



Plant growth regulators improve growth, photosynthesis, mineral nutrient and antioxidant system under cadmium stress in menthol mint (*Mentha arvensis* L.)

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Abstract Menthol mint (*Mentha arvensis* L.) cultivation is significantly affected by the heavy metals like cadmium (Cd) which also imposes severe health hazards. Two menthol mint cultivars namely Kosi and Kushal were evaluated under Cd stress conditions. Impact of plant growth regulators (PGRs) like salicylic acid (SA), gibberellic acid (GA₃) and triacontanol (Tria) on Cd stress tolerance was assessed. Reduced growth, photosynthetic parameters, mineral nutrient concentration, and increased oxidative stress biomarkers like electrolyte leakage, malondialdehyde, and hydrogen peroxide contents were observed under Cd stress. Differential upregulation of proline content and antioxidant activities under Cd stress was observed in both the cultivars. Interestingly, low electrolyte leakage, lipid peroxidation, hydrogen peroxide and Cd concentration in leaves were observed in Kushal compared to Kosi. Among all the PGRs tested, SA proved to be the best in improving Cd-stress tolerance in both the cultivars but Kushal responded better than Kosi.

Keywords Cadmium toxicity · Cadmium tolerance · *Mentha arvensis* · Phytoremediation · Plant growth regulators

Introduction

Heavy metal (HM) contamination of agricultural lands is becoming a significant threat to the sustainability of agroecosystems (Ashraf et al. 2017; Wang et al. 2017; Wani et al. 2018; Ali et al. 2018; Vardhan et al. 2019; Zaid et al. 2019b). Cadmium (Cd), one of the HMs, is a non-essential element, very toxic to plants (Wang et al. 2018; Nouairi et al. 2019; Rizwan et al. 2019; Zaid et al. 2019b). Nearly 20 million hectares of cultivable land worldwide are known to be contaminated by Cd (Liu et al. 2015; Dong et al. 2017). Once taken up by the plants, Cd retards growth, photosynthetic capacity and induces oxidative stress unexpectedly even at low doses (Lux et al. 2010; Qayyum et al. 2017). Presence of Cd in the plants greatly inhibits the photosynthetic capacity by impairing chlorophyll biosynthesis and thereby inhibiting the efficiency of proteins associated with pigment system II (PSII) and activity of carbon dioxide trapping enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Li et al. 2015; Silva et al. 2017). Cadmium also decreases the concentration of essential mineral nutrients by decreasing their uptake and transport rate, in contrast to promoting the accumulation of osmolytes, oxidation of proteins, lipids, and macromolecules (Ali et al. 2015; Akhtar et al. 2017; Santos et al. 2017). During adaptation, plants have evolved complex strategies to cope with the adverse effects of elevated reactive oxygen species (ROS) generated due to Cd exposure in order to maintain internal redox homeostasis. The plant response to Cd stress is mainly mediated through immobilization of Cd through the cell wall and synthesis of chelators such as phytochelatin, metallothionein and organic acids (Hasan et al. 2015). Cadmium detoxification also involves vacuolar compartmentalization and synthesis of heat shock proteins (HSPs) (Hall 2002). The adaptive

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strategies in the plants consist of the induction of enzymatic and non-enzymatic antioxidants (Ahmad et al. 2015) and enhanced accumulation of osmolytes (Khan et al. 2015). In plants' abiotic stress tolerance mechanisms, several plant growth regulators (PGRs) play a critical role in enhancing tolerance against various stresses and also in phytoremediation of contaminated habitats (Wani et al. 2016; Sharma et al. 2019; Neill et al. 2019; Rostami and Azhdarpoor 2019). However, very few efforts have been made to understand the hormone crosstalk involved in the plant response to Cd stress in menthol mint plants. Exogenous applications of PGRs like salicylic acid (SA) (Liu et al. 2011; Li et al. 2019; Faraz et al. 2019), gibberellic acid (GA₃) (Masood et al. 2016), and triacontanol (Tria) (Maresca et al. 2017) have been found to modulate plants' defense responses to Cd stress in various plant species. Salicylic acid has been reported to ameliorate Ni toxicity in periwinkle (Idrees et al. 2013), mustard (Zaid et al. 2019a), Pb stress in wheat (Alamri et al. 2018), mustard (Hasanuzzaman et al. 2019), As toxicity in rice (Singh et al. 2015), Cd in wheat (Shakirova et al. 2016; Kovács et al. 2014), maize (Gondor et al. 2016), radish (Raza and Shafiq 2013), potato (Li et al. 2019) and mustard (Ahmad et al. 2011; Faraz et al. 2019). Similarly, GA₃ has been reported for mitigating the adverse effects of Cd stress in mustard, Pb stress in English ryegrass (Masood and Khan 2013; He et al. 2015; Masood et al. 2016). Triacontanol is a potent primary alcoholic PGR known to promote growth, photosynthetic activity, mineral nutrient contents (Li et al. 2016) and also acts as an antioxidative agent by inhibiting enzymatic and non-enzymatic mediated peroxidative damage of cellular proteins and lipids (Asadi Karam et al. 2017).

Menthol mint (*Mentha arvensis* L.) an important industrial, medicinal and aromatic plant, have numerous applications in the flavorings, food, cosmetic and pharmaceutical industries (Lal 2013). Menthol, as principal secondary metabolite is extracted commercially from this plant (Rao et al. 2000; Naeem et al. 2014). However, a little is known about the differential phytoremediation capacity of cultivars of menthol mint in relation to Cd stress. Among the numerous strategies used for reducing the concentration of Cd from HM challenged soils, phytoremediation is regarded as an efficient, eco-friendly, cost-effective, economical, and aesthetically acceptable remediation strategy to reduce/minimize the danger of contamination of soil by HMs (Baudhdh and Singh 2012; Guo et al. 2016). The phytoremediation potential of different plant species has been extensively studied. Manikandan and Venkatachalam (2011) worked out the tolerance capacity of menthol mint in relation to Hg stress. However, the phytoremediation capacity of menthol mint cultivars in relation to Cd stress has not been studied in detail.

Moreover, the effect of Cd stress on growth, photosynthetic, mineral nutrient, antioxidants and stress responses of menthol mint is largely unknown. Therefore, the present study was planned to study the effect of Cd in two different cultivars, the applicability of PGRs to improve tolerance and differential phytoremediation capability of menthol mint cultivars.

