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HINA KANWAL

IRAM SHOAIB

ALI NOMAN

MUHAMMAD FAISAL MAQSOOD

RASHDA NAHEED

See next page for additional authors

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HINA KANWAL, IRAM SHOAIB, ALI NOMAN, MUHAMMAD FAISAL MAQSOOD, RASHDA NAHEED, OMAR MAHMOUD ALZOUBI, MOHAMED HASHEM, REHAB ELNOUR, FAHAD MOHAMMED ALZUAIBR, NOREEN KHALID, MUHAMMAD KASHIF IRSHAD, and MUHAMMAD AQEEL

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Biostimulant-mediated cellular repair by improving antioxidant dynamics and osmoregulation against metal stress in canola

Hina KANWAL[1,†], Iram SHOAIB[1,†], Ali NOMAN[2,†], Muhammad Faisal MAQSOOD[3], Rashda NAHEED[1], Omar Mahmoud ALZOUBI[4], Mohamed HASHEM[5], Rehab ELNOUR[6], Fahad Mohammed ALZUAIBR[7], Noreen KHALID[8], Muhammad Kashif IRSHAD[9,10], Muhammad AQEEL[11,*]

[1]Department of Botany, Government College Women University, Faisalabad, Pakistan
[2]Department of Botany, Government College University, Faisalabad, Pakistan
[3]Department of Botany, The Islamia University of Bahawalpur, Pakistan
[4]Biology Department, Faculty of Science Yanbu, Taibah University, Yanbu El-Bahr, Saudi Arabia
[5]Department of Botany and Microbiology, Faculty of Science, Assiut University, Assiut, 71516, Egypt
[6]Biology Department, Faculty of Arts and Science, King Khalid University, Dahran Al-Janoub, Saudi Arabia
[7]Department of Biology, Faculty of Science, University of Tabuk, Tabuk, Saudi Arabia
[8]Department of Botany, Government College Women University Sialkot, Pakistan
[9]Department of Environmental Sciences, Government College University Faisalabad, Pakistan
[10]Department of Environmental and Energy Engineering, Yonsei University, Wonju, Republic of Korea
[11]State Key Laboratory of Herbage Improvement and Grassland Agroecosystems (SKLHIGA), College of Ecology, Lanzhou University, Lanzhou, Gansu, P.R. China

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Abstract: Global crop yield and growth are severely impacted by hazardous heavy metal contamination of the soil and water. Hence, this study examined the effects of three different levels of cadmium (Cd) stress (concentrations of 0, 1.5, and 2.25 mM) and the foliar application of a biostimulant, i.e. Moringa leaf extract (MLE), on a range of physiological, biochemical, and morphological parameters of two canola (*Brassica napus* L.) cultivars, Punjab (V_1) and Super (V_2). More Cd was accumulated in both the aerial and subterranean sections of V_1 than in V_2. The morphological and physiological indicators showed a substantial ($p < 0.05$) deterioration as a result of the Cd buildup. The ascorbic acid, malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) levels were considerably ($p < 0.05$) elevated under Cd stress. V_1 had considerably higher H_2O_2 levels than V_2, which suggests that the oxidative stress levels were higher. Additionally, the activity of some antioxidant enzymes, such as catalase and peroxidase (POD) (65% and 40%, respectively), were decreased by the higher concentration of Cd (2.25 mM). V_2 demonstrated stronger antioxidant defense mechanisms than V_1, as seen by a 115% increase in POD activity, a 70% increase in shoot fresh weight, and a 49% increase in amino acids following the MLE application. Despite this, V_2 was still able to withstand the Cd. Under Cd stress, the foliar treatment of MLE increased the yield, growth, biochemical, and physiological characteristics of both V_1 and V_2. The findings indicated that applying MLE foliar spray can improve crop productivity and lessen the impacts of metals on *B. napus* L.

Key words: Brassica, metals, plants, pollution, remediation, soil

1. Introduction

Cadmium (Cd) contamination of soil presents a significant risk to the environment and public health because of its tenacity and endless circulation. Both natural and manmade sources can release lead into the environment, which is harmful to both plants and animals. Rock weathering is one example of a natural source, whereas industrial processes, mining, fertilizers, waste disposal, sewage irrigation, etc., are examples of anthropogenic means (Irshad et al., 2022; Zhu et al., 2022). Due to its greater mobility and extreme toxicity to living things even at very low concentrations, Cd is eighth on the 2017 priority list of hazardous substances (Cheng et al., 2023). Cd can still be absorbed by the roots, accumulate, and move to aerial plant tissues even though it is not necessary for plants (Irshad et al., 2020; Javad et al., 2023).

Growing plants in soil contaminated with Cd is a major environmental management concern and a substantial hazard to plant growth (Huang et al., 2023). In plants, Cd causes oxidative stress that results in DNA damage,

* Correspondence: aqeelbutt99@gmail.com
† These authors contributed equally to this publication work

protein denaturation, and inhibition of the photosynthetic machinery. Moreover, it suppresses shoot and root growth as well as seed germination (Yu et al., 2023). In addition to biochemical and physiological changes, the absorption of Cd causes intricate changes in the features of plant development (Haider et al., 2021). By preventing mitosis, harming the Golgi apparatus, and interfering with the synthesis of cell wall components and polysaccharide metabolism in the root cortex, Cd reduces root growth (Shah et al., 2020).

Moreover, it interferes with respiration, photosynthesis, protein metabolism, and the intake, transport, and consumption of essential nutrients (Clemente et al., 2019). In addition to altering the equilibrium of water, it also suppresses the function of specific enzymes and results in oxidative damage, all of which decrease plant growth (Ghori et al., 2019; Haider et al., 2021). Chlorosis, wilting, and changed leaf orientation brought on by the suppression of chlorophyll production are among the harmful effects of Cd on plants that eventually cause them to die (Hassan et al., 2020). It is a nonredox reactive metal that interacts with the antioxidative system of the defense mechanism, disrupts electron transport, and inhibits important functional groups in biomolecules to produce oxidative stress in plants (Mishra et al., 2014).

According to Irshad et al. (2020), exposure to Cd through the food chain is known to induce several forms of cancer and Itai-itai disease. Thus, it is essential to restrict the amount of Cd that both plants and people absorb. Canola (*Brassica napus* L.), which is rich in phenolics and antioxidants, is the most important source of edible oil in the world and a crop with great economic value (Sanjari et al., 2019). Because canola seeds contain a large amount of oleic acid, a crucial unsaturated fatty acid, its oil is known for having excellent quality and quantity (Mamnabi et al., 2020). Canola oil has a different composition depending on the genotype that is grown. Generally speaking, it is made up of 27% polyunsaturated fatty acids, 66% monounsaturated fatty acids, and 7% saturated fatty acids (Shaaban et al., 2023; Shen et al., 2023). Growing canola in soil contaminated with Cd increases the Cd uptake and accumulation in the plant's components.

