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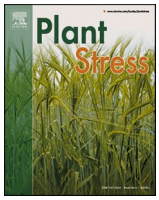

Dose-dependent regulation of morphological, physio-biochemical, nutritional, and metabolic responses by cobalt in *Tagestes erecta* L. plants exposed to salinity stress

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ABSTRACT

Salinity is a major concern globally and causing reduction in crop growth and development thereby lowering food production. Interestingly, cobalt (Co), a multifunctional non-essential micro-element, has an important role in improving growth and development under salinity stress. In the current study, the effects of Co on the morphological, biochemical, nutritional, and metabolic changes of African marigold plants at two salinity levels were assessed. Two concentrations of Co (C1; 10 mg/L and C2; 20 mg/L) were applied as foliar application to the marigold plants under salinity (S1; 300 mM and S2; 600 mM) stress. The results indicated that salinity substantially reduced the growth by negatively affecting the nutritional and metabolic profile. Interestingly, the C1 application mitigated the salinity effects and improved all the studied parameters including vegetative, nutritional, biochemical and metabolites (amino acids, fatty acids, organic acids, sugars, carotenoids, flavonoids, and terpenoids) by reducing the reactive oxygen species (ROS) and methylglyoxal (MG)-induced oxidative stress, at two salinity levels. The overall results revealed that C1was an ideal dose for marigold plants undergoing salinity stress since C2 application showed some toxicity symptoms under both the salinity levels; with reduced growth and impaired development. Based on the observations, the two different oxidative stress scavenging pathways in marigold are discussed i.e. ROS-scavenging by ascorbate-glutathione (AsA-GSH) cycle and the MG-detoxification by glyoxalase. With the limitations imposed upon the glutathione (GSH) pool size and redox homeostasis, the study indicated that GSH played a critical role in both ROS- and MG-detoxification in marigold plants at both salinity levels.

1. Introduction

Soil salinity, a major abiotic stress worldwide, reduces crop yield by lowering plant growth and development through osmotic, ionic, and nutritional imbalance (Isayenkov and Maathuis, 2019; Munns and Tester, 2008; Wu et al., 2023). Excessive salt ion exposure, such as sodium (Na^+), magnesium (Mg^{2+}), potassium (K^+), chloride (Cl^-), carbonate (CO_3^{-2}), and calcium (Ca^{2+}), will cause a negative impact on plant growth and development (de Bang et al., 2021; He et al., 2023; Munns and Gilliham, 2015; Wu et al., 2023). Osmotic stress, another immediate non-ionic stress response, causes stomatal closure and decreased evapotranspiration of water, resulting in the formation of reactive oxygen species (ROS). Excessive ROS generation including hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl ion (OH^-),

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causes membrane damage, protein, and DNA denaturation, and eventually, cell death. In order to maintain the ROS homeostasis, plants have robust and diverse antioxidant defense system called the glutathione-ascorbate (GSH-AsA) pathway, consisting of both enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione (GSH),oxidized glutathione (GSSG), glutathione peroxidase (GPX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), dehydroascorbate (DHA) guaiacol peroxidase (POD), glutathione S-transferase (GST), ascorbic acid (AsA), glutathione reductase (GR), flavonoids, and carotenoids (Riyazuddin et al., 2020). Subtle changes in ROS levels have both toxic and stress-signaling effects in plants; however, the toxic and signaling behavior of these reactive species are entirely dependent on the ROS concentration. In addition to methylglyoxal (MG) reactive nature, studies revealed that at lower concentrations, MG can act as stress-signaling molecule. Conversely at higher concentration, MG causes negative effects in many species (Mishra, 2023). The over accumulation of MG resulted in the denaturation of phospholipids, nucleic acids, proteins, and amino acids such as arginine, lysine, and cysteine to form advanced glycation end products (AGEs) that impair the biological functions in plants under environmental stresses (Mishra, 2023; Nowicka et al., 2023). Previous research revealed that MG is extremely toxic at high concentrations while act as a signaling molecule at low levels (Dorion et al., 2021; Li, 2019). In addition, MG contributes to the depletion of O_2, resulting in oxidative stress in plants (Dorion et al., 2021). Like ROS, plants possess a well-defined two-step MG-detoxification system comprised of two metalloenzymes: glyoxalase I (Gly I) and glyoxalase II (Gly II). In this pathway, Gly I and Gly II use GSH as a cofactor to convert the toxic MG into the nontoxic d-lactate. Successful ROS and MG detoxification is one of the most important variables in salinity stress tolerance, and GSH appears to play a key role in this regard (Batth et al., 2020; Hasanuzzaman et al., 2019).

Exogenous salt-protectants, such as osmolytes, trace elements, biostimulants, and phytohormones, have been found to improve salt tolerance in many plant species (Alhammad et al., 2023; Ma et al., 2022; Wong et al., 2020). Some recent studies revealed the potential role of cobalt (Co), an integral part of many enzymes and co-enzymes, as salt-protectant in different plant species (Brengi et al., 2022). Some previous recent studies revealed that Co application, at a very low concentration enhanced plant growth, development, yield and ameliorated salt stress (Akeel and Jahan, 2020) in tomato (Gad et al., 2017), maize (Gad and El–Metwally, 2015), cucumber (Brengi et al., 2022; Gad et al., 2018), and onion (Gad et al., 2020) and improved nitrogen fixation in some legume crops. Nevertheless, its impact effectiveness is entirely dependent on the concentration and the duration of the application. Despite its potential involvement in the mitigation of salt stress in plants, its function in plant stress physiology, however, is still not fully understood. *Tagetes erecta* L. (Marigold), an important potted cut flowers known for its medicinal, aromatic, ornamental and pharmaceutical characteristics (Abbas et al., 2019; Ferdosi et al., 2022). This multipurpose annual flower is widely grown in many countries including Pakistan and India commercially (Ferdosi et al., 2022). Apart from its medicinal properties, Marigold, is a rich source of metabolites such as carotenoids and flavonoids, and is used as a substitute for saffron in edible dye (Sowndharya and Giri, 2020; Zhang et al., 2020). Previous studies revealed that Marigold growth and development negatively impacted by the salinity stress (Al-Mazroui et al., 2020), however, no evidences were found on the use of exogenous Co application on marigold's overall growth and development, mitigation of ROS-MG-induced oxidative stress, and their detoxification mechanism under salinity stress.

Keeping in view of the significance of marigold and its performance under saline conditions with the plausible mitigation properties of Co, a study was conducted with the following objectives: i) To investigate the effects of salinity on morphological, nutritional, biochemical, and metabolic profile of marigold and how Co could be effective to mitigate the negative effects of high salinity. ii) To examine the negative effects caused by the combination of salinity and Co (high concentration) in marigold tissues. iii) To explore the MG-induced oxidative stress in marigold plants and its detoxification mechanism using Co as salinity-alleviator micronutrient.

2. Materials and methods

2.1. Planting site, soil preparation and seedling transplant

A pot experiment was conducted at research area of Department of Biology, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia. Sand and garden soil in a ratio of 1:1 was used as planting medium. During the experiment, greenhouse average maximum and minimum temperature were 30 °C/20 °C, relative humidity of 15% and 60% (day and night) with an average total daily photosynthetic photon flux density (PPFD) of 12.8 mol. $day^{-1}.m^{-2}$. The soil physio-chemical properties were evaluated using established protocols and the findings were reported earlier (Elnaggar et al., 2024;Wong et al., 2022;Yong et al., 2010). Initially, three seedlings of African marigold were transplanted (at 2–4 true leaf stage) into the pots containing 3 kg of properly sieved garden soil; later, the plants were thinned to one seedling/pot when the transplanted seedlings attained uniform height. All the experiments were done in compliance with relevant institutional, national, and international guidelines and legislation. High research standards were maintained throughout the experiments and following the various established scientific protocols (Wong et al., 2022; Cornelissen et al., 2003; Ma et al., 2022).

2.2. Treatments

There were 9 treatments in the experiment consisting of two salinity levels i.e. 300 mM (S1) and 600 mM (S2) in the form of NaCl, while 0 mM (S0) was taken as control. Similarly, two levels of Co i.e. 10.0 mg/L (C1) and 20.0 mg/L (C2), whereas C0 (0.0 mg/L) was taken as control treatment. Co was taken in the form of $CoSO_4$. Treatment arrangements were as follows: T0= S0+C0, T1= S0+C1, T2= S0+C2, T3= S1+C0, T4= S1+C1, T5= S1+C2, T6= S2+C0, T7= S2+C1, T8= S2+C2. S0 and C0 were taken as control treatments each treatment consisted of 3 replications. Saline conditions were provided to the soil in the form of irrigation water after ten days of transplantation. While the Co was applied to the transplanted seedlings in the form of foliar application twice a week for four consecutive weeks. During the 6th week when the flower attained their maximum size were harvested and the below mentioned parameters were studied according to their standard protocols.