Materials and methods

Experimental setup and plant growth regulators preparation

Menthol mint plants were grown in the earthen pots in the naturally illuminated environmental conditions of the net house at the Department of Botany, Aligarh Muslim University, Aligarh, India (27°52'N, 78°51'E, and 187.45 m altitude, average night/day temperatures, 15 ± 2 °C, 25 ± 3 °C respectively; relative humidity, 60 ± 5%; photosynthetically active radiation (PAR), 755 ± 20 μmol m⁻² s⁻¹; critical photoperiod, 10–12 h). Suckers of *Mentha arvensis* Linn. cv. Kosi and Kushal were procured from the Central Institute of Medicinal and Aromatic Plants, Lucknow, Uttar Pradesh (India). Prior to transplanting, suckers were surface sterilized with 0.01% of the HgCl₂ solution and then washed vigorously with double distilled water (DDW) followed by thorough washing with deionized water. Each earthen pot used for the experiment contained 5 kg mixture of upper soil layer (0–30 cm) which was collected from an uncontaminated paddy field from nearby areas and mixed with farmyard manure (4:1). Physicochemical characteristics of the soil were: sandy loam in texture, pH (1:2) 8.0, EC (1:2) 0.59 mmhos cm⁻¹, and 93.1, 9.4, and 155.5 mg available critical mineral nutrients namely N, P, and K per kg of soil, respectively. A uniform dose of N, P, and K (each 25 mg/kg soil) was applied. Half of the N and full of P and K were applied at the time of transplanting and the remaining N was top-dressed in two splits, the first at 35 days after transplantation (DAT) and the second at 70 DAT. Urea, single superphosphate, and muriate of potash were used as the source of N, P and K, respectively. After a fortnight of transplantation, manual thinning was carried out and 3 healthy plants per pot of uniform size were maintained. At 30 DAT, 50 μM Cd was given in the form of dilute aqueous solution of cadmium chloride (CdCl₂) through roots. Three growth regulators namely SA, GA₃, and Tria were sprayed at 60 DAT thrice at an interval of 15 days by using Tween-20 as a surfactant. The concentration of each growth regulator was based on earlier findings of Khan et al. (2010) and Khanam and Mohammad (2018). Stock solutions of each plant growth regulator were prepared by

dissolving them in DDW and ethanol. From the stock solution, the desired quantity of each plant growth regulator (10^{-6} M) was made by using DDW. Each plant was sprayed with of 3 mL of PGR solution by a sprayer. In one sprinkle, the nozzle of the sprayer was adjusted in such a manner that it ejected 1 mL (approx.) of the required solution and each plant was sprinkled thrice. Each experimental pot was irrigated on alternate days with 100–150 mL of DDW. The experimental pots were arranged in complete randomized block design and were conducted in five replications. The pattern of treatments was as follows: (T1: control; T2: 10^{-6} M GA₃; T3: 10^{-6} M Tria; T4: 10^{-6} M SA; T5: 50 μ M CdCl₂; T6: 50 μ M CdCl₂ + 10^{-6} M GA₃; T7: 50 μ M CdCl₂ + 10^{-6} M Tria; T8: 50 μ M CdCl₂ + 10^{-6} M SA).

The plants were sampled at 100 DAT to assess various physio-biochemical attributes.

Measurement of growth and photosynthetic parameters

Growth characteristics in terms of fresh and dry mass of shoot and leaf area were measured. Plants were uprooted from pots, washed thoroughly and dried by using blotting paper and their fresh masses were noted. After recording fresh mass, plant samples were kept at 72 °C in a hot air oven until completely dried and then weighed to obtain DM. The leaf area was measured by using a leaf area meter (ADC Bioscientific, UK). The SPAD values of Chl in the leaves were estimated under natural environmental conditions by using the SPAD Chl meter (SPAD-502; Konica, Minolta Sensing, Inc., Japan). Gas exchange traits [net photosynthesis (P_N), stomatal conductance (g_s) and intercellular CO₂ concentration (C_i)] were measured in uppermost fully expanded leaves of all plants using infrared gas analyzer (LI-COR 6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA). The measurements were done on a cloudless sunny day at ambient air temperature 25 °C, light saturating intensity; PAR 760 μ mol m⁻² s⁻¹, relative humidity 85% and atmospheric CO₂ concentrations 370 ± 5 μ mol mol⁻¹. The activity of Rubisco (EC 4.1.1.39) was determined by the standard method of Usuda (1985) and expressed as [μ mol (CO₂) m⁻² s⁻¹] Yusuf et al. (2017).

Determination of tolerance index (TI)

Tolerance index was calculated as the ratio of the mean value of treated plant dry mass to mean dry mass of control plants and expressed in percentage points.

Analysis of leaf primary mineral nutrient concentrations

Quantification of N, P and, K concentrations

Fresh leaf samples were oven dried at 72 °C for a period of 48 h. The dried material was made into fine powder and passed through a sieve of 0.42 mm in size. Samples (100 mg) were digested in H₂SO₄ at 110 °C for 2 h in temperature controlled Kjeldahl assembly. The contents were cooled at room temperature for 20 min and a few drops of 30% H₂O₂ were added. Heating and cooling were again repeated. Acid-peroxide digested leaf material (10 mL) was used for determining leaf N, P and K concentrations.

The method of Lindner (1944) was employed for N determination. The procedure of Fiske and Subbarow (1925) was adopted for P determination. The intensity of color was read at 620 nm.

The acid-peroxide digested aliquot was taken and the concentrations of K were determined by using flame photometer (Model, ELICO, CL22D, India). A blank sample was also run along and all the concentration of K was expressed in mg g⁻¹ (DM).

Determination of oxidative stress biomarkers

Thiobarbituric acid reactive substances were determined by measuring the malondialdehyde equivalents according to the protocol of Hodges et al. (1999). The absorbance of the samples was read at 440, 532 and 600 nm and the values were expressed in (nmol TBARS g⁻¹ (FM)).

Sullivan and Ross (1979) method was used for calculating the electrolyte leakage (EL). The electrolyte leakage was calculated by using the given formula:

$$\text{Electrolyte leakage(\%)} = [(EC_b - EC_a)/(EC_c) \times 100]$$

The method of Jana and Choudhari (1981) was used for the quantification of H₂O₂. The absorbance of the reaction mixture was read at 410 nm, on a spectrophotometer (ELICO SL 171 MINI SPEC, India).

Assay of antioxidants and proline

Preparation of enzyme extract

The leaf tissue was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone. The homogenate was centrifuged at 27,600 \times g for 10 min at 4 °C and the supernatant obtained was used as an extract for superoxide dismutase, catalase and, peroxidase.

Determination of superoxide dismutase (SOD, EC 1.15.1.1) activity

The SOD activity was determined by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). The absorbance was read at 650 nm and one unit of SOD activity was defined as the amount of enzyme causing a 50% decrease of NBT.

Estimation of activities of catalase (CAT, EC 1.11.1.6), peroxidase (POX, EC 1.11.1.7) and glutathione reductase (GR, EC 1.6.4.2.)

The activity of CAT was measured by following the procedures of Chance and Maehly (1956). For CAT estimation, 3 mL of 0.1 M phosphate buffer (pH 6.8), 1 mL of H₂O₂ (0.1 M), 10–20 µL of enzyme extract and 1% of guaiacol were mixed. The reaction mixture was left undisturbed for one min at room temperature. The mixture was then titrated against 0.1 N potassium permanganate to find residual H₂O₂ until a joint purple color appeared for at least 15 min. Similarly, a control set was also maintained in which enzyme activity was stopped by adding H₂SO₄ to enzyme extract before its addition to the test tube.

Peroxidase activity was calculated according to the method described by Chance and Maehly (1956). Units of CAT and POX were expressed as [mM (H₂O₂) decomposed mg⁻¹ (FM) and enzyme units (EU) g⁻¹ (FM)], respectively.

Glutathione reductase activity was estimated by the method of Smith et al. (1988).

Determination of carotenoids

The contents of carotenoids were estimated by using the standard method of Maclachlan and Zalik (1963). The contents were calculated using the formula:

$$\text{carotenoid content (mg g}^{-1}\text{ FW)} = 7.6(\text{OD } 480) - 1.49(\text{OD } 510) \frac{V}{d \times W \times 1000}$$

where OD = optical density of the extract at the given wavelengths (480 and 510 nm), V = final volume of extract in 80% acetone, W = fresh mass of leaf tissue (g), and d = length of the light path (1 cm).