Globally, a range of techniques, including foliar treatments and seed priming, have been used to lessen the effects of Cd stressors on plants (Aqeel et al., 2021). Sustainable methods that support plant productivity, preserve soil quality, and are environmentally benign are desperately needed to counteract the Cd threat (Arshad et al., 2016; El Rasafi et al., 2022). A relatively novel approach in agriculture is the use of plant extracts as plant growth regulators (PGRs) (Noman and Aqeel 2017; Noman et al., 2020). The multipurpose plant *Moringa oleifera* L. is used for a variety of reasons and has also been utilized to stimulate crop growth. Studies on plants have shown that applying Moringa leaf extract (MLE) via the seeds or foliage has a positive effect on emergence, seedling/plant growth, and eventually, economic yield (Khan et al., 2020). Natural antioxidants, including ascorbate (AsA) and phenolics, abound in Moringa leaves. Additionally, several growth hormones, such as auxin, cytokinins, and abscisic acid, are enhanced in MLE (Soares et al., 2021). Numerous bioactive substances, such as vitamin A, B_1 (thiamine), C (ascorbic acid), E (tocopherol), carotenoids, phenolic acids, and flavonoids, are present in Moringa leaves (Leone et al., 2015). According to Biswas et al. (2016), MLE may therefore function as a biostimulant and aid in fostering growth in a variety of crops. It has been shown to accelerate root growth, encourage seed germination, and enhance canola yield characteristics (Iqbal et al., 2014; Khan et al., 2020). Furthermore, it has been demonstrated that applying MLE improves crops' resistance to severe stress. Furthermore, MLE-treated plants have shown good resistance to biotic stress (Desoky et al., 2019; Yuniati et al., 2022).

Thus, the hypothesis investigated in this work was that by decreasing Cd accumulation, increasing nutrient and osmoprotectant levels, and boosting nonenzymatic and enzymatic antioxidant activities in canola plants, the foliar application of MLE can improve plant development and yield. For plants to experience less stress from heavy metals, these systems are essential.

2. Materials and methods

2.1. Plant growth experiment

The pot experiment was carried out in September 2021 using 2 canola cultivars, Punjab (V1) and Super (V2), in a 3-factor factorial completely randomized design at the Government College Women University, Faisalabad, Pakistan (31°42′ latitude, 73°08′ longitude). There were 3 replicates of every treatment. An average light/dark photoperiod of 11:13 was used during the experiment. First, 7 kg of a 1:1:1 soil-sand-compost mixture was put into each pot. After 30 days of germination, the plants were treated with 3 concentrations of Cd using Cd chloride solution ($CdCl_2$) at 0, 1.5, and 2.25 mM. A control without Cd was also included. After 4 weeks of Cd stress, MLE (1:30) was sprayed on the plant leaves. The plants were harvested 30 days after the MLE application. The physiobiochemical and other growth traits of the plants were then measured.

2.2. Estimation of the morphological parameters

Before estimating the fresh shoot (Biswas et al., 2016) and root weights, the shoots and roots were cleaned with distilled water and then dried. The plant root length (RL), shoot length (SL), and leaf area were measured with a ruler. Subsequently, they were dried at 70 °C for 48 h in

an oven. The root and shoot dry weights (DWs) were then calculated.

2.3. Estimation of the photosynthetic pigments

Using the Arnon (1949) approach, the contents of photosynthetic pigments, such as chlorophyll *a* and *b*, and carotenoids, were measured and approximated. After homogenizing 0.5 g of the fresh leaves in 10 mL of cold acetone (80% V/V), they were centrifuged at 5,000 rpm × *g* for 10 min. The absorbance of each supernatant was measured at wavelengths of 663, 645, and 480 nm.

Chlorophyll *a* (mg/g) = (12.7 × A663) − (2.59 × A645)

Chlorophyll *b* (mg/g) = (22.9 × A645) − (4.7 × A663)

$$\text{Carotenoids content } (\mu g/g) = \frac{A \times V\,(mL) \times 104}{A_{1\,cm}^{1\%} \times P\,(g)}$$

Here, A is the absorbance, V is the total extract volume, P is the sample weight, and $A_{1cm}^{1\%}$ is 2592.

2.4. Total free amino acids

To grind the fresh leaf material, 0.2 M of phosphate buffer (pH 7.0) was utilized. Following the method of Hamilton and Van Slyke (1943), 1 mL of buffer-ground extract was mixed with 1 mL of 70% pyridine and 1 mL of acid ninhydrin. The test tubes holding the reaction mixture were submerged in a water bath set at 100 °C for 30 min. Using distilled water, the final volume of the solution was 50 mL. The absorbance was measured at 570 nm with a Hitachi 220 spectrophotometer (Hitachi Ltd., Chiyoda-ku, Tokyo, Japan).

2.5. Total soluble sugars

The total soluble sugars were measured in accordance with the method of Yemm and Willis (1954). Briefly, 80% acetone was used to grind a 100-mg dried plant sample. The extract was kept in an incubator at 60 °C for 6 h. Anthrone reagent (6 mL) was added to the extracted mixture. Then, 150 mg of anthrone was added to 72 mL of H_2SO_4. The samples were incubated at room temperature for 10 min after being heated for 10 min in a water bath. The optical density was read with a spectrophotometer at 625 nm.

2.6. Proline determination

The proline content was calculated in accordance with the method of Bates et al. (1973). First, 0.5 g of fresh leaf material was ground using 10 mL of 3% sulfosalicylic acid. Next, 2.25 g of ninhydrin was dissolved in 2 mL of glacial acetic acid and 20 mL of orthophosphoric acid, and the mixture was combined with 2 mL of the solution and left to sit at 100 °C for 1 h. After adding 10 mL of toluene to the reaction mixture, it was well mixed while an air stream was continuously circulated through it for 1 to 2 min in an ice bath. Following its separation from the aqueous phase and warming to room temperature, the optical density of the toluene in the chromophore was measured at 520 nm. Using a standard curve as a guide, the proline concentration was determined and calculated using the fresh weight (FW).

(Proline mol/g FW = (proline g/mL × mL of toluene / 115.5) / (g of sample / 5)

2.7. Total soluble proteins

The method of proposed by Lowry et al. (1951) for estimation of the total soluble proteins was followed. In the preparation of the phosphate buffer, the following chemicals were used:

1. Molar solution was prepared as stock solution from $NaH_2PO_4.2H_2O$ (156.01 g/L).

2. Molar solution was prepared as stock solution from disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) (177.99 g/L).

2.7.1. Copper reagents

Solution A: The copper reagent comprised 2 g of Na_2CO_3, 0.2 g of NaOH, and 1.0 g of sodium potassium tartrate. Distilled water was added to all the 3 chemicals to maintain the volume up to 100 mL.

Solution B: To prepare the $CuSO_4.5H_2O$ solution, 0.5 g $CuSO_4.5H_2O$ was dissolved in distilled water (100 mL).

Solution C: Solution B (1 mL) and solution A (50 mL) were mixed to prepare solution C, as an alkaline solution. This solution was freshly prepared.

2.7.2. Standard bovine serum albumin (BSA) solution (1 mg/mL)

First, 1 g of BSA was dissolved in 10 mL of distilled water to prepare the stock solution. The different standards were prepared from the stock solution below:

0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 M, respectively.

2.7.3. Extraction

The fresh leaf material was weighed to 0.1 g, and 10 mL of 0.2 M pH 7.0 phosphate buffer was used to crush it. Next, a leaf sample weighing 5000 × *g* was centrifuged for 5 min. The supernatant was collected in order to determine the protein content.