2.3. Parameters studied

2.3.1. Morphological parameters

Leaf number/plant (LN/plant) and flower number/plant (FN/plant) were counted manually, and means were recorded. Leaf area (LA) was recorded by using leaf area meter and measurements were taken in mm^2, while flower diameter (FD) was measured using a digital vernier caliper and the data was taken in (mm). Furthermore, after harvesting, the plant parts (roots, leaves, and flowers) were carefully separated. Roots were thoroughly washed with distilled water and air dried. Following which, the flower fresh weight (FFW), shoot fresh weight (SFW), and root fresh weight (RFW) in "mg" were recorded using a digital weighting balance. For dry biomass, plants samples were packed and labeled in paper bags and dried at 70 °C for 72 h in a hot dry oven. After the prescribed period of time, the following parameters were measured in "mg"; flower dry weight (FDW), shoot dry weight (SDW), and root dry weight (RDW) and means were recorded.

2.3.2. Nutrients

The contents of sodium (Na^+), chloride (Cl^-), potassium (K^+), and calcium (Ca^{2+}) were measured from dry leaves and roots tissues by previously reported method (Parvin et al., 2020; Rahman et al., 2016; Wu et al., 2023). From grinded homogenous dry plant samples, 0.1 g was digested with the acid mixture (HNO_3: $HClO_4$; 5:1). The Na^+, Cl^-, K^+, and Ca^{2+} contents were determined from the digested solution by using atomic absorption spectrophotometer (AA-7000, Shimadzu, Japan).

2.3.3. Reactive oxygen species

Hydrogen peroxide (H_2O_2) content was determined by a peroxidase dependent assay adopting the earlier method (Okuda et al., 1991). H_2O_2 was extracted with 0.1% trichloroacetic acid (TCA) followed by incubation with 0.1 mM potassium phosphate (K_3PO_4) buffer (pH 7.0) and 1 M potassium iodide (KI). Absorbance was measured at 390 nm and the concentration of H_2O_2 was determined from the standard curve.

Superoxide radicals (O_2^-) were detected by transferring the plant samples into 0.2% nitro blue tetrazolium chloride (NBT), dissolved in 50 mM sodium phosphate buffer ($Na_3(PO_4)_2$) at pH 7.5. An insoluble formazan compound of dark-blue color appears when NBT reacts with O_2^-. The seedlings were then moved to a bleaching solution to remove the chlorophyll. Afterwards, leaf and root tissues were ground in 0.1% acetic acid solution and centrifuged at 10,000 rpm for 10 min and the absorbance was noted at 560 nm (Kumar et al., 2014).

The presence of hydroxyl radicals (OH^-) in the seedlings was determined by following the earlier method (Aruoma, 1994). Plants samples were homogenized in 1.2 mL of 50 mM sodium phosphate buffer solution at pH 7.0 and centrifuged at 12,000 rpm for 10 min at 4 °C. Afterwards, 0.5 mL supernatant was mixed in 1 mL of 25 mM of sodium phosphate buffer solution containing 2.5 mM 2-deoxyribose solution and incubated at 35 °C in the dark for 60 min. After that, the mixture was mixed with 1 mL glacial acetic acid and 1 mL of 1% thiobarbituric acid (TBA; Sigma, USA) and boiled for 10 min before immediately cooling in an ice bath. The absorbance was measured at 532 nm.

For MG quantification, 100 g fresh samples of both roots and leaves tissues were grounded in distilled water and the water extract centrifuged at 11,000 rpm for 10 min at 4 °C. After that, 100 µL of 5 M of $HClO_4$solution and 250 µL of 7.2 mM 1,2-diaminobenzene solution were added to 650 µL of supernatant. The absorbance at 336 nm was recorded with a spectrophotometer(AA-7000, Shimadzu, Japan) (Nisarga et al., 2017; Vijayaraghavareddy et al., 2020).

2.3.4. Antioxidant activities

About 0.5 g of fresh leaves and roots were homogenized in 100 mM of (K_3PO_4) buffer (pH 7.0) having 0.5% Triton and 0.1% polyvinylpyrrolidone (PVP) using a pre-chilled pestle and mortar. Further the homogenate was centrifuged at 15,000 g for 20 min at 4 °C and the supernatant was used for enzymes assays.

SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT), according to the previous protocol (Dhindsa et al., 1981). One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the NBT reaction.

The activity of APX was determined by previously developed protocol (Nakano and Asada, 1981). The APX activity was calculated by using an extinction coefficient of 2.8/(mM cm). One unit of enzyme was the amount necessary to decompose 1.0 µmol of substrate per min at 25 °C.

The GR was determined as described earlier (Foyer and Halliwell, 1976) by monitoring the GSH-dependent oxidation of NADPH. The GR activity was calculated by using an extinction coefficient of 6.2/ (mM cm). One unit of enzyme was the amount necessary to decompose 1.0 µmol of NADPH per minute at 25 °C.

The DHAR activity was measured following the increase in absorbance at 265 nm due to the GSH-dependent production of AsA as described by Foyer et al. (1989).The reaction mixture contained K-phosphate buffer (0.1 M, pH 6.2), 2 mM GSH, and 50–100 g of proteins (Bradford, 1976). The reaction started upon addition of 1 mM DHA. One enzyme unit was equivalent to nmol/g FW.

For APX, the homogenizing buffer was supplemented with 2 mM ascorbate. The MDHAR activity was measured in the supernatant as described previously (Hussain and Asada, 1984). Its activity was measured spectrophotometrically by following the decrease in absorbance at 340 nm due to NADPH oxidation.

The reduced ascorbate (AsA) and oxidized dehydroascorbate (DHA) contents were determined by the earlier method (Law et al., 1983).0.5 g of fresh leaves and roots were homogenized in 2.0 mL of K-phosphate buffer (100 mM, pH 7.0)and centrifuged at 1000 g for 10 min. To 1.0 mL of the supernatant, 0.5mLof 10% (w/v) TCA was added, thoroughly mixed, and incubated for 5 min at 4 °C. The 1.5mLof above solution and 0.5 mLof NaOH were thoroughly mixed and centrifuged at 5000 *g* for 10 min at 20 °C. The aliquot thus obtained was equally distributed into two separate microfuge tubes (750µL each). For the estimation of AsA, 200 µL of K-phosphate buffer (150 mM,pH 7.4) was added to 750µl of the aliquot, while for DHA estimation, 750 µL of the aliquot was added to100 µL of dithiotheritol (DTT) followed by vortex mixing, incubation for 15 min at 20 °C, and addition of 100µl of 0.5% of N-Ethylmaleimide (NEM). Then, both microfuge tubes were incubated for 30 s at room temperature. To each sampletube, 400µl of 10% TCA, 400µl of H_3PO_4, 400µl of 4% bipyridyl dye (N',N-dimethyl bipyridyl), and 200µL of 3% $FeCl_3$ were added and thoroughly mixed. The absorbance was recorded at 525 nm after incubation for 1 h at 37 °C.

The contents of reduced glutathione (GSH) and oxidized (GSSG) were estimated following the earlier method (Griffith, 1980). Fresh leaves and roots (0.5 g) were homogenized in 2.0 mL of 5% sulfosalicylic acid under low temperature. The homogenate was centrifuged at 10,000 rpm for 10 min. To 0.5 mL of the supernatant, 0.6 mL of K-phosphate buffer (100 mM, pH 7.0) and 40µL of 5′,5′-dithiobis-2-nitrobenzoic acid (DTNB) were added. After 2 min, the absorbance was recorded at 412 nm on a spectrophotometer (AA-7000, Shimadzu, Japan). GSSG was assayed by the same method in the presence of 2-vinylpyridine, and the GSH concentration was calculated from the difference between total glutathione and GSSG.

The GST activity was determined by measuring the formation of the glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) conjugate, according to previously developed method (Habig and Jakoby, 1981). Appearance of GS-DNB (*S*-2,4-dinitrophenyl-glutathione, ϵ_{340} =9.6 mM^{-1}/cm) was followed at 340 nm and pH 6.5. Final concentrations of 1 mM CDNB (stock in ethanol) and 1 mM GSH were used in 0.1 M K-buffer, pH 6.5, and 1 mM EDTA. Cuvettes were incubated for 2 min, and then CDNB was added to start the reaction, which was recorded for 2 min. Rate measurements obtained were corrected for the spontaneous reaction of CDNB without enzyme.

The catalase (CAT) activity was determined according to the previous method (Chandlee and Scandalios, 1984) with some modifications. Frozen samples (0.5 g) were ground in a pestle and mortar with 5 mL of 50 mM ice-cold $Na_3(PO_4)_2$buffer solution having pH 7.5 containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Afterwards, the extract was centrifuged at 12,500 rpm for 20 min at 4 °C. The supernatants were extracted for enzyme examination. The reaction mixture comprised 2.6 mL of 50 mM K_3PO_4 solution (pH 7.0), 40 µL enzyme extract, and 400 µL of 15 mM H_2O_2. The absorbance was recorded at 240 nm and the CAT activity was measured using the previously describe method of (Bradford, 1976).

The glutathione peroxidase (GPX) (Flohé and Günzler, 1984) and peroxidase (POD) (Lobarzewski and Ginalska, 1995) activities were determined according to the tetraguaiacol formation and the measurements were made in nmol/g FW.