Estimation of proline content

The method described by Bates et al. (1973) was used for the determination of proline content.

Atomic absorption spectrophotometer (AAS) analysis of root and leaf Cd

The concentrations of Cd in the root and leaf were then determined by atomic absorption spectrophotometer (GBC, 932 plus; GBC Scientific Instruments, Braeside, Australia) according to the method of Zaid and Mohammad (2018).

Determination of nitrate reductase (NR) and carbonic anhydrase (CA) activity

The activity of the NR enzyme was determined by the method of Jaworski (1971). The method of Dwivedi and Randhawa (1974) was used for measuring the activity of CA. The absorption of the reaction mixtures was read at 540 nm by spectrophotometer (ELICO SL 171 MINI SPEC, India).

Statistical analysis

The statistical analysis was performed by using by SPSS 17.0 software tool for windows 10. The percentage data and the significant differences between the means of two cultivars were determined by Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ and presented as mean \pm standard error ($n = 5$). Each value of mean and standard error in the table and figures respectively represent five replicates.

Results

Growth characteristics

Growth traits such as the fresh and dry mass of plants and leaf area were decreased by Cd stress in both cultivars. The fresh and dry mass of shoot also followed a similar trend (Figs. 1A–D, 2B). The Cd stress decreased FM of plants by 15.34% in Kosi and 18.89% in Kushal with respect to control. The plant DM decreased by 14.30% in Kosi and 6.69% in Kushal respectively, with respect to control. The FM of the shoot in Kosi was decreased by 15.07%, and 14.49% in Kushal. Cadmium also decreased DM of shoot by 41.76% in Kosi whereas, in Kushal, a 34.27% decrease was noted. Leaf area in Kosi cultivar decreased by 8.02% and 7.79% in Kushal by Cd stress as compared to respective controls. Treatment of plants with PGRs improved all the growth traits in unstressed plants. Moreover, the follow-up treatments after Cd stress with SA, GA₃, and Tria successfully countered the damage caused by Cd stress. The response of the plants treated with SA in Kushal was more effective than Kosi. Of the two cultivars, Kushal was found more responsive to SA under Cd stress as it possessed higher plant FM, DM as well as high shoot FM, DM and leaf area than Kosi cultivar.

Fig. 1 Effect of plant growth regulators (PGRs); gibberellic acid (GA₃), triacontanol (Tria) or salicylic acid (SA) grown with or without cadmium (Cd) on (A) fresh mass of plant, (B) dry mass of plant, (C) shoot fresh mass and, (D) shoot dry mass in two different cultivars of menthol mint. Data are presented as treatments mean ± SE (n = 5). The bar shows standard error and different capital and small letters represent significant differences in Kushal and Kosi cultivars among various treatments by DMRT test at $P \leq 0.05$

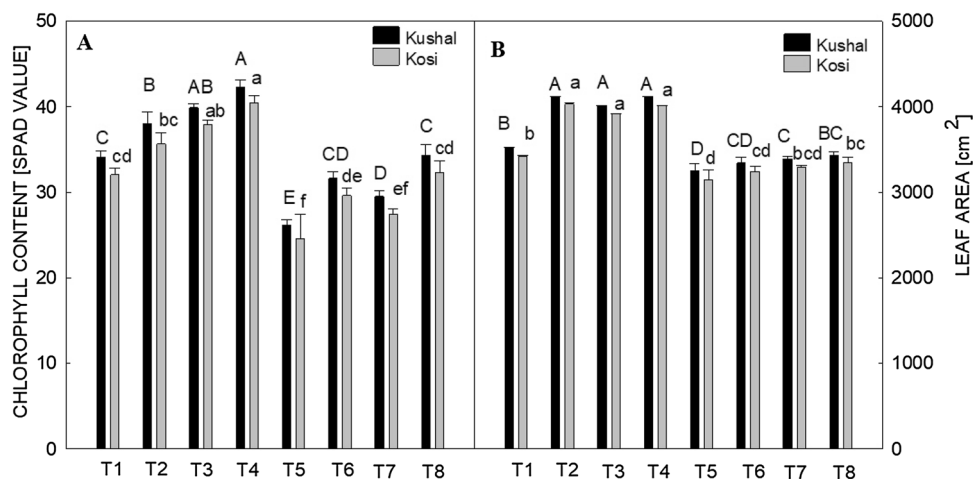
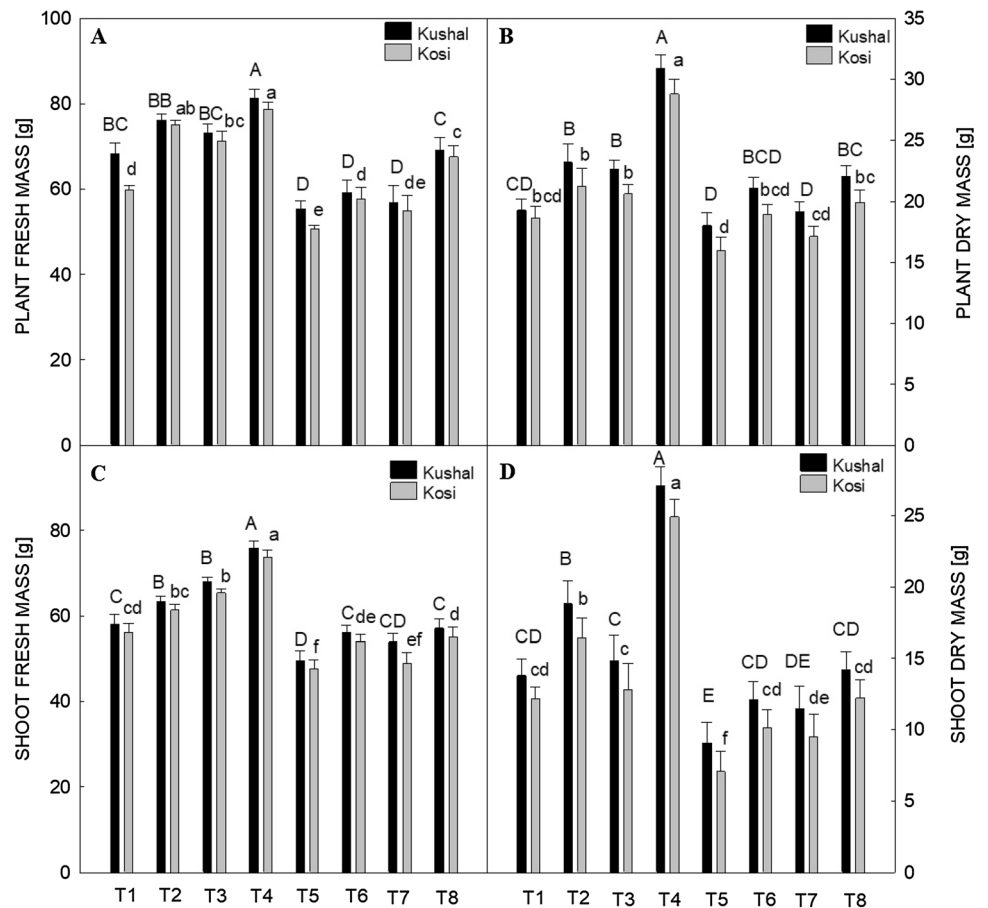