2.7.4. Procedure

First, 1 mL of the leaf extract, 1 mL of phosphate buffer (pH 7.0), and a blank were placed in a test tube. Next, 1 mL of solution C was added, and the mixture was then carefully stirred and allowed to sit at room temperature for 30 min. Then, 0.5 mL of a 1:1 diluted Folin–Ciocalteu phenol reagent was added, completely dissolved, and allowed to sit at room temperature for almost 30 min. Ultimately, a spectrophotometer set to measure wavelengths of 620 nm was used to determine the optical density of the resultant solution (Hitachi Ltd.).

2.8. Glycine betaine

To determine the amount of glycine betaine in the leaf material, 0.5 g of the leaf sample was homogenized in 5 mL of distilled water. After filtering, 0.5 mL of the supernatant

was combined with 10 mL of a 2 N hydrochloric acid (HCl) solution. Next, 0.1 mL of potassium triiodide was combined with 500 µL of the mixture. To make this solution, 5 g of potassium iodide and 3.75 g of iodine were combined with 50 mL of 1 N HCl and vigorously shaken for 30 min. After gently shaking the combination for 90 min, it was cooled in a water bath. Then, 10 mL of 1,2-dichloroethane was added to the mixture, after 1 mL of ice-cold distilled water had been added. The test tubes were submerged in a water bath with a constant stream of air flowing over them for 1 to 2 min, and the temperature of the ice bath was adjusted to 4 °C. Consequently, 2 separate layers developed. The organic layer that remained after the upper aqueous layer was removed was utilized to measure the optical density at 365 nm (Grieve and Grattan, 1983).

2.9. Malondialdehyde (MDA)

First, 0.5 g of fresh leaf material was homogenized in 5 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 2000 × *g* for 15 min, following the procedure described by Carmak and Horsts (1991). Then, 1 mL of supernatant and 4 mL of 0.5% thiobarbituric acid (TBA produced in 20% TCA) were dissolved to create a reaction mixture. The reaction mixture was brought to a boil using a water bath, and the reaction was quickly stopped by setting the samples on ice. After another 10 min of centrifuging the samples at 10,000 × *g*, the absorbance of the supernatant was measured at 532 and 600 nm using a spectrophotometer.

2.10. Ascorbic acid

Fresh leaf material (0.25 g) was ground using 10 mL of 6% TCA. Then, 2 mL of dinitrophenyl hydrazine (2%) and 4 mL of leaf extract were combined, and 1 drop of thiourea (10%, made in 70% ethanol) was added. The solution was boiled in a water bath for 15 min. It was then mixed with 5 mL (80%) of H_2SO_4. The optical density of each sample was measured at 530 nm using an IRMECO U2020 spectrophotometer (IRMECO GmbH & Co. KG, Lütjensee, Germany).

2.11. Hydrogen peroxide (H_2O_2)

A prechilled mortar was used to grind 0.5 g of fresh leaf material using 5 mL of 0.1% (w/v) TCA. Then, it was centrifuged at 12,000 × *g* for 15 min. Next, 0.5 mL of supernatant was mixed with 1 mL of KI and 0.5 mL of potassium phosphate buffer. After that, it was vortexed, and a spectrophotometer (IRMECO U2020) was used to read the absorbance at 390 nm (Velikova et al., 2000).

2.12. Antioxidant enzyme activities

Using a sterile, ice-cold pestle and mortar, 0.1 g of fresh leaf material was ground in 10 mL of 7.8 pH phosphate buffer (50 mM). After that, the sample was centrifuged for 15 min at 15,000 × *g* at room temperature. The enzymatic peroxidase (POD) and catalase (CAT) activities were measured in the supernatant that was collected after centrifugation (Chance and Maehly, 1955). To generate a 3-mL reaction mixture for the CAT activity, 1 mL of H_2O_2 (5.9 mM), 1.9 mL of potassium phosphate buffer (50 mM, pH 7.8), and 0.1 mL of the sample extract were combined. The reduction in absorbance was read at 240 nm. Next, 0.1 mL of H_2O_2 (40 mM), 0.1 mL of guaiacol (20 mM), 0.75 mL of phosphate buffer (50 mM, 7.8 pH), and 0.05 mL of sample extract were added to 1 mL of reaction mixture to determine the POD activity. The change in absorbance was read at 470 nm using a spectrophotometer (IRMECO U2020).

2.13. Ionic contents of the roots and shoots

2.13.1. Digestion methods

After adding 0.1 g of dried plant material and 3 mL of H_2SO_4 to a flask, the mixture was allowed to sit at room temperature for 1 night. A few drops of H_2O_2 were added, and the digestive flask was heated on a hot plate to 65 °C until fumes began to form. Every 10 min, a drop of H_2O_2 was added until the liquid became transparent. After the digested material was filtered, distilled water was added to bring the volume up to 50 mL. The concentrations of many ions, including calcium (Ca^{2+}), potassium (K^+), and sodium (Na^+), were measured using this extracted solution. Following digestion, the amounts of Ca^{2+}, K^+, and Na^+ were measured using a flame photometer (PFP7, Jenway, London, UK) in the filtered extracts.

2.13.2. Shoot and root Cd ions (Cd^{+2})

The Cd^{+2} contents in the roots and shoots were determined via their acid digests using an atomic absorption spectrophotometer (AAnalyst-300, PerkinElmer LAS (Germany) GmbH, Rodgau, Germany).

2.14. Total phenolics

The mixture was allowed to sit at room temperature for 1 night after 0.1 g of dried plant material and 3 mL of H_2SO_4 were added to the flask. With a few drops of H_2O_2 added, the digestive flasks were heated to 65 °C on a hot plate until vapors began to form. Drop by drop, H_2O_2 was added to the mixture every 10 min until it became transparent. Following the digestion process, the material was filtered, and distilled water was added to bring the volume up to 50 mL. The $Ca2^+$, K^+, and Na^+ contents were measured using this extracted solution. Using a flame photometer, the amounts of $Ca2^+$, K^+, and Na^+ were measured in the filtered extracts following digestion.

2.15. Statistical analysis

Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and Meander Statistics Toolbox (MSTAT; University of Wisconsin–Madison, Madison, WI, USA) were used to analyze the data. MSTAT was utilized to carry out the analysis of variance (ANOVA). The least significant differences test was used to compare the means at a 0.05 probability level if the F-test revealed significant differences between the means.

3. Results

3.1. Growth attributes

Under stress from varying concentrations of Cd (0, 1.5, and 2.25 mM), both V_1 and V_2 showed a notable reduction in the root and shoot FWs and DWs, as well as in the RL and SL, and the leaf area. Under both Cd-stressed and nonstressed conditions, the foliar treatment of MLE (1:30) improved these growth parameters significantly ($p < 0.001$). After applying the MLE foliar spray, V_1 demonstrated increases in the root FW (40%), shoot FW (70%), root DW (34%), shoot DW (41%), RL (60%), SL (64%), and leaf area (67%) with 1.5 and 2.25 mM of Cd. After the MLE treatment, V_2 exhibited root and shoot FW values that were higher than those of V_1. Between the two cultivars, the decrease in these growth parameters was the greatest in V_1, while that for V_2 was less pronounced (Figure 1, Table).