To understand the effect of Co treatment under salinity stress on MG-detoxification system, Gly I and Gly II activities in both leaves and roots were studied. The extraction buffer and procedure of Gly I and Gly II was the same as the ROS-scavenging enzymes. The determination of Gly I

and Gly II was referred to the previous method (Li et al., 2019) and their activities were calculated using the molar absorption coefficient of 3.37 $\times 10^3$ M^{-1} cm^{-1} (for S-d-lactoylglutathione) and 1.36 $\times 10^4$ M^{-1} cm^{-1} (2-nitro-5-thiobenzoic acid), respectively, and expressed as μg/g FW.

2.3.5. Metabolites extraction

All metabolites such as sugars (glucose, sucrose, maltose, ribose, fructose, galactose, xylose, arabinose, galacturonic acid, and rahmnose), amino acids (proline, alanine, lysine, leucine, phenylanalynine, histidine, serine, valine, tyrosine, arginine, aspargine, glutamate, methionine, and aspartate), fatty acids (lauric acids, myristic acid, oleic acid, stearic acid, palmitic acid, lenolic acid, margaric acid, palmitolic acid, aracdic acid, elaidic acid, and linoelaidicic acid), carotenoids (α-carotene, β-carotene, lutein, lycopene, zeaxanthin, lactucaxanthin, mutatoxanthin, antheraxanthin, neoxanthin, luteoxanthin, flavoxanthin, and rubixanthin), terpenoids (sitosterol, monoterpene, thiophene, stigmasterol, brein, lupeol, erythrodiol, aranidiol-3-O-myristate, aranidiol-3-O-lurate, butyl ester, methyl ester, and carnulacic acid acetate), falvonoids (rutin, narcissin, quercentin, isorhamnetin, isorhamnetin-3-O-β-D glycoside, and isoquercitrin) and organic acids (lactate, fumarate, succinate, citrate, pyruvate, and butyrate) in roots, leaves, and flowers of *Tagestes erecta* L. were studied by the earlier documented procedure (Müller et al., 2015). Sample volumes of 1μL were analyzed with a Trace gas chromatograph (GC) coupled to a PolarisQ ion trap mass spectrometer (GC–MS) equipped with an AS2000auto sampler (Thermo Electron, Dreieich, Germany). Derivatized metabolites were evaporated at 250 °C in the splitless mode and separated on a 30 m × 0.25 mm RTX-5MS capillary column with a 0.25 mm coating equipped with an integrated 10 m guard column (Restak, Bad Homburg, Germany). Helium carrier gas flow was adjusted to 1 mL/m. The interface temperature was set to 250 °C and the ion source temperature to 220 °C. The oven temperature was kept constant for 3 min at 80 °C after each analysis. Mass spectra were recorded at 1 scan/s with a scanning range of 50 to 750 *m/z*. Metabolites were identified by comparison with pure standard (Sigma-Aldrich). In addition, the freely available Golm Metabolome Database (Kopka et al., 2005) was of particular help in identifying several metabolites. All identified compounds matched the references by mass spectral data and chromatographic retention time. Relative levels of selected metabolites were determined automatically by integrating the peak areas of selective ions (Fiehn et al., 2000) with the processing setup implemented in Xcalibur 1.4 software (Thermo Electron, Dreieich, Germany). Relative response ratio was calculated by normalizing the respective peak areas to the peak area of the internal standard ribitol and dividing the value by the dry weight of the sample. Measurements were performed in technical duplicates for each of the three replicates of control and the C1 and C2 treated plants.

2.4. Statistical analysis

A pot experiment was conducted with nine treatments in total (three replications each). All the data was analyzed statistically by using analyses of variance technique (ANOVA) under complete randomized design (CRD). Treatment means were analyzed statistically by the least significance difference (LSD) test at a 5% level of probability by using SPSS software. The TBTools software was used to develop the heatmaps, and PCA analyses were performed using Canoco 5 software.

3. Results

3.1. Dose-dependent regulation of the growth indices by cobalt under salinity stress

Salinity stress negatively impacted the growth indices of *Tagestes eracta* L. which is indicated by the 6, 9, 11, 20, 15, 19, 10, 15, 23, and 41% reduction at 300 mM of salt-stress (moderate stress), while 9, 26, 31, 3, 36, 31, 33, 41, 34, and 55% decline following 600 mM of salinity-stress (severe stress) in LN/plant, LA, FN/plant, FD, FFW, FDW, RFW, RDW, SFW, and SDW, respectively. However, the Co application (C1) alleviated the saline stress by improving the LN/plant, LA, FN/plant, FD, FFW, FDW, RFW, RDW, SFW, and SDW up to 1.12-, 1.43-, 1.29-, 1.05-, 1.19-, 1.35-, 1.22-, 1.16-, 1.79-, and 2.86-fold at moderate stress (300 mM), respectively. Additionally, C1 at severe salinity stress (600 mM), except FD, all morphological parameters showed improved growth up to 1.05-, 1.58-, 1.59-, 1.28-, 1.15-, 1.46-, 1.52-, 2.14-, and 2.95-fold, respectively. Interestingly, the results revealed that C2 application negatively impacted the growth indices at both the salinity stress levels (Table 1).

3.2. Dose-dependent modulation of the nutritional profileby cobalt under salinity stress

Likewise other parameters, nutrients such as Na^+, Cl^-, K^+, Ca^{2+} adversely affected by the salt stress. In marigold roots, $Na^+$and Cl^- increased up to 1.26- and 1.48-fold at 300 mM, while 1.59- and 2.04-fold under 600 mM salinity stress, respectively. Whereas K^+ and Ca^{2+} reduced by 22 and 17% under 300 mM and 52 and 58%at 600 mM salinity stress, respectively. Furthermore, C1 application reduced Na^+ and Cl^- by 21 and 17% under moderate stress (300 mM) and 30 and 34% under severe salinity stress (600 mM). In contrast, K^+ and Ca^{2+}elevated up to 1.15- and 1.35-fold under 300 mM, whereas 1.31- and 2.68-fold at 600 mM salinity stress compared to untreated plants, respectively. Moreover, C2 application decreased Na^+, K^+ and Ca^{2+} by 26, 22, and 21% and Cl^- increased up to 1.29-fold at 300 mM stress, while C2 reduced $K^+$and Ca^{2+} up to 27 and 23% whereas, Na^+ and Cl^-enhanced up to 1.10- and 1.34-fold at 600 mM salt stress, compared to untreated plants (Fig. 1).

In marigold leaves, Na^+ increased up to 1.22- and 1.59-fold at 300 mM and 600 mM salinity stress, respectively. Whereas Cl^-, K^+ and Ca^{2+} reduced by 10, 11, and 33% at 300 mM and 18, 43 and 54% at 600 mM salt stress, respectively. Nevertheless, C1 application decreased the Na^+ and Cl^- by 10 and 25% under moderate stress (300 mM), whereas 9 and 7% under severe salt stress (600 mM), while K^+ and Ca^{2+} increased up to 1.13- and 1.83-fold at 300 mM stress and 1.18- and 2.57-fold under 600 mM salt stress compared to untreated leaves, respectively. Additionally, C2 application further enhanced Na^+, Cl^-, and K^+levels and reduced Ca^{2+}levels at both the salinity levels (Fig. 1).

3.3. Dose-dependent regulation of the reactive species by cobalt under salinity stress

The current results revealed that reactive species such as H_2O_2, O_2^-, OH^-, and MG showed a slight increase following 300 mM NaCl, while 1.42-, 1.34-, 1.13-, 1.21-fold elevation was noticed after 600 mM salinity in marigold roots. A similar trend was observed in the leaves of *Tagestes eracta* L. where the higher accumulation of reactive species was recorded following 600 mM NaCl-induced saline stress (Fig. 2). However, C1 application considerably reduced the ROS and MG content in both roots and leaves at both the salinity levels. In contrast, the C2 application further increased the oxidative stress by elevating the accumulation of ROSand MG at both the salinity levels in both organs of *Tagestes eracta* L. (Fig. 2).

3.4. Dose-dependent regulation of the antioxidant activities by cobalt under salinity stress

Salinity stress negatively impacted the antioxidants activities in both organs (roots and leaves) of *Tagestes eracta* L. Both treatments (salt and cobalt) affected the activities of enzymes in roots, as shown by the separate cluster of control samples in the hierarchical clustering (Table 2). Specifically, along with the slight reduction in the antioxidant activities of roots under 300 mM NaCl, the 600 mM NaCl resulted in the 19, 20, 29, 28, 22, 4, 13, 16, 29, 34, 14, 39, 35, 86, and 47% decline in

Table 1
The interactive effects of salinity and cobalt on morphological attributes of *Tagestes erecta* L.