Fig. 2 Effect of plant growth regulators (PGRs); gibberellic acid (GA₃), triacontanol (Tria) or salicylic acid (SA) grown with or without cadmium (Cd) on (A) SPAD value and, (B) leaf area of two different menthol mint cultivars. Data are presented as treatments

mean ± SE (n = 5). The bar shows standard error and different capital and small letters represent significant differences in Kushal and Kosi cultivars among various treatments by DMRT test at $P \leq 0.05$

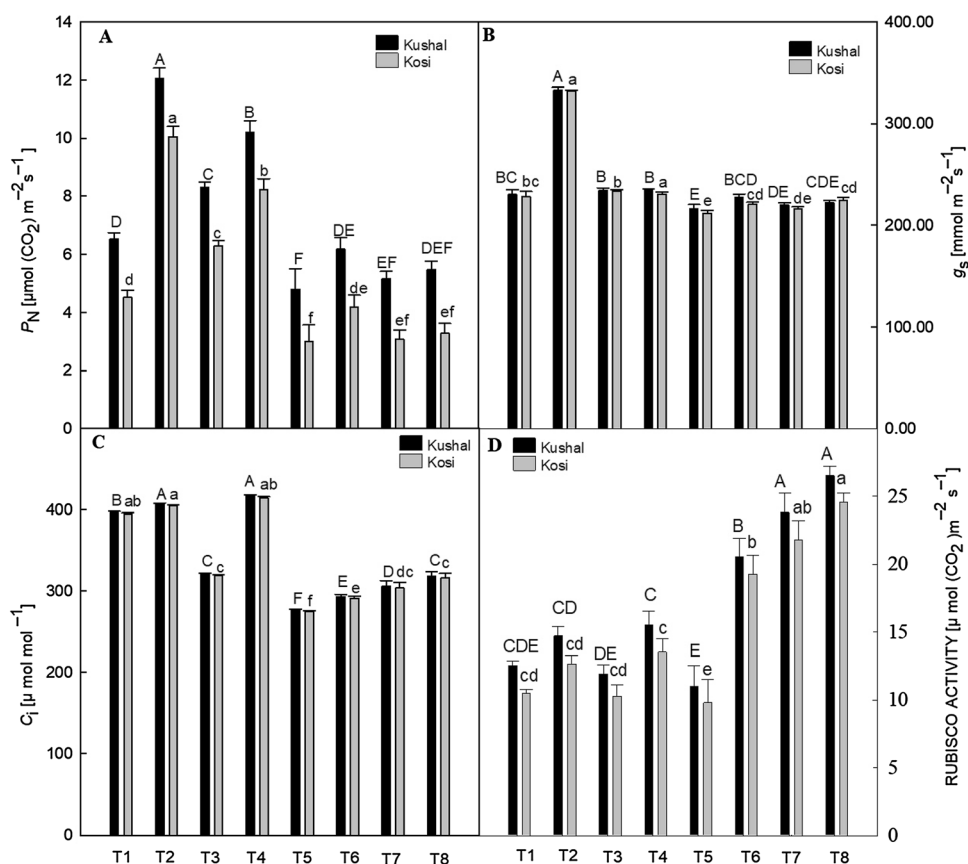
Tolerance index

The cultivar Kushal showed a greater tolerance index than cultivar Kosi (Fig. 6C).

Chlorophyll content and leaf gas exchange parameters

SPAD value, Rubisco activity and all photosynthetic attributes— P_N , g_s , C_i decreased in the plants by the Cd stress, as compared to respective control plants (Figs. 2A,

Fig. 3 Effect of plant growth regulators (PGRs); gibberellic acid (GA_3), triacontanol (Tria) or salicylic acid (SA) on two different cultivars of menthol mint grown with or without cadmium (Cd) on (A) net photosynthetic rate (P_N), (B) stomatal conductance (g_s), (C) internal carbon dioxide concentration (C_i) and, (D) Rubisco activity. Data are presented as treatments mean \pm SE ($n = 5$). The bar shows standard error and different capital and small letters represent significant differences in Kushal and Kosi cultivars among various treatments by DMRT test at $P \leq 0.05$



3A–D). The Chl content diminished significantly ($P \leq 0.05$) by 23.36% in Kosi and 23.31% in Kushal, compared to control plants (Fig. 2A). Cadmium treatment triggered a significant decrease in P_N by 50.66% in Kosi and 26.38% in Kushal, respectively, compared to control plants (Fig. 3A). SA, GA_3 and Tria application to non-stressed plants increased the SPAD value, Rubisco activity, and photosynthetic attributes. All plant growth regulators improved the photosynthetic attributes in Cd-stressed plants and recovered the damage but SA was very effective in improving Chl, Rubisco activity, g_s and C_i in stressed plants. Kushal performed better than Kosi cultivar under Cd stress.

Leaf mineral nutrient contents

All the three principal mineral nutrients N, P and, K in the leaves were decreased by Cd treatments in comparison to control plants in both the cultivars (Fig. 4A–C). Nitrogen concentrations decreased by 50% in Kosi and 57.57% in Kushal, P by 39.53% in Kosi and 14.58% in Kushal and K by 49.76% and 44.97% in Kosi and Kushal, respectively as compared to the respective control plants. GA_3 , Tria, and SA increased all the mineral nutrient concentrations in unstressed plants. However, of all the PGRs, GA_3 triggered

the maximum increase in leaf N in both cultivars but Kushal was more responsive as it retained more N, P, and K concentrations under Cd persistence.