3.2. Yield attributes

In both V_1 and V_2, the application of Cd to the roots resulted in a significant ($p < 0.001$) decrease in the number

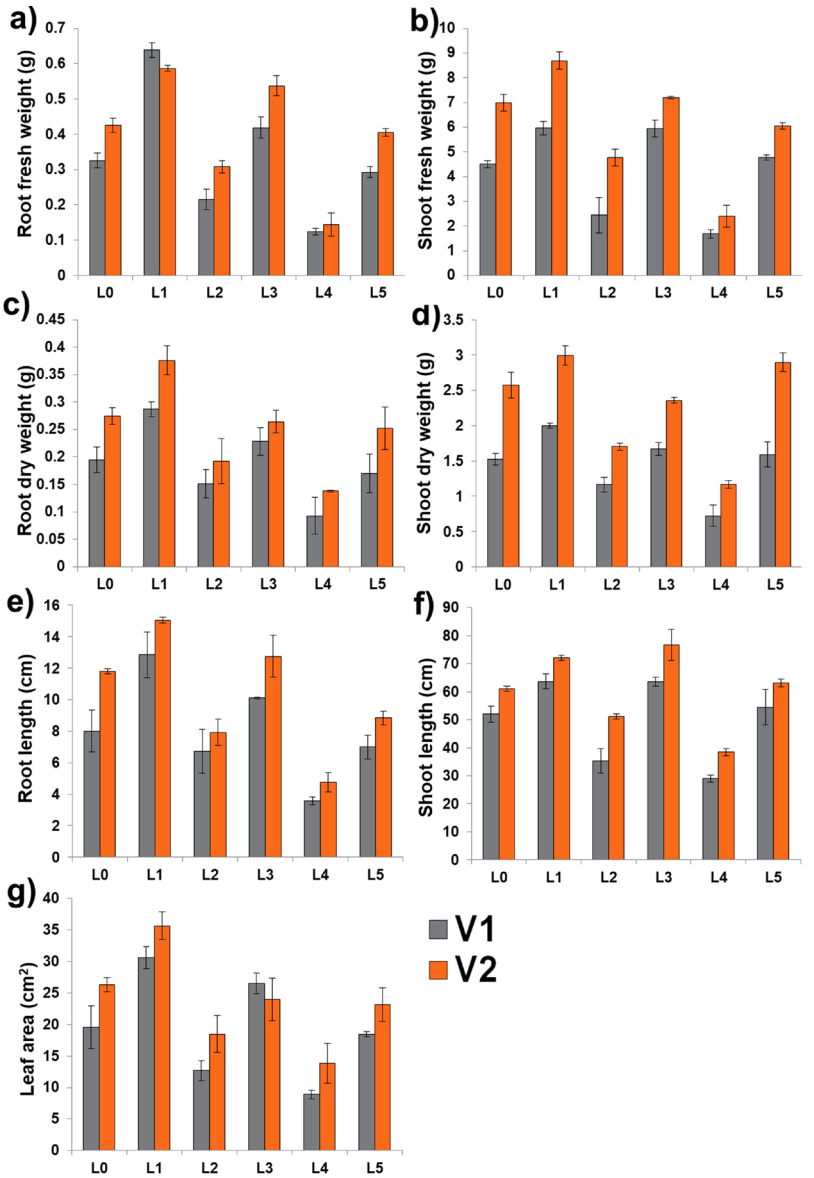

Figure 1. Morphological attributes: (a) root FW, (b) shoot FW, (c) root DW, (d) shoot DW, (e) RL, (f) SL, and (g) leaf area of the two canola varieties under different levels of Cd stress and the foliar application of moringa leaf extract (MLE). L0: control, L1: MLE (1:30), L2: 1.5 mM of Cd, L3: 1.5 mM of Cd + MLE, L4: 2.25 mM of Cd. L5: 2.25 mM of Cd+MLE.

Table. ANOVA table for different parameters of two canola varieties under different levels of Cd stress and foliar application of Moringa leaf extract (MLE).

Source	Variety (V)	MLE	Stress (S)	V*MLE	V*S	MLE*S	V*MLE*S	Error
Degree of freedom	1	1	2	1	2	2	1	26
Root fresh weight	0.038***	0.446**	0.1656583*	2.507e4ns	0.005*	0.003ns	0.019ns	0.057
Shoot fresh weight	30.856***	60.163***	17.993***	0.0931ns	1.295*	7.491ns	0.190ns	1.418
Root dry weight	0.0348444*	0.071289**	0.0395509*	4.27114ns	0.0019007ns	0.0019007*	9.858*	0.007
Shoot dry weight	2.716**	1.927*	1.440*	0.149ns	0.0847ns	0.787ns	0.422*	0.326
Root length	41.473***	41.530***	87.253***	0.061**	1.633ns	4.564*	4.022ns	2.441
Shoot length	1046.57***	4031.93***	514.482***	4.131*	17.069ns	704.759***	0.468ns	34.216
Leaf area	153.499***	857.093***	411.032***	25.737ns	20.125**	4.383*	16.384*	17.547
No. of siliqua per plant	245.444***	469.444***	347.675***	1.777ns	9.865ns	0.429ns	0.048**	9.795
No. of seeds per siliqua	169 ***	802.778***	223.444***	0.195**	0.286**	1.022ns	10.012ns	5.135
Total yield	0.113***	0.353***	0.019***	3.063**	0.019ns	0.039***	5.920ns	9.300
Chlorophyll a	6.246***	0.001***	2.984**	51.068ns	1.250*	7.145ns	1.447ns	4.038
Chlorophyll b	8.414*	0.003***	7.908*	8.890**	2.874ns	9.926ns	6 1.646ns	1.577
Carotenoids	0.001***	0.006***	0.002**	1.734ns	2.882ns	1.617*	9.162ns	2.961
Proline	175.324***	416.199***	733.488***	6.215***	16.793ns	102.089***	0.258ns	5.125
Glycine betaine	359.049***	955.020***	368.353***	0.169ns	43.750*	18.067ns	16.543ns	8.163
Malondialdehyde	344.626***	218.300***	555.268***	1.459ns	42.022**	84.029**	0.110ns	6.218
Ascorbic acid	44.662***	62.631***	63.902***	1.701ns	0.200ns	0.384ns	6.703**	0.651
Total soluble sugars	0.352***	0.115***	0.577***	0.003ns	0.019ns	0.062*	0.061**	0.007
Total phenolic content	0.021**	0.109***	0.029***	0.002ns	2.778ns	0.018*	0.002ns	0.002
Total free amino acids	0.136***	0.185***	0.129***	0.014***	0.007***	0.019***	0.011***	5.145
Hydrogen peroxide	1131.840***	6843.998***	882.757***	1.421*	54.853ns	233.351ns	0.241**	67.353
Root Ca^{+2}content	1.174***	2.507***	0.806*	0.007*	0.0436**	0.190ns	0.190ns	0.455
Shoot Ca^{+2}content	0.340ns	0.007**	5.282ns	0.562 *	2.54E+07	0.027ns	1.860**	0.771
Root K^{+} content	16.674***	27.562***	30.062***	0.174ns	3.299ns	8.896*	17.715***	1.722
Shoot K^{+} content	72.362***	319.115***	116.090***	11.578**	68.396***	68.396*	8.271**	1.382
Root Na^{+} content	50.174***	82.507***	21.049***	0.174ns	24.590***	0.924ns	1.049ns	1.472
Shoot Na^{+} content	23.798**	49.163 ***	9.898*	24.883 **	43.110***	25.048**	34.547***	1.859
Root Cd^{+2} content	12.840***	101.673***	13.907***	8.507**	0.217ns	3.048ns	0.012ns	0.856ns
Shoot Cd^{+2} content	0.008**	0.031***	0.014ns	2.611**	2.602ns	0.001**	3.028ns	0.001
Soluble proteins	0.002***	0.006***	0.002***	5.036**	2.979ns	1.113*	4.056ns	1.455
Catalase	53.845***	80.528***	63.743***	2.881ns	0.793ns	10.754*	0.269*	1.399
Peroxidase	0.138*	0.069ns	0.423***	0.015ns	0.114*	0.112*	0.095ns	0.023