Treatments	0 mM Salinity			300 mM Salinity			600 mM Salinity			p-value
	C0	C1	C2	C0	C1	C2	C0	C1	C2	
LN/plant	225.0 ± 2.0c	230.3 ± 1.5b	201.3 ± 3.5f	210.0 ± 2.0e	236.0 ± 2.0a	191.7 ± 2.5g	204.5 ± 1.5f	216.7 ± 2.0c	167.3 ± 2.1h	0.0001
LA (mm^2)	14.3 ± 0.8d	19.8 ± 0.6a	13.2 ± 0.2e	13.01±0.4e	18.7 ± 0.4b	10.2 ± 0.1f	9.7 ± 0.4cd	15.3 ± 0.3ab	8.7 ± 0.33g	0.0001
FN/plant	14.0 ± 2.0abc	18.0 ± 3.0a	9.7 ± 2.5cd	12.3 ± 3.1bc	16.0 ± 3.6ab	9.0 ± 1.4cd	9.6 ± 2.5±de	7.7 ± 1.4ef	5.0 ± 1.7d h	0.0013
FD (mm)	15.6 ± 2.7ab	17.9 ± 2.9a	6.5 ± 1.3ef	12.3 ± 2.4cd	12.9 ± 2.7bc	5.9 ± 0.4f	8.08±1.1e	10.4 ± 2.01c	2.7 ± 0.4 g	0.0012
FDW (g)	12.8 ± 1.3b	14.2 ± 1.2a	10.0 ±.062cd	10.8 ± 1.1c	13.0 ± 2.01ab	8.9 ± 0.7de	5.6 ± 0.3de	6.4 ± 0.8cd	6.1 ± 0.8f	0.0001
FFW (g)	8.2 ± 1.2b	9.4 ± 1.1a	5.6 ± 0.8e	6.6 ± 0.4c	8.9 ± 1.1ab	3.6 ± 0.4f	3.8 ± 0.2e	5.7 ± 0.4c	2.8 ± 0.02g	0.0001
SFW (g)	8.6 ± 1.1c	12.6 ± 1.6a	7.4 ± 0.8d	6.6 ± 0.3e	11.8 ± 0.9b	6.3 ± 0.2e	2.5 ± 03f	12.0 ± 1.013c	3.6 ± 0.7 g	0.0001
SDW (g)	5.6 ± 0.5d	10.5 ± 1.4a	3.9 ± 0.3e	3.3 ± 0.1ef	9.5 ± 0.7b	3.5 ± 0.03e	2.5 ± 0.3f	7.5 ± 0.6c	1.5 ± 0.02 g	0.0001
RFW (g)	5.8±±0.8c	7.4 ± 0.5a	4.7 ± 0.5d	5.2 ± 0.3d	6.4 ± 0.3b	3.2 ± 0.1f	2.2 ± 0.02e	5.7 ± 0.3c	2.7 ± 0.1g	0.0001
RDW (g)	3.8 ± 0.4b	5.3 ± 1.1a	2.8 ± 0.1d	3.2 ± 0.1c	3.7 ± 0.3b	2.2 ± 0.01e	5.6 ± 0.2f	3.4 ± 0.1ab	1.8 ± 0.02f	0.0001

LN/plant; Leaf Number/plant, LA; leaf area, FN/plant; flower number /plant, FD; flower diameter, FFW; flower fresh weight, FDW; flower dry weight, SFW; shoot fresh weight, SDW; shoot dry weight; RFW; root fresh weight, and RDW; root dry weight, SS; Salinity stress, C0; cobalt 0 mg/L (control treatment), C1; cobalt 10 mg/L, and C2; cobalt 20 mg/L. Values marked with different letters are significantly different from each other at $p \leq 0.05$ levels (ANOVA followed by least significant difference (LSD) test.

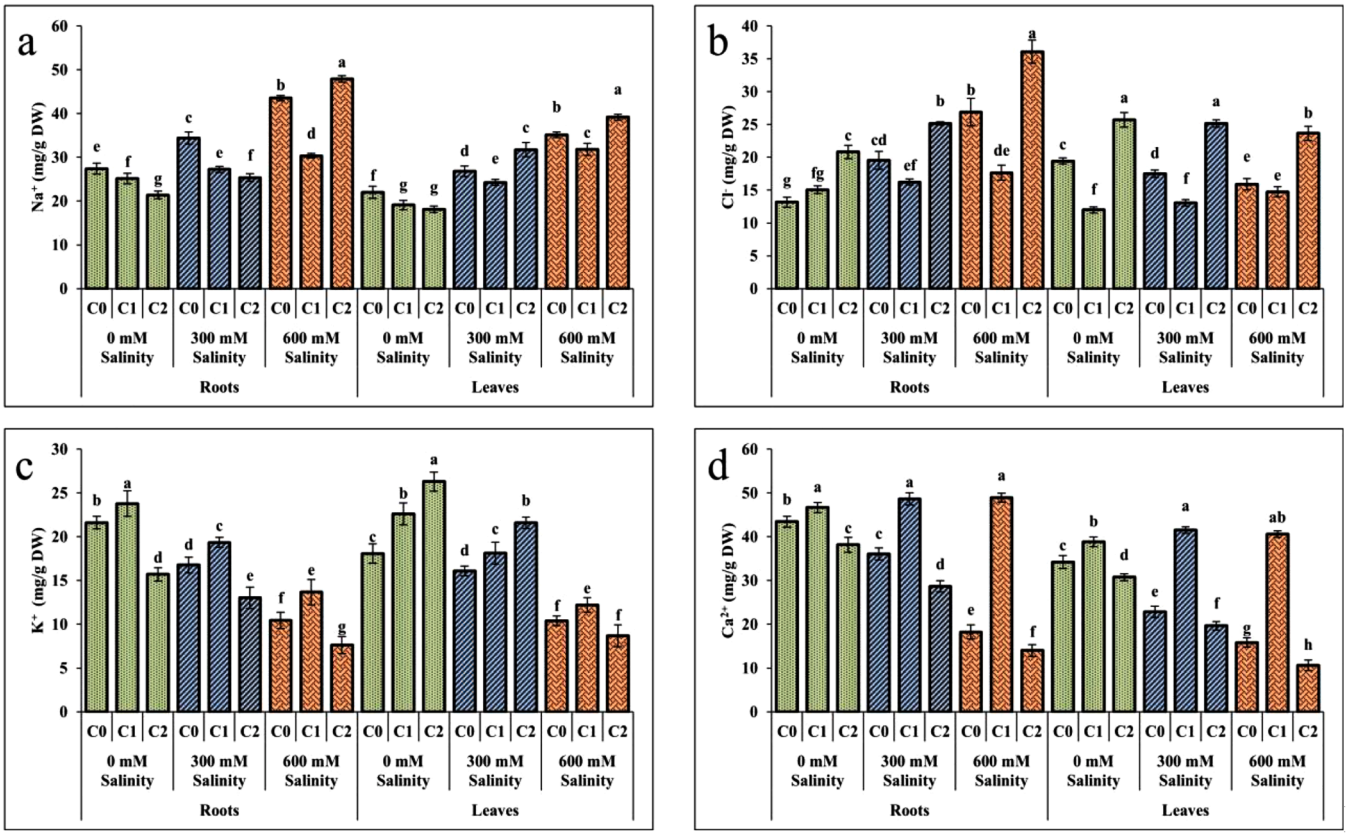

Fig. 1. Interactive effects of saline stress and cobalt on nutritional profile of *Tagestes eracta* L. (a) Na^+; sodium ion, (b) Cl^-; chloride ion, (c) K^+, potassium ion, (d) Ca^{2+}; calcium ion in roots and leaves of *Tagestes erecta* L. SS; salinity stress, C0; cobalt 0 mg/L (control treatment), C1; (cobalt 10 mg/L), and C2; (cobalt 20 mg/L). Values marked with different letters are significantly different from each other at $p \leq 0.05$ levels (ANOVA followed by least significant difference (LSD) test; three independent experiments with parallel each.

the activities of SOD, CAT, POD, APX, GPX, GSH, GSSG, AsA, GR, GST, DHAR, MDHAR, DHA, Gly I, and Gly II, respectively (Table 2). Contrarily, the C1 application considerably enhanced the antioxidants levels in roots at both the salinity stress levels since 1.48-, 1.38-, 1.08-, 1.49-, 1.05-, 1.04-, 1.11-, 1.29-, 1.09-, 1.21-, 1.14-, 1.17-, 1.16-, 8.14-, and 2.18-fold elevation was observed after 300 mM, whereas 1.71-, 1.59-, 1.07-, 1.82-, 1.10-, 1.02-, 1.07-, 1.27-, 1.18-, 1.09-, 1.06-, 1.21-, 1.18-, 28.87-, and 3.45-fold increment was recorded under 600 mM, respectively. Unlike C1, the C2 application substantially reduced the activities of all (except Gly I and Gly II) antioxidant enzymes irrespective of the salinity stress.

