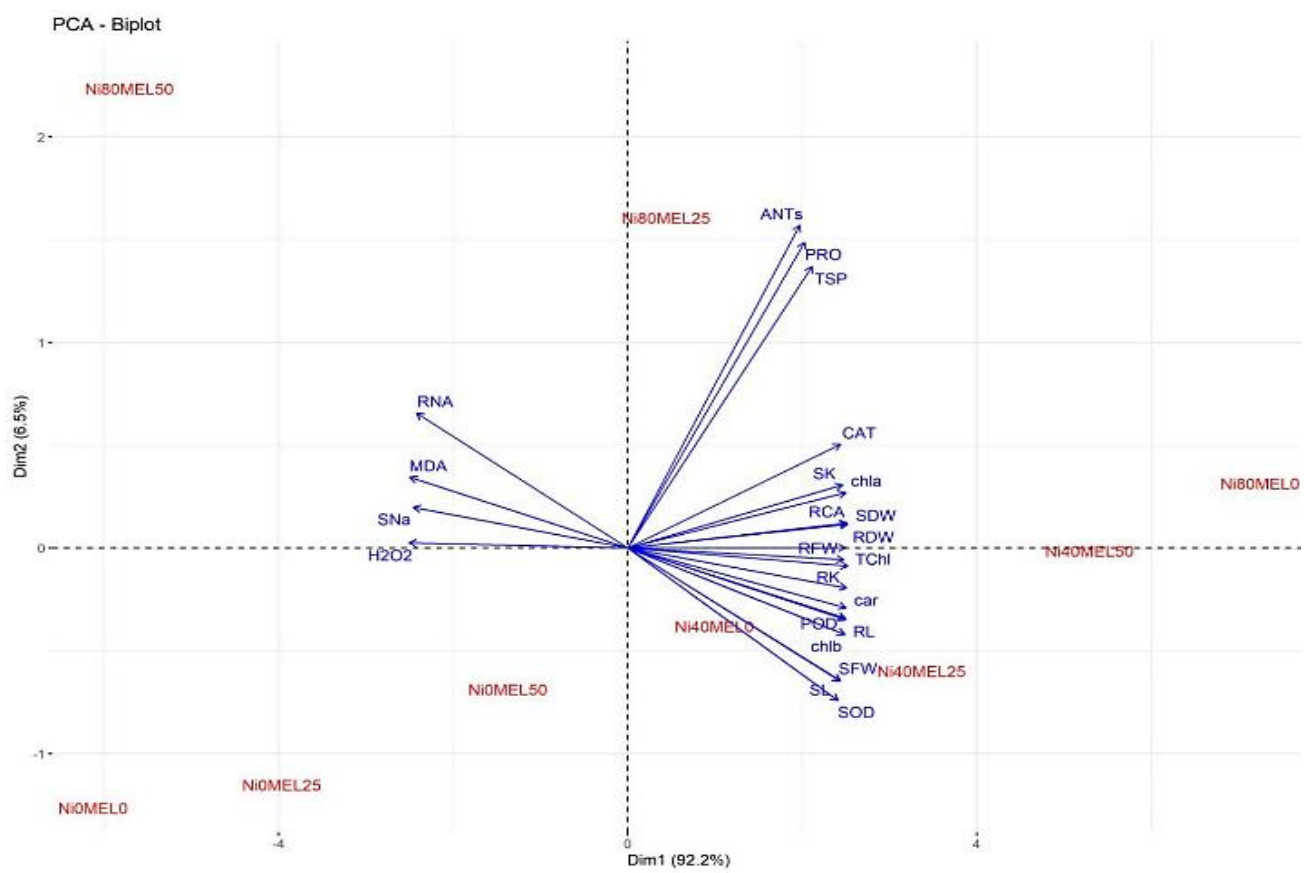


Fig. 15. Principal component analysis **(**PCA)biplot of sunflower (FH-741) between morpho-physiological, biochemical, and ionic characteristics under nickel stress.

Discussion

Sunflower (*Helianthus annuus* L.) growth and yield are adversely affected by rising Ni concentrations in the soil (Ahmad *et al.,* 2023). Because Ni causes serious damage to the root cell membranes, ceases cell elongation, and hinders cell divisions, excessive Ni can cause root development suppression. This results in weak root functions, which prevent plants from absorbing water and vital mineral nutrients to sustain healthy growth (Hassan *et al.,* 2019). In the present study nickel stress prevents shoot growth and reduced FW, DW, SL and sunflower RL. Heavy metal prevents plants from growing as they incited them under ionic and osmotic stress, which disrupts normal cell division, elongation, and functioning (Hoque *et al.,* 2021). MEL is now more than just a plant hormone; it is a powerful growth regulator. According to earlier research, exogenous MEL successfully restored the development and vigor of *T. foenum-graecum* plants by counteracting the negative effects of abiotic stressors such as nickel, and simulated acid rain (Parwez *et al.,* 2021). By encouraging root elongation, preventing damage to the root membrane, and regulating stomatal closure for optimal transpiration, the current study showed that foliar spray of MEL substantially enhanced growth attributes in sunflower under Ni stress. Similar results were observed with exogenous melatonin application in preventing Cd-induced growth inhibition of mallow *(Malva parviflora*) plants (Tousi *et al.,* 2020).