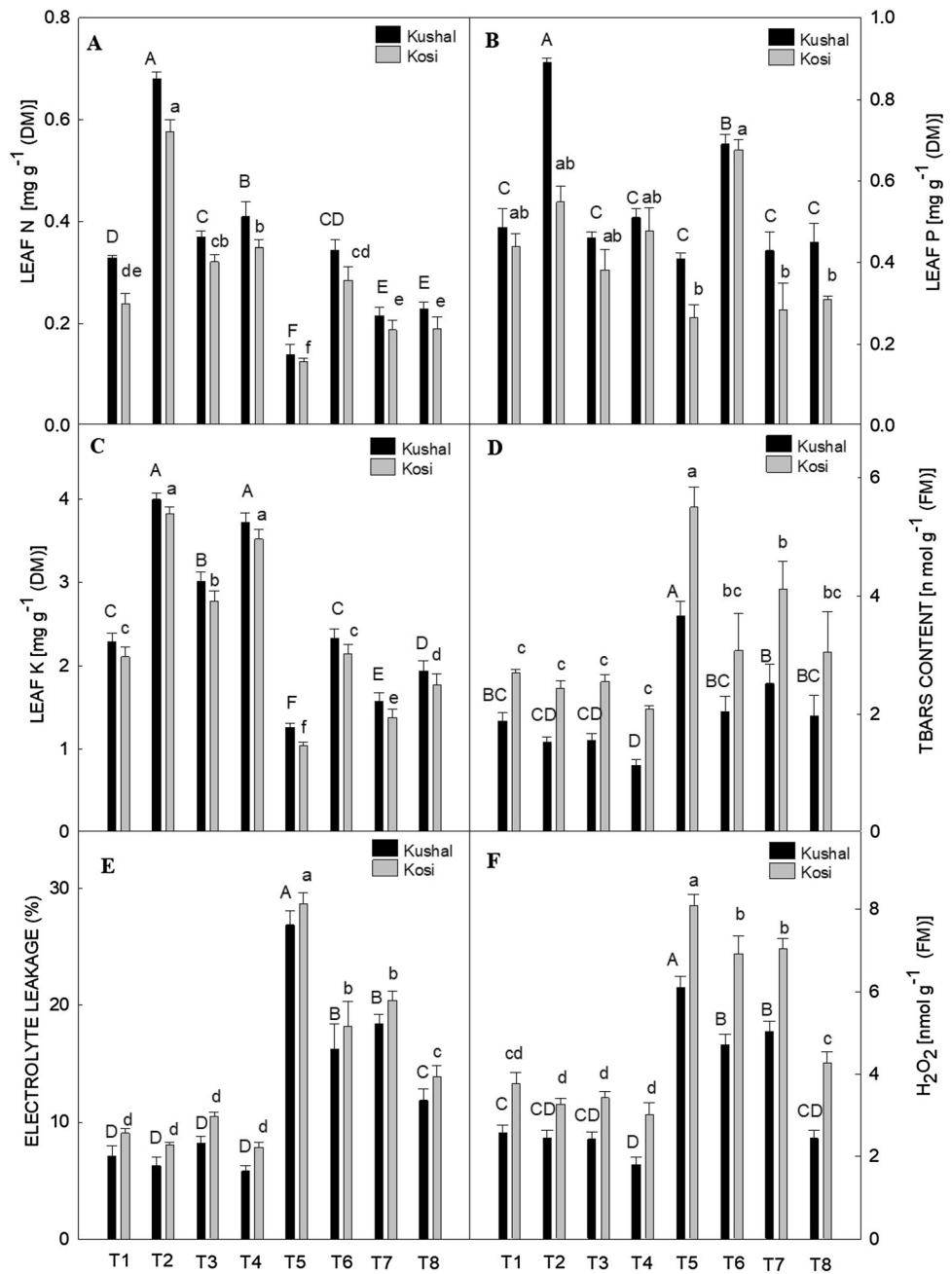
Thiobarbituric acid reactive substances, EL, and H_2O_2 content

Thiobarbituric acid reactive substances, EL, and H_2O_2 content increased significantly under Cd treatments in both the cultivars in comparison to control plants. Cadmium increased TBARS content by 50.81% and 48.76%, H_2O_2 content by 53.45% and 69.83% and EL by 68.38% and 73.72% in Kosi and Kushal, respectively, over the respective control plants (Fig. 4D–F). Moreover, PGRs treatment decreased the aforesaid parameters in Cd-stressed plants and in addition to this, SA proved very effective and lowered down the values of TBARS, EL and H_2O_2 in Cd-stressed plants when applied as follow-up treatment. Kosi was more sensitive to Cd stress than Kushal cultivar.

Impact on the activities of antioxidant enzymes and proline content

Cadmium stress increased the activities of all antioxidant enzymes studied in both the cultivars, as compared to

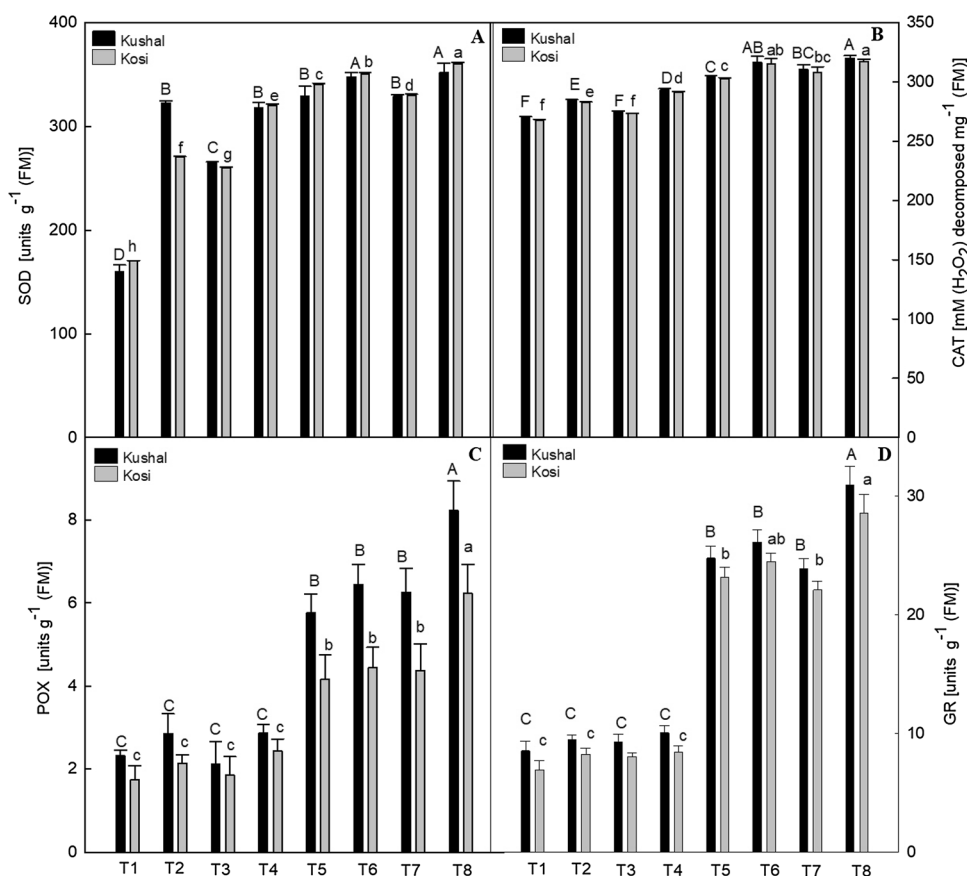
Fig. 4 Effect of plant growth regulators (PGRs); gibberellic acid (GA_3), triacontanol (Tria) or salicylic acid (SA) on two different cultivars of menthol mint grown with or without cadmium (Cd) on (A) Nitrogen (N), (B) Phosphorous (P), (C) Potassium (K), (D) thiobarbituric acid reactive substances (TBARS) content, (E) electrolyte leakage and, (F) hydrogen peroxide (H_2O_2) content. The bar shows standard error and different capital and small letters represent significant differences in Kushal and Kosi cultivars among various treatments by DMRT test at $P \leq 0.05$



control plants (Fig. 5A–D). Superoxide dismutase activity increased by 50.04% in Kosi and 51.28% in Kushal, CAT by 11.46% in Kosi and 11.19% in Kushal, POX by 58.31% in Kosi and 59.82% in Kushal and GR activity significantly by 70.13% and 65.57%, respectively, in Kosi and Kushal, over the respective control plants. Treatment of PGRs to Cd stressed plants further increased the activities of SOD, CAT, POX, and GR in both the cultivars. Maximum activities of these enzymes were noted in the Cd stressed plants which received SA treatment, such that SOD values increased by 52.77% in Kosi and 54.42% in Kushal.