of siliquae per plant, seeds per siliqua, and overall yield. A maximum reduction in these characteristics was observed when 2.25 mM of Cd was applied. Regarding these characteristics, a varied reaction was seen when Cd was applied at the various concentrations (1.5 and 2.25 mM). V_1 outperformed V_2 in terms of the decrease between the two cultivars. After applying the MLE foliar spray (1:30) and varying concentrations of Cd, the number of siliquae per plant and other yield parameters significantly ($p < 0.05$) increased in both cultivars (Figure 2).

3.3. Photosynthetic attributes

The application of varying concentrations of Cd to the roots resulted in a significant ($p < 0.001$) decrease in the carotenoid, and chlorophyll *a* and *b* levels in both V_1 and V_2. A maximum reduction in these pigments was seen when 2.25 mM of Cd was applied. The amount of chlorophyll *a* and *b*, and carotenoids in the leaves increased significantly after applying the MLE foliar spray (1:30) with varying concentrations of Cd. Following the application of MLE, V_2 outperformed V_1 in terms of a more notable increase in these values (Figure 3).

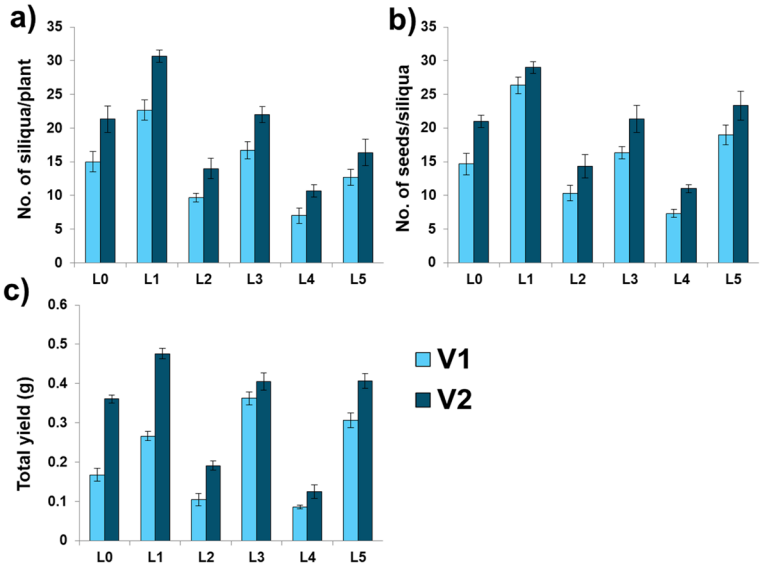

Figure 2. Yield attributes: (a) number of siliqua\plant, (b) number of seeds\ siliqua, and (c) total yield of the two canola varieties under different levels of Cd stress and the foliar application of MLE.

3.4. Physiological attributes

The ANOVA results demonstrated the significant impact of the Cd and MLE treatments on the physiological variables as well as the significant ($p < 0.01$) differences between the two cultivars. For the glycinebetaine (GB), MDA, AsA, sugars, and total soluble proteins, the variety × MLE relationship was not significant. Under Cd stress, the addition of MLE resulted in a considerable increase in the GB, AsA, sugars, and total phenolic levels. As an osmoticum, proline prevents the cytosol from becoming dehydrated due to Cd stress. The ANOVA for the proline content showed significant differences between the two cultivars, Cd treatments, and variety × MLE interaction. The average proline concentration increased significantly ($p \leq 0.01$) in both cultivars when the concentration of Cd was increased from 0 to 1.5, and 2.25 mM. The greatest increase in the proline content was observed with 2.25 mM of Cd, suggesting that cellular functions were negatively impacted by Cd stress. The proline content decreased after applying the MLE foliar spray, and the MDA concentration increased when the stress levels were increased compared to the healthy control plants. MLE was sprayed on the leaves in order to decrease the MDA content. Following the MLE treatment, V_1 demonstrated reductions of 41% and 43%, respectively, in the MDA content with 1.5 and 2.25 mM of Cd, whereas V_2 demonstrated reductions of 31% and 34% (Figure 4).

3.5. Total free amino acids

When the concentration of Cd was increased from 0 to 1.5 and 2.25 mM, the average proline content of both cultivars increased significantly ($p \leq 0.01$). The proline levels increased the most with 2.25 mM of Cd, suggesting that cellular functions were negatively impacted by Cd stress. The proline content decreased after applying the MLE foliar spray, and the MDA concentration increased in response to higher Cd stress levels when compared to the control plants. The MLE foliar spray was applied to decrease the MDA levels. After applying the MLE, the MDA content in V_1 decreased by 41% and 43% with 1.5 and 2.25 mM of Cd, respectively, whereas V_2 showed reductions of 31% and 34% (Figure 4).

3.6. Total soluble proteins

Under Cd stress, a considerable decrease in the total soluble protein was seen in both cultivars. This decrease with the increasing concentration of Cd was the lowest in V_2 and was the most noticeable in V_1. The maximum decrease was recorded with 2.25 mM of Cd, but both cultivars showed a significant but lessened decrease with 1.5 mM of Cd. The protein concentration was triggered by the MLE spray. However, this increase was less pronounced in V_1 because the variety was less resistant than V_2. V_2 also exhibited notable growth as a result of the MLE spray (Figure 4).