Salt and cobalt affected the activities of enzymes in leaves as displayed by their separate groups in the hierarchical cluster analysis from the control plants (Table 3). Specifically, the antioxidants activities in the leaves of *Tagestes eracta* L. also showed the similar trend as in the root since the slight reduction was noticed after 300 mM NaCl-induced salt stress, however, the application of 600 mM NaCl-induced saline stress resulted in 32, 22, 47, 40, 31, 14, 38, 33, 36, 27, 20, 49, 52, 25,

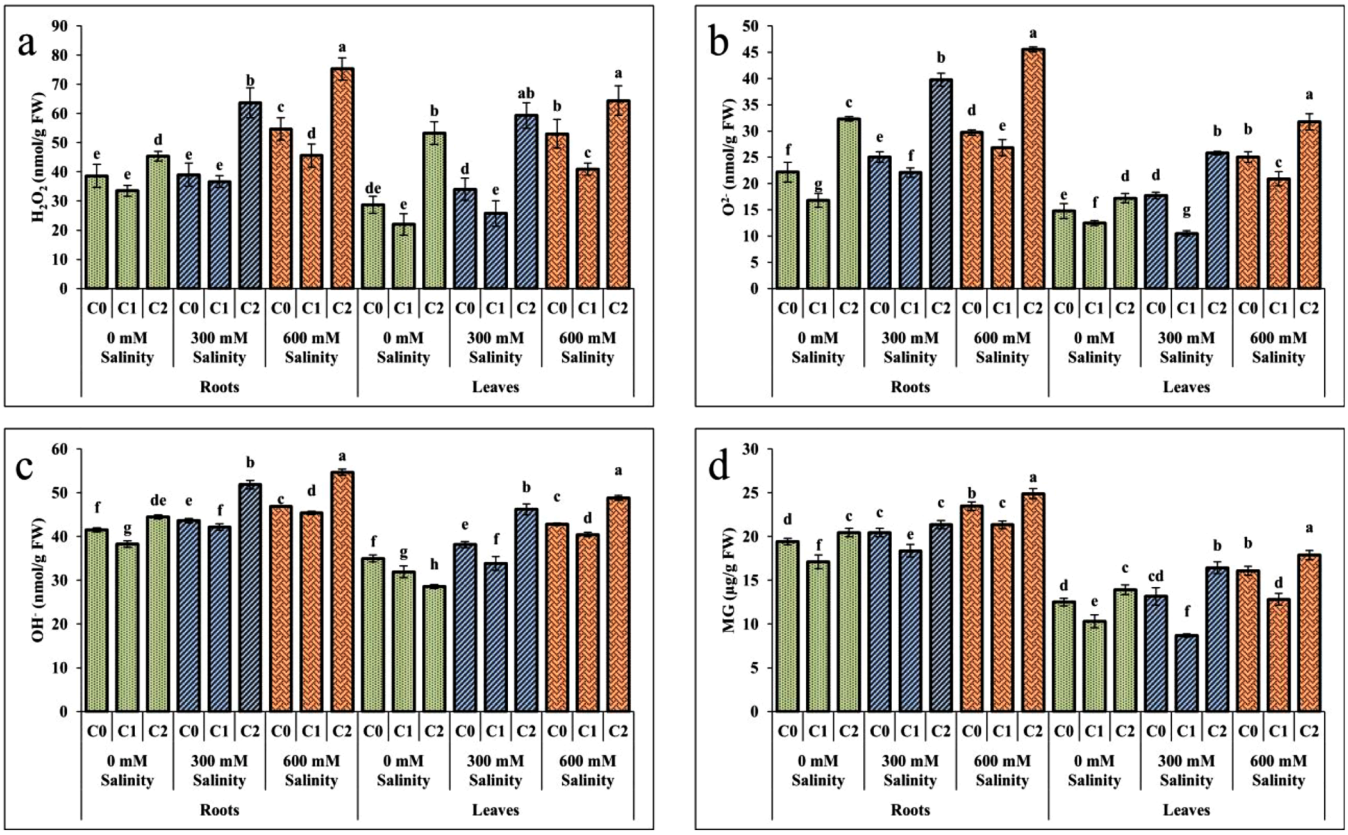

Fig. 2. Interactive effects of saline stress and cobalt on reactive species levels of *Tagestes eracta* L. (a) H_2O_2; hydrogen peroxide, (b) O_2^-; superoxide ion, (c) OH^-, hydroxyl radical, and (d) MG; methylglyoxal, C0; cobalt 0 mg/L (control treatment), C1; (cobalt 10 mg/L), and C2; (cobalt 20 mg/L). Values marked with different letters are significantly different from each other at $p \leq 0.05$ levels (ANOVA followed by least significant difference (LSD) test; three independent experiments with parallel each.

Table 2
The interactive effects of salinity and cobalt on antioxidant activity in roots of the *Tagestes erecta* L. plants.

Treatments	0 mM Salinity			300 mM Salinity			600 mM Salinity			p-value
	C0	C1	C2	C0	C1	C2	C0	C1	C2	
SOD	46.7 ± 1.17d	54.6 ± 0.80c	45.8 ± 1.18d	40.7 ± 0.74e	60.6 ± 0.71b	37.8 ± 0.92f	37.7 ± 2.14f	64.4 ± 1.10a	33.7 ± 1.66g	0.0001
CAT	29.3 ± 0.55d	34.4 ± 0.48c	26.6 ± 0.29e	25.7 ± 0.76e	35.8 ± 0.73b	23.3 ± 0.34f	23.4 ± 0.49f	37.4 ± 0.52a	21.8 ± 0.31g	0.0001
POD	48.2 ± 0.47b	52.7 ± 0.58a	42.8 ± 0.55c	35.3 ± 0.74e	38.1 ± 0.92d	30.2 ± 0.86g	33.9 ± 0.87f	36.1 ± 0.72e	27.0 ± 0.69h	0.0001
APX	38.1 ± 0.38d	44.0 ± 0.80c	35.1 ± 0.72e	32.2 ± 0.88f	48.1 ± 0.80b	29.5 ± 0.45g	27.4 ± 0.67h	49.8 ± 0.40a	22.1 ± 0.91i	0.0001
GPX	74.4 ± 1.12b	79.7 ± 1.02a	71.7 ± 1.10c	65.6 ± 1.22e	68.8 ± 1.10d	60.5 ± 0.60f	57.7 ± 1.09g	63.7 ± 1.67e	43.7 ± 1.67h	0.0001
GSH	143.9 ± 0.93c	156.0 ± 0.64a	141.3 ± 0.40d	141.3 ± 0.57d	146.3 ± 0.61b	136.7 ± 0.92e	136.8 ± 0.85e	140.0 ± 0.89d	128.7 ± 1.36f	0.0001
GSSG	65.1 ± 0.40c	78.7 ± 0.51a	63.1 ± 0.82d	61.0 ± 0.13e	67.8 ± 0.30b	56.7 ± 0.97f	56.0 ± 0.64f	60.1 ± 0.78e	49.2 ± 0.64g	0.0001
AsA	45.2 ± 0.97d	58.5 ± 1.28a	42.1 ± 0.88e	41.3 ± 0.48e	53.6 ± 1.25b	39.1 ± 0.76f	37.9 ± 0.84f	48.3 ± 1.46c	30.7 ± 0.69g	0.0001
GR	39.1 ± 0.76b	45.5 ± 1.36a	29.4 ± 0.92e	33.3 ± 1.26d	36.5 ± 1.23c	22.8 ± 0.90f	27.5 ± 0.79e	32.4 ± 0.55d	12.7 ± 1.76g	0.0001
GST	32.9 ± 0.58b	36.4 ± 0.32a	18.8 ± 0.26f	29.5 ± 0.50c	35.9 ± 0.94a	18.0 ± 0.72f	21.4 ± 0.51e	23.3 ± 0.78d	14.3 ± 0.70g	0.0001
DHAR	22.9 ± 0.75c	26.5 ± 0.42a	21.2 ± 0.21de	21.4 ± 0.46d	24.5 ± 0.42b	19.1 ± 0.14f	19.5 ± 0.43f	20.6 ± 0.30e	15.5 ± 0.25g	0.0001
MDHAR	45.5 ± 0.42b	53.2 ± 0.85a	41.7 ± 0.44d	36.8 ± 0.91e	43.2 ± 1.19c	33.0 ± 0.82f	27.3 ± 0.38g	32.9 ± 0.92f	25.2 ± 0.98h	0.0001
DHA	54.5 ± 1.30b	66.3 ± 0.77a	51.0 ± 1.13c	42.9 ± 1.86d	49.9 ± 1.04c	38.6 ± 1.42e	35.1 ± 0.76f	41.5 ± 1.13d	28.8 ± 1.24g	0.0001
Gly I	0.6 ± 0.30d	1.3 ± 0.21c	2.2 ± 0.075b	0.2 ± 0.12de	1.7 ± 0.29c	3.2 ± 0.16a	0.08±0.04e	2.3 ± 0.26b	3.3 ± 0.40a	0.0001
Gly II	2.3 ± 0.28d	3.4 ± 0.18c	3.8 ± 0.40bc	1.7 ± 0.14de	3.7 ± 0.17bc	4.1 ± 0.81ab	1.2 ± 0.19e	4.2 ± 0.27ab	4.5 ± 0.46a	0.0001

C0; cobalt 0 mg/L (control treatment), C1; cobalt 10 mg/L, and C2; cobalt 20 mg/L. SOD; superoxide dismutase, CAT; catalase, APX; ascorbate peroxidase, GSH; glutathione,GSSG; oxidized glutathione,GPX; glutathione peroxidase,MDHAR; monodehydroascorbate reductase,DHAR; dehydroascorbate reductase, DHA; dehydroascorbate,POD; guaiacol peroxidase,GST; glutathione S-transferase, AsA; ascorbic acid, GR; glutathione reductase, Gly I; Gloxalase I, and Gly II; Gloxalase II.

and 39% reduction, respectively. Nevertheless, the exogenous application of Co (C1) significantly elevated the salinity stress by improving the antioxidant activities (Table 3). Nevertheless, C2 application reduced all (except CAT, Gly I and Gly II) the antioxidants at both the salinity levels.