Catalase values increased by 15.64% in Kosi and 15.68% in Kushal. Salicylic acid treatment to Cd stressed plants increased POX values by 72.18% in Kosi and 71.89% in Kushal. At the same treatments, GR values in Kosi and Kushal were increased by 75.76% and 72.39% respectively over the respective control plants. Carotenoid content increased significantly by Cd stress in both cultivars. In Kushal, Cd stress increased the Car content by 76.92% and 77.47% in Kosi with respect to unstressed plants (Fig. 6A). Moreover, PGRs treatment to Cd stressed plants increased the Car contents in both the cultivars, but out of three

Fig. 5 Effect of plant growth regulators (PGRs); gibberellic acid (GA_3), triacontanol (Tria) or salicylic acid (SA) on two different cultivars of menthol mint grown with or without cadmium (Cd) on activities of (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) peroxidase (POX) and, (D) glutathione reductase (GR). Data are presented as treatments mean \pm SE ($n=5$). The bar shows standard error and different capital and small letters represent significant differences in Kushal and Kosi cultivars among various treatments by DMRT test at $P \leq 0.05$



PGRs, SA triggered a maximum increase of 91.83% in Kosi and 91.45% in Kushal, over control plants.

Proline content in Cd-stressed plants in both the cultivars increased significantly by 38.63% in Kosi and 42.60% in Kushal, over the respective control plants (Fig. 6B). Salicylic acid, GA_3 , and Tria had an additive effect on proline accumulation in both the cultivars, GA_3 increased the proline content by 6.19% in Kosi, whereas, Tria increased the proline content by 3.53% with respect to Cd-treated plants. In Kushal, GA_3 increased the proline content by 1.60%, and 5.83% by Tria application with respect to Cd stressed plants. However, the SA application increased the proline content maximally in both unstressed and stressed plants. In unstressed plants, SA increased proline content by 27.87% in Kosi and 37.50% in Kushal over the control plants. Nevertheless, SA in Cd-stressed plants increased proline content by 65.61% in Kosi and 61.45% in Kushal cultivars, over the control plants. Kushal accumulated high concentrations of proline in control as well in all treatments than the Kosi.

Heavy metal concentrations in root and leaf

Accumulation of Cd significantly increased in root and leaf of the Cd-treated plants in both cultivars, as compared to

unstressed control (Table 1). The root Cd increased by 31.21 fold in Kosi with respect to control plants. All PGRs decreased the Cd concentrations in the root of both the cultivars, but SA decreased the Cd concentrations by 67.73% in Kosi and 58.63% in Kushal with respect to Cd stressed plants. In contrast, Cd concentrations in leaves of the Cd stressed plants increased by 20.71 fold in Kosi and 17.33 fold in Kushal with respect to non-stressed control plants (Table 1). Among the three PGR tested, SA decreased Cd concentrations in the leaves significantly by 72.41% in Kosi and 71.63% in Kushal as compared to Cd stressed plants. Kushal accumulated more Cd in roots but less in leaves and was found more responsive to SA treatment.

Activities of nitrate reductase and carbonic anhydrase

Cadmium stressed plants had decreased activities of NR and CA in the leaves, as compared to control plants. Cadmium stress diminished NR activity by 38.46% in Kosi and 26.66% in Kushal respectively with respect to control plants (Table 2). Cadmium stress also decreased CA activity by 10.68% in Kosi and 12.34% in Kushal, compared to control plants. Treatment of plants with SA, GA_3 ,

Fig. 6 Effect of plant growth regulators (PGRs); gibberellic acid (GA₃), triacontanol (Tria) or salicylic acid (SA) on two different cultivars of menthol mint grown with or without cadmium (Cd) on (A) carotenoids content, (B) proline content and, (C) tolerance index (TI). Data are presented as treatments mean ± SE (n = 5). The bar shows standard error and different capital and small letters represent significant differences in Kushal and Kosi cultivars among various treatments by DMRT test at P ≤ 0.05

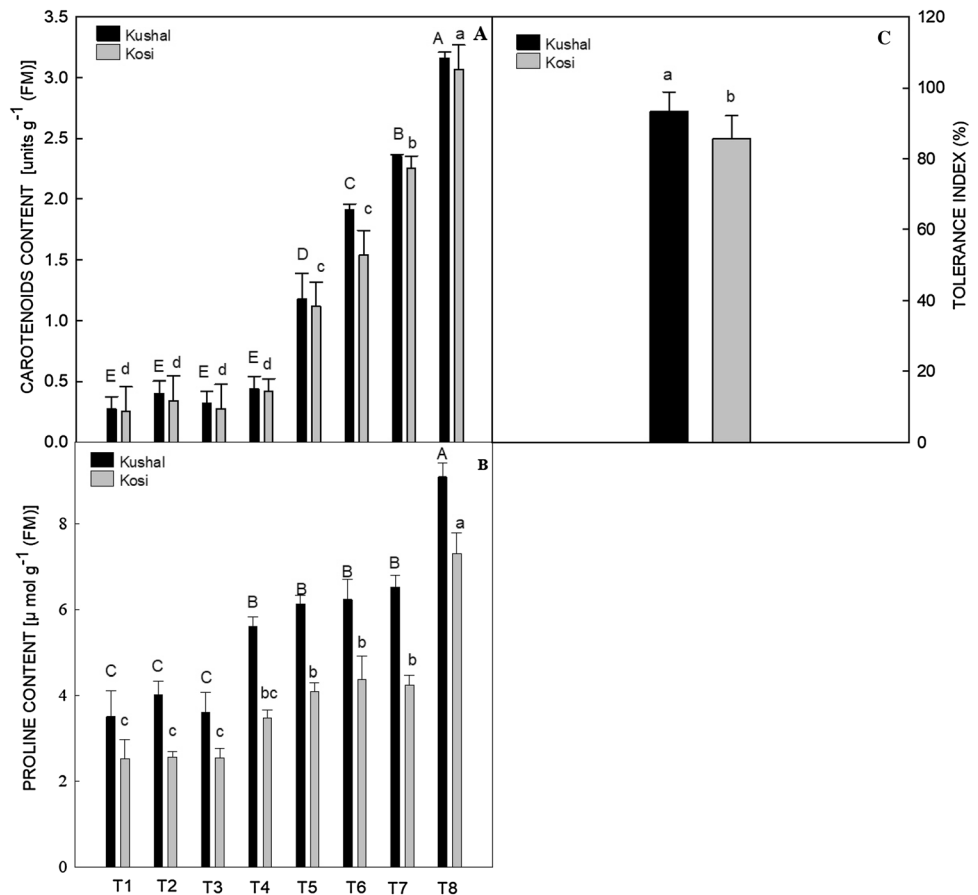


Table 1 Effect of plant growth regulators (PGRs); gibberellic acid (GA₃), triacontanol (Tria) or salicylic acid (SA) on cadmium (Cd) concentrations of root and leaf of two *Mentha arvensis* L. cultivars at 100 DAT