Significant deterioration of photosynthetic pigments and major chlorophyll alterations, which suggest a decrease in photosynthetic activity, are signs of nickel phytotoxicity in sunflowers (Ahmad *et al.,* 2023). In the current study, Ni stress reduced photosynthetic pigments, while exogenous melatonin increased photosynthetic pigments. This protective effect may additionally be found from melatonin's ability to maintain chloroplast integrity, reduce oxidative damage, and maintain chlorophyll biosynthesis. The use of MEL increased the generation of photo-synthetic pigments in the current study (Fig. 3). By maintaining the integrity of the chloroplasts, down-regulating a number of enzymes that break down chlorophyll, and inhibiting the activity of enzymes that catabolize chlorophyll, MEL was shown to prevent the loss of chlorophyll (Du *et al.,* 2024). Greater tolerance was determined in FH-741, as reflected through improved vigor, lower oxidative damage, better nutrient stability, and decreased Ni accumulation as compared to FH-701 which indicates a more potent ability to maintain physiological homeostasis under Ni stress.

Primary harmful ions that excessively build up in cytoplasm are sodium ions (Na^+), which causes ionic stress, which is the basic range of heavy metal stress. By moving potassium ions from cytoplasm to ectoplasm causes this action. As a result, it is necessary to maintain cytoplasmic K^+/Na^+ balance for plant heavy metal stress tolerance (Kumar *et al.,* 2022). The current research findings are shown that Na^+ aggregation and an ongoing reduction in K^+ levels are caused by nickel stress in sunflowers. FH-741 accumulated more Ni in roots but maintained lower Ni concentrations in shoots than FH-701, this would indicate that FH-741 restricts Ni movement to photosynthetically active tissues. As a result, the entry of K^+ and Na^+ into plant cells was successfully blocked. When too many Na^+ ions enter plant cells, they immediately disrupt cellular metabolism, which causes K^+ ions to leak out (Demidchik, 2014). The Ca^{+2} binding will be displaced on the Ca^{+2} binding site, resulting in high levels of sodium ions in plants, which will reduce the pectin cross-linking and limit cell development (Celus *et al.,* 2018). Even if treatment was stress or non-stress, it decrease (Na^+) due to MEL treatment and level of potassium ions (K^+) in the roots increased *(Siddiqui et al.,* 2021).The current study shows that FH-741 maintained a better K^+ / Na^+ ratio than FH-701, due to greater Ni tolerance which may result from more efficient ion differentiation and homeostasis and FH-741 does not tolerate higher Ni stress yet actively maintains a more favorable cellular ionic environment.

The root system serves as main organ of plant for resistance to adverseness. Based on this, we concluded that melatonin mainly affected the roots of sunflower, improving ion homeostasis and reduced the harmful effects of ionic stress on the plant. An essential component of the cell wall is calcium, most of which serves as a pectineus structural element that preserves the structure and functionality of the cell wall. Additionally, Ca^{+2}, K^+ can play an important role in preventing salt damage by increasing the absorption and reducing the Na^+ toxicity (Younis & Mansour, 2023).

The most direct result of Ni toxicity in sunflower at the biochemical level is an increase in MDA content, which is caused by enhanced membrane lipid peroxidation carried by oxidative bursts produced by H_2O_2 concentrations (Ahmad *et al.,* 2023). In previous study by Jiang *et al.,* (2024), an increase in MDA levels alters membrane functions, causing electrolyte extravasation, disrupting ion homeostasis, and resulting in osmotic stress. Eliminating the harmful ROS effects caused by excess Ni through increased activity of antioxidant enzymes that decrease ROS is an innate mechanism (Rizwan *et al.,* 2018). Similarly, in response to Ni-generated ROS production in sunflower, my findings show an increase in SOD, POX, and CAT activities at 80 mg kg^{-1} Ni soil which suggesting that an endogenous defense response cause to detoxify excessive ROS. By significantly lowering ROS species concentrations like O_2 and H_2O_2 through direct scavenging and increasing the expression of antioxidant enzyme genes to support vital metabolic processes, MEL application in plants exposed to heavy metal toxicity reverses the related oxidative burst (Younis & Mansour, 2023).