3.5. Dose-dependent modifications in the metabolic profile by cobalt under salinity stress

The metabolic profile of *Tagestes eracta* L. plants was modulated by the interactive effect of saline stress and cobalt in all the studied organs i.e. roots, leaves and flowers. According to hierarchical clustering, most of

Table 3
The interactive effects of salinity and cobalt on antioxidant activity in leaves of the *Tagestes erecta* L. plants.

Treatments	0 mM Salinity			300 mM Salinity			600 mM Salinity			
	C0	C1	C2	C0	C1	C2	C0	C1	C2	p-value
SOD	38.1 ± 1.66d	45.1 ± 1.69c	18.9 ± 1.10f	27.2 ± 1.56e	49.4 ± 1.48b	16.6 ± 1.08f	25.6 ± 1.18e	51.8 ± 1.35a	12.5 ± 0.76g	0.0001
CAT	23.9 ± 0.81c	27±0.90b	21.4 ± 0.41de	22.1 ± 0.87d	28.4 ± 0.49a	22.1 ± 1.01d	18.5 ± 0.44f	20.5 ± 0.42e	20.5 ± 0.47e	0.0001
POD	44.2 ± 0.76b	46.1 ± 0.67a	38.8 ± 0.89c	33.2 ± 0.40e	37.0 ± 0.51d	29.9 ± 0.98f	23.2 ± 0.753h	28.0 ± 0.82g	20.1 ± 0.68i	0.0001
APX	32.7 ± 0.62d	35.4 ± 0.32c	30.0 ± 0.83e	26.5 ± 0.54f	39.2 ± 0.63b	23.3 ± 0.58g	19.4 ± 0.50h	41.0 ± 0.67a	15.3 ± 0.82i	0.0001
GPX	59.5 ± 0.77b	64.4 ± 1.06a	54.5 ± 1.27c	43.5 ± 1.14f	48.2 ± 1.63e	37.9 ± 1.95h	40.5 ± 0.57g	52.2 ± 1.07d	34.2 ± 1.68i	0.0001
GSH	139.2 ± 0.67b	146.7 ± 1.11a	137.0 ± 0.66c	126.9 ± 0.82e	130.9 ± 0.75d	123.2 ± 0.89f	119.1 ± 1.12g	123.2 ± 0.74f	114.8 ± 1.03h	0.0001
GSSG	53.1 ± 0.92b	57.2 ± 0.45a	51.3 ± 0.84c	48.4 ± 0.46d	49.1 ± 0.76d	45.1 ± 0.65e	32.8 ± 0.68g	36.9 ± 0.96f	29.3 ± 0.70h	0.0001
AsA	35.2 ± 0.71b	40.2 ± 0.42a	32.4 ± 0.48c	29.4 ± 0.58e	30.8 ± 0.62d	24.9 ± 0.63g	23.4 ± 0.51h	28.0 ± 0.75f	18.4 ± 0.64i	0.0001
GR	22.3 ± 0.50c	26.9 ± 0.97a	20.2 ± 0.44d	19.0 ± 0.69e	24.9 ± 0.79b	16.2 ± 0.50f	14.3 ± 0.63g	27.2 ± 0.48a	9.8 ± 0.34h	0.0001
GST	25.0 ± 0.58c	30.3 ± 0.58a	17.2 ± 0.59f	22.4 ± 0.49d	28.0 ± 0.87b	15.3 ± 0.83g	18.1 ± 0.65ef	19.2 ± 0.72e	12.2 ± 0.80h	0.0001
DHAR	19.4 ± 0.47c	22.6 ± 0.46a	19.0 ± 0.18cd	18.5 ± 0.47d	20.2 ± 0.61b	13.5 ± 0.41g	15.4 ± 0.39f	17.5 ± 0.43e	10.4 ± 0.43h	0.0001
MDHAR	37.5 ± 0.40b	43.4 ± 0.37a	35.9 ± 0.77c	25.2 ± 1.02e	28.0 ± 0.97d	20.1 ± 0.74g	19.0 ± 0.63g	23.0 ± 0.8f	15.3 ± 0.71h	0.0001
DHA	47.7 ± 2.09b	56.1 ± 1.51a	43.9 ± 1.37c	34.6 ± 1.06e	38.6 ± 1.19d	26.6 ± 0.99g	22.7 ± 1.033h	28.9 ± 1.06f	17.7 ± 1.13i	0.0001
Gly I	3.5 ± 0.30g	4.7 ± 0.25f	6.9 ± 0.32c	3.0 ± 0.03h	5.7 ± 0.13e	7.5 ± 0.32b	2.6 ± 0.18h	6.2 ± 0.29d	8.3 ± 0.35a	0.0001
Gly II	2.6 ± 0.30cde	2.9 ± 0.79cd	4.7 ± 1.15ab	2.1 ± 0.45de	3.5 ± 0.11bc	5.5 ± 0.83a	1.5 ± 0.55e	4.2 ± 1.15ab	5.1 ± 0.92a	0.0001

C0; cobalt 0 mg/L (control treatment), C1; cobalt 10 mg/L, and C2; cobalt 20 mg/L. SOD; superoxide dismutase, CAT; catalase, APX; ascorbate peroxidase, GSH; glutathione,GSSG; oxidized glutathione,GPX; glutathione peroxidase,MDHAR; monodehydroascorbate reductase,DHAR; dehydroascorbate reductase, DHA; dehydroascorbate,POD; guaiacol peroxidase,GST; glutathione S-transferase, AsA; ascorbic acid, GR; glutathione reductase, Gly I; Gloxalase I, and Gly II; Gloxalase II.

the studied (note that only the most significant metabolites are explained here and the data for all the studied metabolites are provided in tabular form: Table S2) metabolites were influenced by the salt and cobalt treatments. The separated cluster of higher NaCl and cobalt concentrations (600 mM-C2) indicated that the significant influence of the combined effects of these exogenously applied compounds.

3.5.1. *Metabolite profile of roots*

Along with the slight reduction in the sugar levels of roots after 300 mM NaCl-induced saline stress, the application of 600 mM saline stress resulted in the down regulation of glucose, sucrose, maltose, ribose, and fructose by 31, 19, 40, 18, and 14%, respectively. Nevertheless, the exogenous application of Co (C1) substantially improved the studied sugar contents. While C2 had no significant effect when applied under 300 mM saline stress, it reduced the sugar levels in 600 mM NaCl-treated plants.

Similarly, the carotenoids including lutein, lycopene, zeaxanthin, and flavoxanthin were slightly reduced after 300 mM stress, while this reduction was substantial as shown by the 56, 49, 67, and 43% reduction after 600 mM, respectively. However, the application of C1 resulted in the improved of studied carotenoids acids. Interestingly, the C2 application even declined the carotenoids levels in roots at both the salinity levels (Fig. 3).

The terpenoids (brein, lupeol, and erythrodiol) also displayed similar trend as of carotenoids since it was also slightly reduced by 300 mM saline stress, but its reduction was considerable following 600 mM saline stress. Nevertheless, C1 application enhanced the specific terpenoids including brein, lupeol, and erythrodiol up to 2.05-, 1.36-, and 1.56-fold after 600 mM salinity stress compared to untreated marigold plants, respectively. In contrast, the C2 application significantly decreased the terpenoids such as brein, lupeol, and erythrodiol by 28, 21, and 26% under severe saline stress compared to untreated plants, respectively (Fig. 3).

Likewise, others, flavonoids contents (rutin, narcissin, and quercentin) were also down-regulated by both levels of saline stress. Whereas they significantly decreased by 34, 58, and 56% under severe salinity stress. Whereas the flavonoids contents were up-regulated by the foliar application of Co (C1) under both salinity levels. Nonetheless, the C2 application had further reduced the flavonoids contents at both salinity levels (Fig. 3).

Like sugars and linked products, the root organic acids including lactate, citrate, pyruvate, and butyrate were decreased by both the salinity levels especially under 600 mM NaCl-induced saline stress i.e. 19, 26, 36, and 66%, respectively. In contrast, the plants of *Tagestes eracta* L. showed elevation in the organic acids' levels when treated with Co (C1) i.e. 1.14-, 1.25-, 1.23-, and 1.87-fold after 300 mM while 1.1-, 1.29-, 1.48-, and 3.49-fold under600mM salinity stress, respectively (Fig. 3). Interestingly, the C2 application considerably reduced the lactate, citrate, pyruvate, and butyrate contents by 12, 10, 15, and 33% at 600 mM saline stress. While no significant change was noticed in organic acids contents under 300 mM saline stress in marigold roots.