3.7. H_2O_2

There was a significant ($p < 0.01$) increase in the H_2O_2 level with both concentrations of Cd when compared to the control plants. After examining this parameter in the plants treated with MLE under Cd stress, a discernible response pattern was seen. When compared to plants growing under stress alone, the exogenous MLE considerably ($p \leq 0.05$) decreased the amount of H_2O_2 by 37% and 32% ($p \leq 0.05$) under Cd stress in V_1. When 1.5 and 2.25 mM

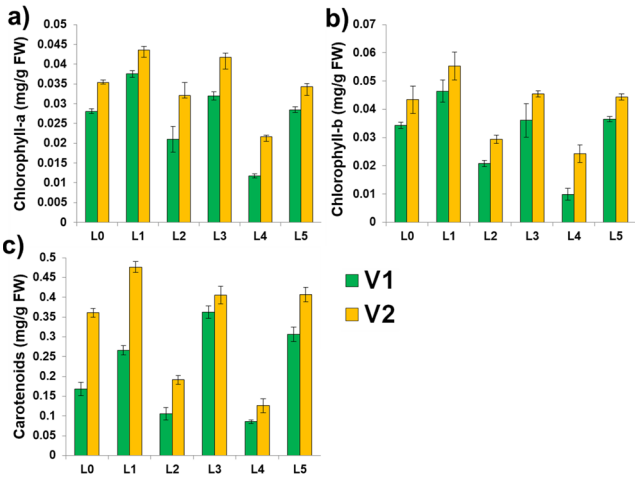

Figure 3. Morphological attributes: (a) chlorophyll-a, (b) chlorophyll-b, (c) carotenoids of the two canola varieties under different levels of Cd stress and the foliar application of MLE.

of Cd were present in V_2, the MLE (1:30) dramatically decreased the H_2O_2 buildup by 38% and 33%, respectively, in comparison to Cd treatment without using the MLE as a PGR (Figure 4).

3.8. Ionic contents of the roots and shoots

With an increasing amount of Cd stress, the root Ca^{+2} contents were considerably increased in both cultivars ($p < 0.01$), while a sharp decline was seen with 1.5 mM of Cd. The Ca^{+2} contents steadily decreased in the V_1 shoots as the concentration of Cd increased. This did not exhibit a discernible trend as the Cd concentration increased to 1.5 and 2.25 mM in V_2. Significant increases in the shoot Ca^{+2} contents were seen in both V_1 and V_2; however, this increase was accompanied by higher levels of Cd imposition. The Na^+ contents of both cultivars changed as the concentration of Cd was increased. Increasing the concentration of Cd had a significant impact on the Na^+ content of the shoots in both cultivars. As the concentration of Cd increased, V_2 exhibited a slight decrease, with V_1 exhibiting the greatest reduction (Figure 5).

An increase in the concentration of Cd in the soil resulted in a notable increase in the root K^+. The shoot K^+ were shown to increase in V_2 with 1.5 mM of Cd and decrease with the highest concentration of 2.25 mM. Additionally, V_1 displayed a 1.5-mM shoot K^+ deficiency. There were notable variations in the ionic contents of both cultivars when the plants were treated exogenously with the MLE (Figure 5).

In both cultivars, there was a significant ($p < 0.01$) increase in the Cd^{+2} contents in the roots and shoots, and the concentration was higher in roots. The Cd^{+2} contents increased in line with the concentration of Cd, showing the greatest increase with 2.25 mM of Cd, with V_1 displaying a greater increase than V_2. The MLE decreased the ion activity in both cultivars, but it was very important in V_2 since it was less impacted by the Cd stress.

3.9. Antioxidants

For both the cultivars and treatments, the statistical data for POD revealed highly significant ($p < 0.01$) differences. The leaf POD activity significantly changed when they were subjected to 1.5 and 2.25 mM of Cd. When compared to the control plants, the POD activities in V_1 were roughly 60% and 65% with 1.5 and 2.25 mM of Cd, respectively. However, when compared to the control plants, V_2 showed a 115% and 66% increase in POD activity. After applying the MLE foliar spray to both cultivars with Cd concentrations of 1.5 and 2.25 mM, the POD activity increased considerably in comparison to the control plants (Figure 6).

The statistical data related to POD revealed differences for both the cultivars and treatments that were extremely significant ($p < 0.01$). The leaf POD activity significantly changed in response to 1.5 and 2.25 mM of Cd. The POD activities in V_1 were roughly 60% and 65% with 1.5 and 2.25 mM of Cd, respectively, in comparison with the control plants. Conversely, V_2 exhibited a 115% and 66% increase in POD activity when compared to the control plants. The POD activity in both cultivars increased significantly with the MLE foliar spray with both concentrations of Cd (1.5 and 2.25 mM) when compared to the control plants (Figure 6).

4. Discussion

Previous studies have documented the vulnerability of crop plants to various forms of metal stress, such as that

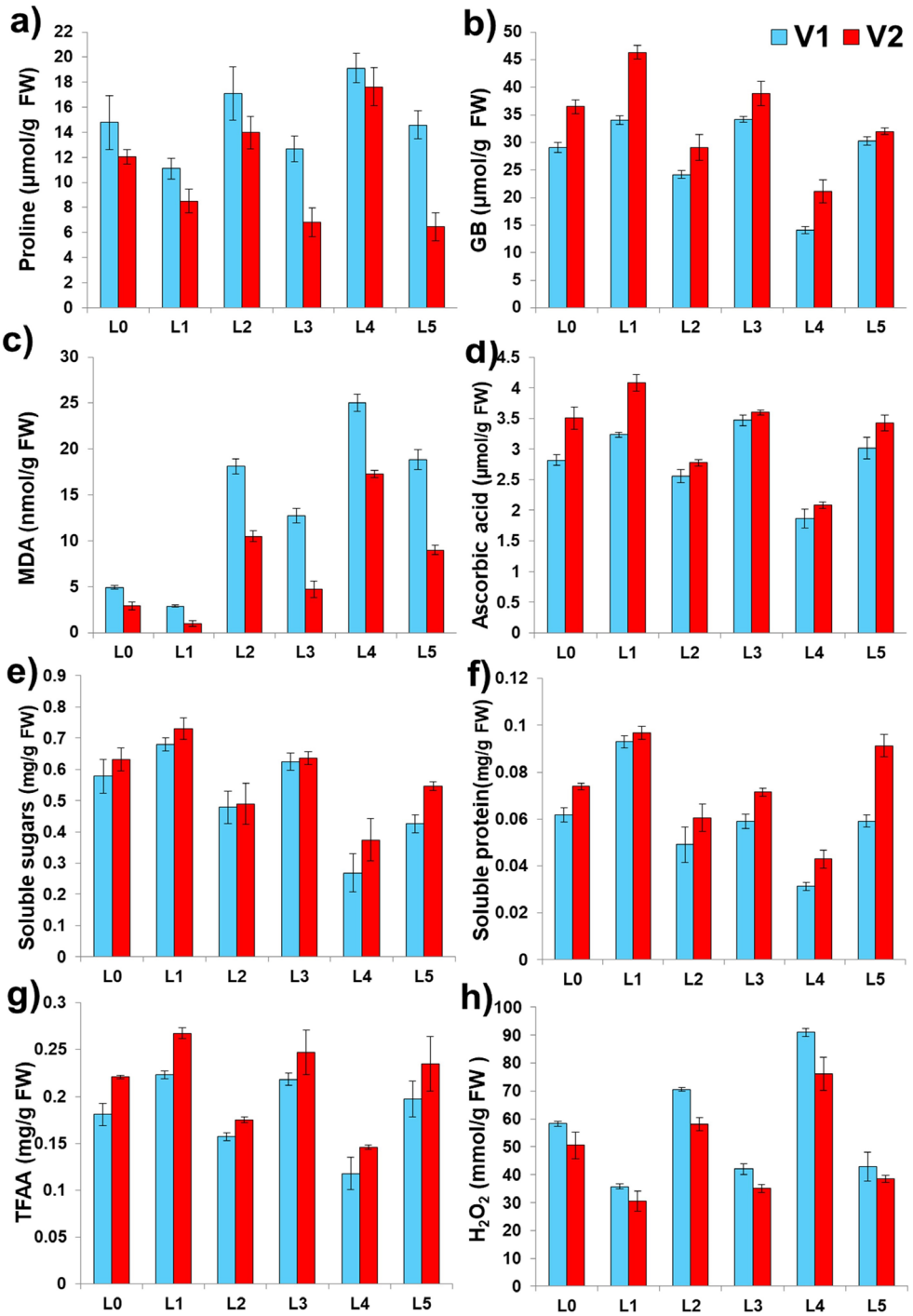

Figure 4. Physiological attributes: (a) proline, (b) glycine betaine, (c) MDA, (d) ascorbic acid, (e) soluble sugars, (f) soluble protein, (g) total free amino acids, and (h) H_2O_2 of the two canola varieties under different levels of Cd stress and the foliar application of MLE.