Treatment	Root Cd [$\mu\text{g g}^{-1}$ (DM)]		Leaf Cd [$\mu\text{g g}^{-1}$ (DM)]	
	Kosi	Kushal	Kosi	Kushal
Control	0.42 ± 0.03 ^c	0.44 ± 0.04 ^D	0.14 ± 0.03 ^d	0.12 ± 0.02 ^C
GA ₃ [10^{-6}]	0.32 ± 0.09 ^c	0.36 ± 0.08 ^D	0.08 ± 0.01 ^d	0.06 ± 0.01 ^C
TRIA [10^{-6}]	0.38 ± 0.01 ^c	0.40 ± 0.05 ^D	0.12 ± 0.02 ^d	0.10 ± 0.02 ^C
SA [10^{-6}]	0.27 ± 0.02 ^c	0.31 ± 0.01 ^D	0.06 ± 0.01 ^d	0.05 ± 0.01 ^C
Cd [50 μM]	13.11 ± 0.75 ^a	15.11 ± 0.74 ^A	2.90 ± 0.42 ^a	2.08 ± 0.26 ^A
Cd + GA ₃	10.25 ± 0.86 ^b	12.25 ± 0.86 ^B	1.60 ± 0.48 ^b	1.41 ± 0.47 ^{BC}
Cd + TRIA	11.42 ± 0.39 ^b	13.06 ± 0.78 ^B	1.00 ± 0.36 ^{bc}	0.77 ± 0.34 ^{BC}
Cd + SA	4.23 ± 0.81 ^b	6.25 ± 0.82 ^C	0.80 ± 0.05 ^{cd}	0.59 ± 0.14 ^C

Data are presented as treatment mean ± SE (n = 5). Data followed by the same letter are not significantly different by Duncan’s Multiple Range Test (DMRT) at P ≤ 0.05. Capital letters indicate a significant difference between Kushal and small letters of Kosi cultivars respectively

and Tria improved the activities of NR and CA in unstressed and stressed plants with respect to control plants. However, SA treatment completely recovered the damage caused by Cd stress and increased the activities of NR by 44.44% in Kushal and 35% in Kosi and CA by 26.91% in Kosi and 24.19% in Kushal respectively, over control plants (Table 2). Kushal cultivar under Cd stress was more responsive to SA treatment than Kosi.

Discussion

The present study was conducted to elucidate the responses in menthol mint cultivars that differ in Cd tolerance and to test the efficacy of PGRs in modulating Cd-induced changes. In the present study, Cd significantly reduced (P ≤ 0.05) all the plant growth attributes in both cultivars of menthol mint. The suppression of growth in both cultivars under Cd toxicity could be due to restricted uptake of

Table 2 Effect of plant growth regulators (PGRs); gibberellic acid (GA₃), triacontanol (Tria) or salicylic acid (SA) on leaf nitrate reductase (NR) and carbonic anhydrase (CA) activities of two *Mentha arvensis* L. cultivars at 100 DAT

Treatment	NR activity [μ mole (NO ₂) g ⁻¹ (FM) s ⁻¹]		CA activity [mole (CO ₂) kg ⁻¹ (FM) s ⁻¹]	
	Kosi	Kushal	Kosi	Kushal
Control	0.13 ± 0.03 ^{bc}	0.15 ± 0.04 ^C	4.40 ± 0.21 ^c	4.70 ± 0.35 ^{CD}
GA ₃ [10 ⁻⁶]	0.15 ± 0.09 ^{bc}	0.16 ± 0.05 ^{BC}	6.40 ± 0.53 ^b	6.92 ± 0.69 ^B
TRIA [10 ⁻⁶]	0.12 ± 0.02 ^{bc}	0.14 ± 0.03 ^C	5.99 ± 0.61 ^b	6.60 ± 0.85 ^B
SA [10 ⁻⁶]	0.26 ± 0.01 ^a	0.30 ± 0.02 ^A	8.82 ± 0.43 ^a	9.02 ± 0.50 ^A
Cd [50 μ M]	0.08 ± 0.75 ^c	0.11 ± 0.01 ^C	3.93 ± 0.77 ^c	4.12 ± 0.74 ^D
Cd + GA ₃	0.12 ± 0.86 ^{bc}	0.17 ± 0.03 ^{BC}	4.19 ± 0.29 ^c	4.34 ± 0.49 ^{CD}
Cd + TRIA	0.14 ± 0.39 ^{bc}	0.14 ± 0.02 ^C	4.03 ± 0.39 ^c	4.13 ± 0.38 ^D
Cd + SA	0.20 ± 0.81 ^{ab}	0.27 ± 0.04 ^{AB}	6.02 ± 0.61 ^b	6.20 ± 0.73 ^{BC}

Data are presented as treatment mean ± SE (n = 5). Data followed by the same letter are not significantly different by Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$. Capital letters indicate a significant difference between Kushal and small letters of Kosi cultivars respectively

water and essential mineral nutrients by roots (Dinakar et al. 2009), altered rate of cell division, cell growth and cellular structures (Ashraf et al. 2017), denaturation of protein by disruption of H–S (hydrogen-sulfur) bond. It has been revealed that Cd stress cause altered growth and development of plants (Akhtar et al. 2017), inhibition of photosynthesis (Masood et al. 2012), changes in components of the cell wall and in the metabolism of carbohydrates (Abdel Latef 2013). The cumulative ill effect of Cd stress on overall metabolism decreased growth traits in both the cultivars but the maximum reduction was noted in Kosi. The Kushal cultivar had more leaf area than Kosi which could have helped to efficiently utilize solar radiation, resulting in higher photosynthetic rate and other plant growth related attributes. The inhibitory effects of Cd stress on growth might be due to the interference of vital physio-biochemical processes including photosynthesis and translocation of mineral nutrient elements inside plant tissues (Hou et al. 2007). Cadmium-induced growth reduction in the present study was further corroborated by studies in Indian mustard (Gill et al. 2011), maize (Akhtar et al. 2017), *Miscanthus* spp. (Guo et al. 2016), spinach (Younis et al. 2016), chickpea (Ahmad et al. 2016). Similar Cd-induced decrease in growth traits has been reported in peppermint plants by Ahmad et al. (2018) and in menthol mint plants (Zaid and Mohammad 2018). In the present report, the decrease in plant DM in both cultivars was due to the decrease in the rate of P_N , which exerted an important role in the accumulation of biomass in menthol mint plant during Cd toxicity. Reduction in the rate of photosynthesis under Cd stress is also attributed to the low biosynthesis of Chl and decreased the scavenging activity of Rubisco protein (Khan et al. 2016). However, SA improved growth traits and leaf gas exchange parameters in both cultivars (Figs. 1, 2, 3). Salicylic acid-mediated increase in the rate of photosynthesis in presence of Cd stress could have improved due to increase in Rubisco

activity, which is also in accordance with the finding of Krantev et al. (2008) in maize plants under Cd stress. The salicylic acid application also modulated Cd-induced changes in both cultivars of menthol mint and these results are in line with the findings of Choudhury and Panda (2004) in rice plants. A similar increase in leaf gas exchange traits and Rubisco activity by exogenous SA application in response to Cd stress has been reported in *Mentha piperita* (Ahmad et al. 2018).

Findings of the present work indicated that Cd stress decreased the uptake of the mineral nutrient elements in both the cultivars (Fig. 4A–C). Nutrient imbalance by Cd stress generated negative impacts on diverse physio-biochemical processes in plants. The pronounced inhibitory effects of Cd on the essential mineral concentrations of the leaf was due to their reduced uptake and translocation which corroborates with the earlier results obtained by Gill et al. (2012); Zouari et al. (2016); Ahmad et al. (2015, 2016). The high mineral nutrient concentrations in Kushal suggest that mineral metabolism of nutrients played a vital role in the capacity of plants to maximally tolerate Cd-induced oxidative changes. However, Cd-mediated variation in mineral nutrient concentration in both cultivars could be due to prevailing genetic variation (Anjum et al. 2008). Salicylic acid application optimized the nutrient status and increased mineral nutrient concentrations in both the cultivars under Cd stress (Fig. 4A–C). A similar increase in mineral nutrient concentrations by SA in barley plants under Cd stress has been reported by Metwally et al. (2003).