The similar trend was shown by amino acids (proline, alanine, lysine, leucine, serine, and valine) in the roots of *Tagestes eracta* L. plants since those were slightly down-regulated under 300 mM salinity, and this reduction was 25, 24, 66, 48, 39, and 31% under 600 mM saline stress, respectively. In contrast, the exogenous application of Co (C1) improved all the amino acids' levels following both the salinity levels, but C2 application had no such effects.

Similarly, the fatty acids (oleic, stearic, palmitic, lenolic,) contents had significant reduction (38, 45, 24, and 54%) after 600 mM of salinity stress, respectively. Whereas the application of C1 resulted in the improved growth of studied fatty acids. Interestingly, the C2 application declined the fatty acids in roots at both the salinity levels, especially under 600 mM NaCl (Fig. 3).

3.5.2. *Metabolite profile of leaves*

As observed in the roots, the sugar contents in leaves of *Tagestes erecta* L. also declined under both salinity levels. Along with the slight reduction in the sugar (glucose, sucrose, maltose, ribose, and fructose) levels of roots after 300 mM NaCl-induced saline stress, the application of 600 mM saline stress induced the 22, 28, 36, 20, and 22% reduction under 600 mM salinity stress, respectively.

However, C1 treated leaves showed a significant improvement in sugar content at both the salinity levels. C1 increased glucose, sucrose, maltose, ribose, and fructose up to 1.11-, 1.20-, 1.18-, 1.09-, 1.08-, and 1.17-fold under moderate while 1.19-, 1.25-, 1.08-, 1.14-, and 1.09-fold

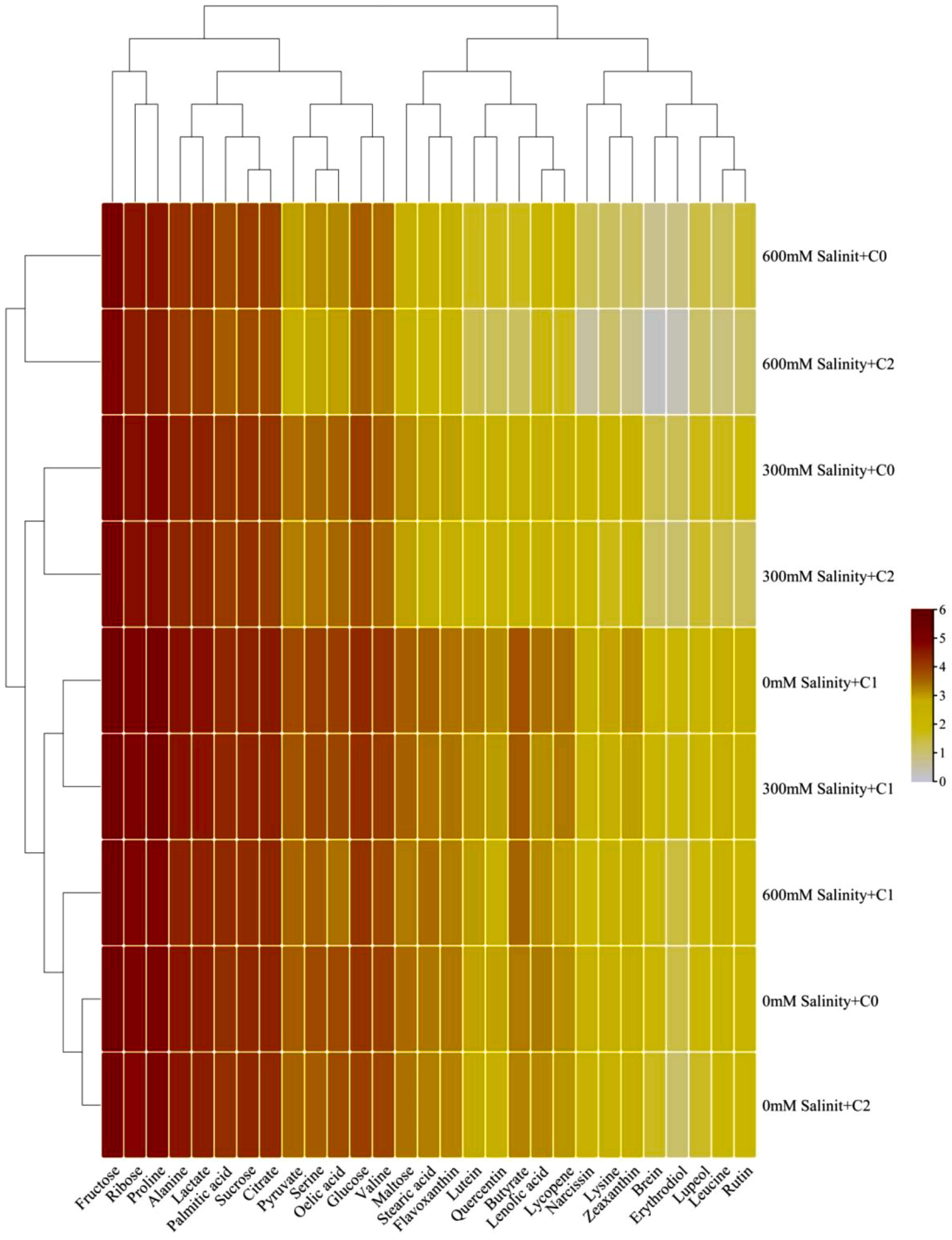

Fig. 3. The synergistic effects of salinity and cobalt on marigolds' root metabolic profile. SS; salinity stress, C0; cobalt 0 mg/L (control treatment), C1; cobalt 10 mg/L, and C2; cobalt 20 mg/L. The heatmap was developed by TbTools software by using log-2 values.

under severe salinity stress compared to untreated plants, respectively. Contrary to C1, C2 application reduced the sugar contents by 7, 1, 18, 8, and 8% at 300 mM whereas, 13, 11, 15, 1, and 8% at 600 mM saline stress compared to untreated plants, respectively (Fig. 4).

Both the salinity stress levels down regulated the carotenoids such as lutein, lycopene, zeaxanthin, and flavoxanthin, While C1 application significantly enhanced carotenoid content at both the salinity levels. Interestingly, the C2 application further reduced the carotenoids levels thereby enhancing the oxidative stress.

Like other metabolites in marigold leaves, terpenoids such as brein, lupeol, and erythrodiol were declined by both salinity levels especially 58, 39, and 55% reduction was noticed after under severe saline stress (600 mM), respectively. Nevertheless, C1 application improved terpenoids content under both saline conditions as compared to untreated plants, respectively. Though, the C2 application decreased the terpenopids concentration when compared with untreated leaves, respectively.

Flavonoids (rutin, narcissin, and quercentin), another important class of metabolites, also faced a substantial reduction saline stress. However, C1 application reduced the salinity stress by improving their contents under both saline conditions. The C2 application showed a different trend though (Fig. 4).