caused by Cd (El Rasafi et al., 2022). Plant biomass and leaf area output decreased as a result of varying degrees of Cd toxicity (Hussain et al., 2021). Several plant growth metrics, such as the root and shoot dry mass, leaf area, and root and shoot FWs, have been employed as useful indicators to measure metal contamination. The morphological characteristics and yield components of V_2 can be used to rate it as the tolerant variety. By increasing

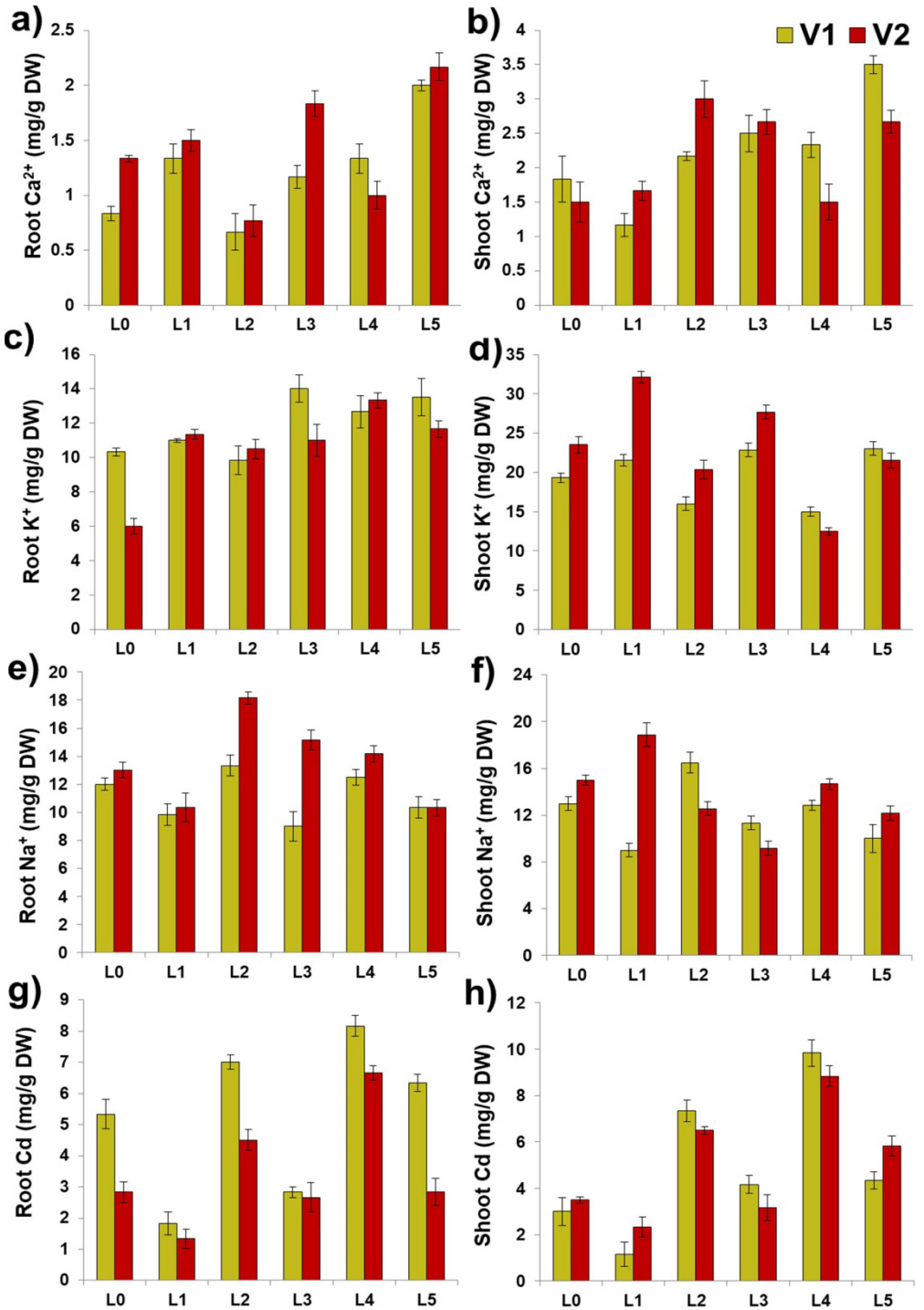

Figure 5. Ionic content: (a) root Ca^{2+}, (b) shoot Ca^{2+}, (c) root K^{+}, (d) shoot K^{+}, (e) root Na^{+}, (f) shoot Na^{+}, (g) root Cd, and (h) shoot Cd of the two canola varieties under different levels of Cd stress and the foliar application of MLE.

the level of Cd in V_2, the shoot FW and dried biomass output revealed more resistance compared to the roots, indicating its superior tolerance under the various Cd stress levels utilized. The lowest concentration of Cd (1.5 mM) showed the least amount of effect on the root and shoot FWs in both cultivars.

The higher concentration of Cd decreased the biomass output and yield in V_1. The obstruction of ion channels and slowed cell development rate resulted in a significant

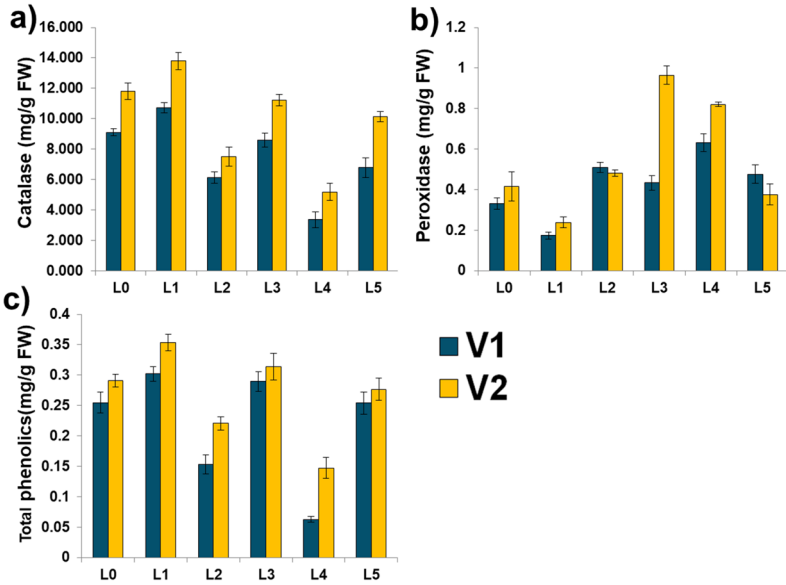

Figure 6. Antioxidants: (a) CAT, (b) POD, and (c) total phenolic contents of the two canola varieties under different levels of Cd stress and the foliar application of MLE.

decrease in several morphological parameters, including the RL, SL, and root and shoot DWs (Bauddh et al., 2016). Previous research has shown that many metals can be hazardous to crops, causing a reduction in the RL and SL as well as an accumulation of phytomass (Souza et al., 2011). In both cultivars, the presence of Cd had a considerable negative impact on the chlorophyll pigment (carotenoids, chlorophyll *a* and *b*) levels, with V_1 being more affected.