The menthol mint cultivars exhibited a sharp decline in the activities of NR and CA in response to Cd stress (Table 2). This might be due to the inhibition or dysfunction of the enzyme protein by metal stress (Orcutt and Nilsen 2000). In addition to this, Cd stress also disturbed the activity of plasma membrane-localized proton pumps (Obata et al. 1996), thus restricting the uptake of nitrate which is the substrate of NR activity (Campbell 1999).

Increased NR activity and N content mediated by SA under Cd stress could be due to the optimized allocation of available N under Cd stress in both cultivars which improved growth and photosynthetic traits (Figs. 1, 2, 3). A similar increase in the activity of NR due to SA treatment under Cd stress has been reported in chickpea plants by Hayat et al. (2014), tomato plants by Koc et al. (2013), peppermint plants (Ahmad et al. 2018), which are in agreement with our present results. The enzyme CA is the second most abundant zinc metalloprotein in C_3 plants (Okabe et al. 1984), which catalyzes the reversible inter-conversion of HCO_3^- and CO_2 in higher plants for maintaining a constant supply for Rubisco (Fariduddin et al. 2003). Cadmium stress decreased the activities of CA and Rubisco in the present study in both the cultivars. In fact, decreased Rubisco activity by Cd stress has been attributed due to the low activity of CA or Cd-Mg replacement in active centers in Cd-stressed plants (Siedlecka et al. 1998). Similar SA mediated increase in CA activity under Cd stress was reported by Alyemini et al. (2014) and Ahmad et al. (2018).

Plants have well developed antioxidant defense systems which comprise enzymatic and non-enzymatic antioxidants to fight against the ill effects of HMs' stress (Jalmi and Sinha 2015; Rizwan et al. 2017). Exposure to Cd stress triggered the excessive production of ROS in plants which is manifested in the form of increased oxidative stress. Increased TBARS, EL and H_2O_2 content were observed in both cultivars under Cd stress. These parameters were lower in Kushal reflecting their higher tolerance capacity against Cd-induced oxidative stress as compared to Kosi (Figs. 4D–F). In the present study, Cd stress increased the antioxidant activities in Kushal except for SOD which showed higher in Kosi. A similar increase in the activity of SOD has earlier been reported by Syeed et al. (2011) in cultivars of mustard. Higher activities of antioxidant enzymes in Kushal under Cd stress or PGRs treatment reflects efficient detoxification of Cd as reflected in low leaf Cd concentrations and less EL, H_2O_2 production and TBARS content. Enhanced activities of antioxidants maintained redox pool in both cultivars and more prominently in Kushal which ultimately led to the protection of cells against Cd-induced oxidative stress that was in turn reflected in the form of low values of leakage of ions. These results are in conformity with the results of Zhang et al. (2015) in *Cucumis melo*, Ahmad et al. (2016) in *Cicer arietinum*, Han et al. (2015) in *Iris hexagona*. Carotenoids are lipophilic antioxidant agents in plants which have the ability to scavenge excess ROS and show a promotive effect on growth and development of plants exposed to biotic and abiotic environmental pressures (Mourato et al. 2012; El-Beltagi and Mohamed 2013). In addition, they play a principal role in regulating the photoprotection of

the photosynthetic reaction center against auto-oxidation (Ashraf and Harris 2013; Gururani et al. 2015). Cadmium triggered an enhanced biosynthesis of carotenoid contents and the results are in harmony with the results of El-Beltagi and Mohamed (2013) in pea plants. The high carotenoid contents during PGRs treatment in general and during SA application in particular in Cd-stressed plants of menthol mint would be ascribed to its antioxidative and oxidative stress role. Enhanced carotenoid biosynthesis with PGRs supplementation might have reduced the oxidative stress caused by Cd toxicity in the present study. In line with our results, studies suggest that SA is known to increase carotenoid content in *Nigella sativa* (Kabiri et al. 2014), and in *Glycine max* plants (Razmi et al. 2017). Exogenous PGR treatments are known to promote the expression of genes associated with carotenoid biosynthesis pathway in plants (Cruz et al. 2018). In a more recent experiment, Zaid and Mohammad (2018) also found an enhanced carotenoid biosynthesis under Cd stress in *M. arvensis* plants supplied with exogenous phytohormone and mineral.

Cytosolic accumulation of compatible osmolytes is not only involved in the cell acclimation but also for sustaining the regulation of redox homeostasis potential under various environmental stresses including HMs (Ruszkowski et al. 2015; Anjum et al. 2017). Proline is one of the compatible solutes which help plants in the adjustment of the wide range of environmental extremes and serves as an adaptive means to stabilize cellular structures and functions, scavenges excess ROS, regulates redox environment (Ashraf and Foolad 2007; Bhaskara et al. 2015; Per et al. 2017). The present work revealed a differential accumulation of proline concentration in both cultivars under Cd stress. Cadmium-mediated increase in proline could be due to the increased activity of proline biosynthetic enzymes (Khan et al. 2015). Moreover, SA in the present study maintained the highest concentration of proline under stress conditions due to overexpression of genes related to proline biosyntheses such as pyrroline-5-carboxylate reductase and γ -glutamyl kinase (He et al. 2002; Maghsoudi et al. 2018; Lee et al. 2019). Kushal had always a high concentration of proline than Kosi in control as well as in all treatments and was found more tolerant to Cd stress.

It has been reported that Cd accumulates differently among different plant species and cultivars and is absorbed by either apoplastic or symplastic pathways (Kushwaha et al. 2015; Dong et al. 2017). In the present study, it was noted that roots accumulated more Cd as compared to leaves in both cultivars. This might be due to the poor root-shoot Cd translocation rate. Nonetheless, the high root and low leaf accumulation in Kushal contributed greater tolerance and well as high phytoremediation potential towards Cd stress than Kosi. This could be also one of the prime reasons that high leaf Cd concentration in Kosi generated

negative effects on growth, mineral nutrients, and photosynthetic responses and increased oxidative stress. Similar tolerance to Cd stress has also been reported in bluegrass (Dong et al. 2017), miscanthus species (Guo et al. 2016) and in rice plants under Pb stress by Ashraf et al. (2017).

Conclusions

Cadmium toxicity differently hampered the growth, photosynthetic, mineral nutrient uptake in menthol mint cultivars. Out of the three PGR tested to ameliorate the Cd-induced changes, SA treatment found more promising. Enhanced osmolyte accumulation, optimum mineral metabolism, reduced oxidative stress and induced anti-oxidative defense systems were more prominent in Kushal cultivar as compared to Kosi. The effect of SA application in modulating Cd-induced changes was more conspicuous in Kushal cultivar than Kosi. Furthermore, uptaken Cd accumulated more in roots than translocated to leaves. The results showed that the menthol mint cultivars responded differentially to Cd stress tolerance. The findings presented could be helpful in evolving strategies to phytoremediate Cd from challenged habitats by selecting specific cultivar of menthol mint as well as for the development of tolerant menthol mint cultivars.

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