Current study showed that 50 µM MEL had the greatest impact on increasing the activities of CAT, POD, SOD under high Ni (Fig. 5). This, in turn, reduced the concentrations of H_2O_2 that were detected and observed using the NBT strain (Fig. 4). Additionally, as a defense mechanism, nickel supply increased the formation of a non-enzymatic antioxidant like proline, which was further enhanced with all provided quantities of MEL, with the largest rise occurring at 50 µM MEL.

Increases in proline levels promote the growth of Krebs cycle enzymes for the production of alternative energy sources, decrease photoinhibition damage in the photosystem, and immediately lead to the regeneration of chlorophyll (Younis & Mansour, 2023). Additionally, proline either directly neutralizes harmful free radicals generated by stress or indirectly reduces their concentration by being incorporated into the synthesis of

phytochelatins or metallothioneins that combine with HMs (Hosseinifard *et al.,* 2022). The reduction of HM harmful effects in plants is mostly dependent on changes in proline content, anthocyanin, and total soluble protein, in response to environmental changes (Qaderi *et al.,* 2023).

The total concentration of proline, anthocyanin, and total soluble protein in sunflower was reduced by nickel at 80 mg kg^{-1} in the present study. Reducing HM-induced cytoplasmic toxicity is largely dependent on the metal chelating properties of proline, anthocyanin, and total soluble protein for deactivating excess HMs, including Ni, through hydroxyl radical trapping and interfering with ROS generation through direct scavenging (Qaderi *et al.,* 2023). Additionally, these metabolites support the regular metabolic functioning of plants by stabilizing proteins and the membrane lipid bilayer, maintaining cell turgor and water potential under osmotic stress (Salam *et al.,* 2023). Under nickel stress, exogenous MEL at 50 µM level exhibits a notable increase in the accumulation of proline, anthocyanin, and total soluble protein in sunflower leaves. Increasing proline can enhance osmotic adjustment, membrane stabilization, and ROS buffering (Zulfiqar & Ashraf, 2023). Increased anthocyanin accumulation indicates stimulation of the phenylpropanoid/flavonoid pathway, in which MEL may also promote enzymes such as PAL and CHS and downstream anthocyanin biosynthesis and higher TSP may reflect improved protection and maintenance of stress-responsive antioxidant proteins (Zhu *et al.,* 2025). Treatment with exogenous melatonin by overexpressing the melatonin synthetic-related genes stimulated the antioxidant enzymes (including SOD, CAT, POD, PAL) and expanded antioxidant levels (proline, flavonoid, and Anthocyanins), inhibiting ROS production with heavy metal stress in *tomato, Nicotiana tabacum* L., rice, maize, wheat, Azolla imbricata, watermelon seedlings (Gu *et al.,* 2022).

According to present study, plants exposed to Ni showed a dose-dependent buildup of Ni in their roots and shoots, while plants treated with MEL showed a lower concentration of Ni (Fig. 8). By limiting metal uptake at the root site and accumulating in tissues, MEL-mediated restoration of plant growth traits under HM toxicity was consistent with previous findings (Altaf *et al.,* 2023). Different sub-clusters of heatmap analysis illustrate those complex connections between a wide range of characteristics, including how some biochemical pathway such as phenolic compound pathway interact with melatonin and nickel stress treatment. According to El Brouzi *et al.,* (2024), melatonin treatment protect against nickel-induced oxidative stress by boosting enzymatic antioxidant, melatonin may have a protective impact at lower Ni levels. Additionally, the PCA results show how MEL treatment and heavy metal stress change many sets of characteristics, including photosynthetic pigments and growth factors. This is in line with previous research by Mir, Alam *et al.,* (2022) which used PCA to find plant characteristics that are resistant to nickel. Furthermore, the association between stress tolerance-induced secondary metabolites specifically, anthocyanin and total soluble protein emphasize the importance of secondary metabolites in preventing oxidative damage.

Conclusion

The conclusion of my study shows that the use of MEL foliar spray is beneficial to both sunflower cultivars because it eliminates the adverse effects of nickel stress. But cultivar FH-741 has better growth as compared to FH-701 because it is more stress tolerant. This is achieved through enhancing the enzymatic activities that are components of the antioxidant defense system that subsequently lowers the generation of ROS. This was defined by lower markers of H_2O_2 and MDA in the two varieties. The application of foliar MEL to the sunflower plants that were nickel-stressed enhanced plant growth, biomass, and photosynthetic pigment activity. Moreover, ion homeostasis in plants adding melatonin was exhibited since there were more potassium and calcium ions that were concentrated in the root and shoot under nickel stress. Increasing levels of melatonin serve as role of alleviating the extreme effects of nickel stress poses to both sunflower varieties. This is achieved through increase of secondary metabolites synthesis, including total soluble protein, total proline, anthocyanins contents. However, MEL did not only enhance the production in sunflower and also affected flowering and fruit maturity but also inhibited leaf-senescence.

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