Under Cd stress, however, the chlorophyll level in V_2 remained almost constant. Conversely, as the concentration of Cd increased, the level of chlorophyll *b* decreased. As the amount of Cd in the soil increased, the level of carotenoids considerably decreased. However, according to Liu et al. (2020), there has already been a recorded decrease in chlorophyll pigments due to Cd stress. Under heavy metal stress, photosynthetic activity was decreased due to a decrease in chlorophyll production (Islam et al., 2021; Chen et al., 2022). According to Sebastian et al. (2019), a decrease in the chlorophyll content under Cd stress signifies a delay in the production of photosynthetic pigments and a decrease in the effectiveness of photosynthetic machinery.

The proline, MDA, and H_2O_2 levels increased in both cultivars, but a significant decrease in organic osmolytes was noted when the Cd levels increased. The reduction in the amino acid and protein levels under Cd stress indicates a disruption in the cellular protein balance. Under stressful conditions, a general phenomenon reported in many plant species is a decrease in the amounts of soluble sugars, ascorbic acid, GB, total soluble protein, and total free amino acids in different regions of the plant (Hayat et al., 2012; Asare et al., 2023). Proline and H_2O_2 were shown to accumulate in higher amounts in V1, whereas V2 accumulated less of both. Specifically, metal pollution and environmental stressors can lead to a significant reduction in plant yield components and the buildup of organic osmolytes in agricultural plants under Cd stress because this as a defense mechanism in many plant species (Chen et al., 2023b).

Since the disruption of several morphological, physiological, and yield features produced by Cd stress affected the cellular mechanism and resulted in irregular plant growth, MLE was given exogenously to eliminate or minimize the effects of metal stress on the canola. Since it has particular qualities like zeatin, cytokinins, amino acids, and micro and macronutrients, it is an affordable method for increasing crop productivity. Using MLE foliar spray, it was possible to see a considerable increase in the RL and SL, root and shoot FWs, leaf area, dry biomass of the roots, and dry mass of the shoots, as well as in the yield characteristics such as the number of seeds per siliqua, siliquae per plant, and the total weight of the seeds. In previous findings, MLE enhanced the yield and yield-related parameters under stressed conditions (Biswas et al., 2016). Similarly, using MLE under Cd stress produced noteworthy results in the physiological characteristics. The different concentrations of Cd were shown to decrease many organic osmolytes including the total soluble proteins, amino acids, GB, ascorbic acid, soluble sugars, and carotenoids chlorophyll *a* and *b*. MLE significantly increased these characteristics in both V_1 and

V_2; however, this increase was more noticeable with the lower concentration of Cd (1.5 mM), since plant enzyme activity to withstand stressful conditions was disturbed with the higher concentration (2.25 mM).

Increasing the concentration of Cd led to severe ion absorption and accretion, including K^+ and Na^+. The V_2 root K^+ increased with 2.25 mM of Cd, and V_1 showed a similar pattern. With 1.5 mM of Cd, the shoot K^+ contents in V_1 and V_2 decreased. As the amount of Cd increased, the ionic content of the roots decreased and that of shoots increased. Zou et al. (2023) also reported similar results. Nonetheless, the Na^+ content increased with a moderate level of Cd in the majority of the instances, with caustic being the highest level. Conversely, the response of the root and shoot Ca^{+2} to the increasing Cd levels was erratic; regardless of the Cd concentration, an increase was noted in certain parameters for both cultivars and a decrease in others.

MLE contributed significantly to reducing the MDA and H_2O_2 contents due to its enzymatic properties and different necessary and nonessential nutrients (Haider et al., 2021; Hasanuzzaman et al., 2023). Using the MLE also decreased the proline levels. The control plants, which had a Cd concentration of 0 mM, showed the greatest decrease in the proline content. These findings show that both under normal and Cd-stressed conditions, the MLE spray decreased the overproduction of H_2O_2, proline, and MDA levels. Therefore, a decrease in the H_2O_2 contents led to a reduction in oxidative stress, which, in turn, allowed the plants to withstand the stress brought on by Cd.

The antioxidant activity in both cultivars increased with 1.5 mM of Cd but decreased with 2.25 mM. The activity of some antioxidants, such as POD and CAT, fluctuated. It has been documented that when Cd stress levels are lower, antioxidant activities increase because the plants' defense mechanisms become more active (Chen et al., 2023a; Mahamood et al., 2023). Since oxidative stress and reactive oxygen species (ROS) were produced with higher Cd levels, the antioxidant activities were eventually slowed down. The current research revealed that in both canola cultivars, under Cd stress, the POD activity significantly increased, whereas the CAT activity decreased. The homeostatic imbalance of ROS was the cause of the reduced CAT enzyme activity. Prior research on other metal stresses, such as Cd stress, revealed the metal-stressed enzyme activities and the production of ROS (Ramzan et al., 2022; Zulfiqar et al., 2022). When MLE was administered exogenously to both canola cultivars, the activities of several antioxidants increased under stressed conditions. This indicates that the MLE significantly decreased the detrimental effect of Cd on the canola and enhanced crop output, with the V_2 cultivar exhibiting more substantial results.

Finally, the MLE showed greater potential as a biostimulant to mitigate the detrimental effects of soil Cd on *B. napus* plant cultivars. This development is actually mechanical plant support. MLE-mediated alterations in the antioxidant defense system, osmolytes, and photosynthetic pigments showed a significant decrease in the harmful effects of Cd on canola. In addition, the enzymatic properties of MLE and a variety of necessary and nonessential nutrients contributed significantly to reducing the MDA and H_2O_2 levels. Comparative alterations in ionic homeostasis provided more evidence for the advantageous function of MLE in enhancing plant characteristics for increased resilience in a polluted rhizosphere. We advise assessing the commercial applicability of MLE to several crops that include modifications to their transcriptome, phenomics, and genomics.

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Author contribution
HK, AN, MA: Conceived the idea and designed the experiment; MA and OMA: writing, reviewing, editing; IS, RN, MFM: performed the experiments, gathered the literature; MKI, HK,OMA, MH, REN: analyzed the data, and helped in the interpretation of the results; MA, NK, MKI and AN: critically revised the manuscript. All of the authors approved the final version of the manuscript.

Conflict of interest
The authors declare no conflicts of interest.

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