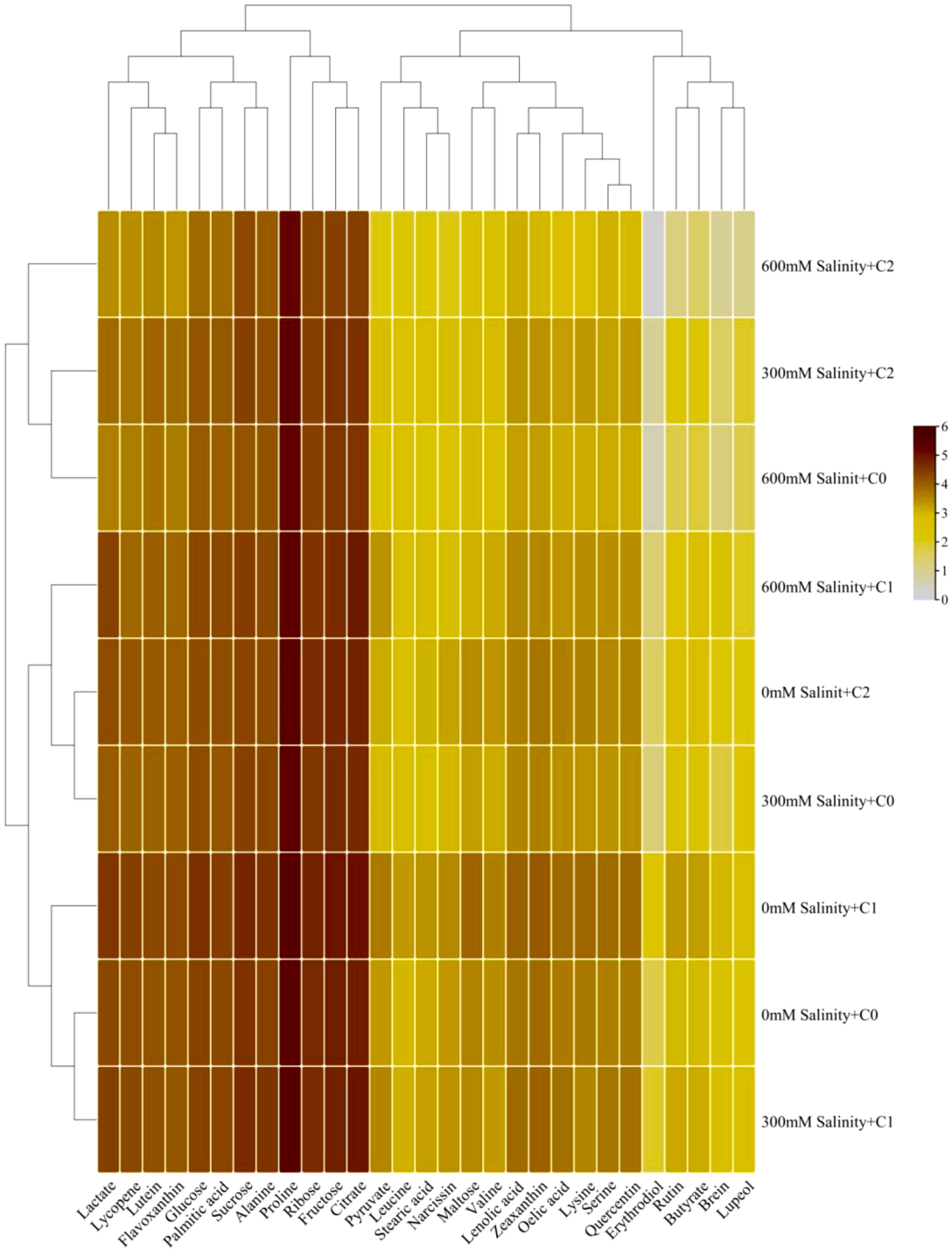

Fig. 4. The synergistic effects of salinity and cobalt on marigolds' leave metabolic profile. SS; salinity stress, C0; cobalt 0 mg/L or (control treatment), C1; cobalt 10 mg/L, and C2; cobalt 20 mg/LSS; salinity stress. The heatmap was developed by TbTools software by using log-2 values.

Organic acids including lactate, citrate, pyruvate, and butyrate also faced a reduction of 17, 11, 24, and 28% at 300 mM whereas, 39, 21, 44, and 53% at 600 mM salinity stress. However, C1 application likewise the other metabolites in marigold leaves, also enhanced lactate, citrate, pyruvate, and butyrate content up to 1.29-, 1.21-, 1.53-, and 1.56-fold under moderate while, 1.71-, 1.29-, 1.88-, and 1.81-foldunder severe salinity stress compared to untreated plants, respectively. Nevertheless, C2 application showed the same reduction pattern of organic acids like other metabolites in marigold leaves and flowers compared to untreated plants (Fig. 4).

Amino acids such as proline, alanine, lysine, leucine, serine, and valine faced a slight reduction following 300 mM, while 13, 16, 33, 14, and 28% decline was noticed in 600 mM saline stress-treated leaves, respectively. Nevertheless, the C1 application enhanced the amino acids under both salinity stress levels compared to untreated plants. Like other metabolites, C2 application substantially reduced amino acids in marigold leaves irrespective of the salinity stress (Fig. 4).

Although 300 mM saline stress-treated plants showed reduction in the contents of various fatty acids (oleic acid, stearic acid, palmitic acid, and lenolic acid), but this reduction was 32, 37, 20, and 28% following

600 mM salinity stress, respectively. However, C1 application reduced salinity stress by improving oleic acid, stearic acid, palmitic acid, and lenolic acid up to 1.29-, 1.45-, 1.17-, and 1.25-fold at 300 mM salinity whereas, 1.21-, 1.37-, 1.25-, and 1.19-fold at 600 mM saline stress compared to untreated plants, respectively. Fascinatingly, the C2 application further reduced fatty acids contents by 9, 13, 3, and 8% at moderate stress while 22, 17, 10, and 11% under severe saline stress compared to untreated plants, respectively (Fig. 4).

3.5.3. Metabolite profile of flowers

Likewise for the roots and leaves, the metabolites in flowers of *Tagestes eracta* L. plants were also affected by the interactive effects of saline stress and Co. Specifically, the sugars (glucose, sucrose, maltose, ribose, and fructose) displayed a slight reduction after 300 mM saline stress level, but this down-regulation was higher following 600 mM saline stress since it reduced the sugar levels by 46, 21, 32, 20, 13, 33, and 52% as compared to control, respectively. Nonetheless, the C1 application substantially up-regulated the levels of those sugars under

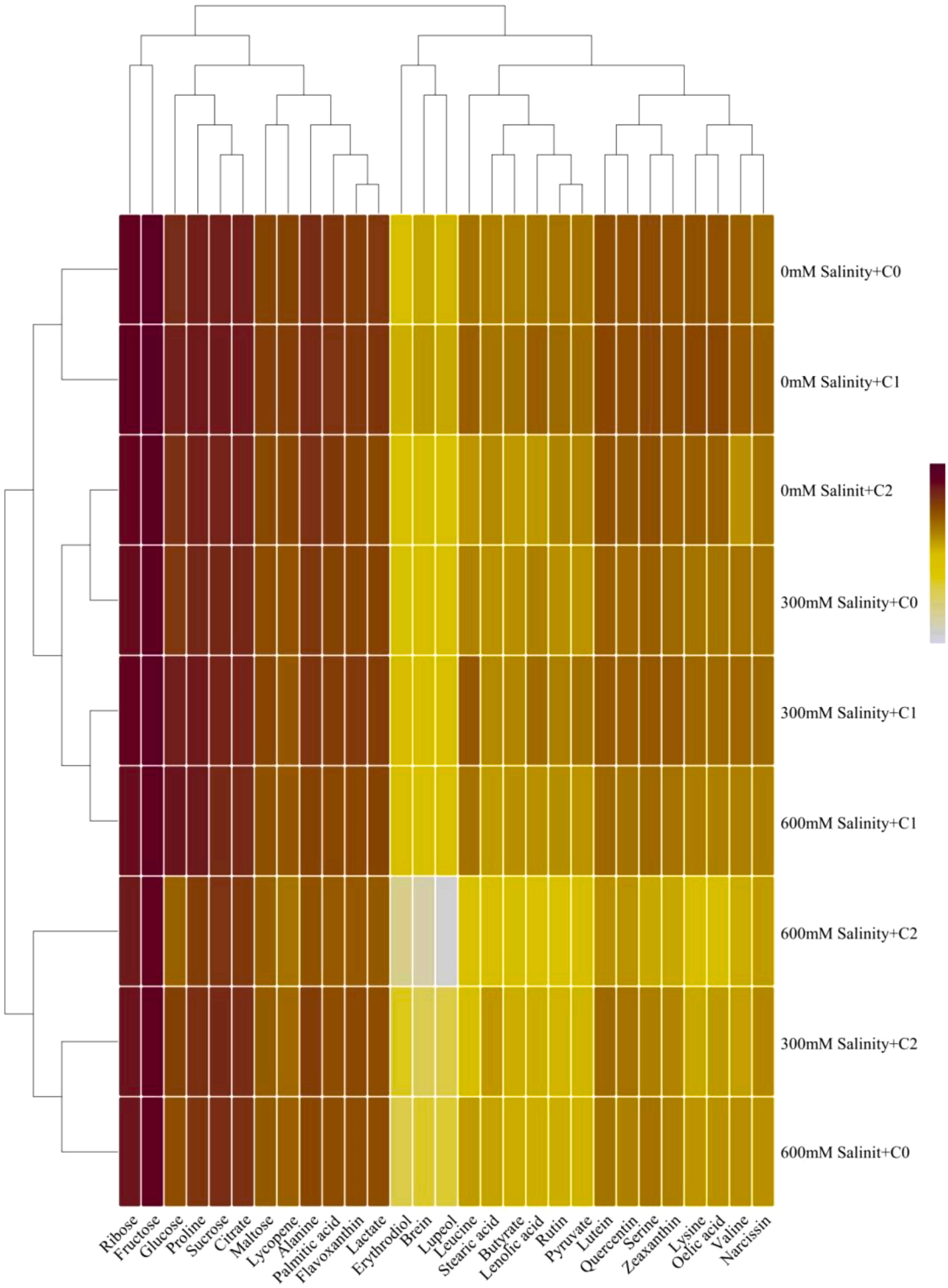

Fig. 5. Salinity and cobalt-dependent alterations in metabolic profile in marigold flowers. SS; salinity stress, C0; cobalt 0 mg/L (control treatment), C1; cobalt 10 mg/L, and C2; cobalt 20 mg/L. The heatmap was developed by TbTools software by using log-2 values.

both saline stress levels. Contrary to C1, the C2 application reduced the sugar contents under the studied saline stress conditions (Fig. 5).

Salinity stress also considerably reduced the carotenoids (lutein, lycopene, zeaxanthin, and flavoxanthin) contents in flowers of *Tagestes eracta* L. plants at both applied concentrations. However, C1 application improved them following both saline conditions. Unlike C1, the C2 application substantially decreased the carotenoids contents by 12, 30, 36, and 8,% at 300 mM salinity, while 34, 27, 44, and 18% after600mM saline stress compared to untreated plants (Fig. 5).

Like other metabolites, terpenoids (brein, lupeol, and erythrodiol,) contents also faced a remarkable reduction under both the salinity levels. In addition to the slight or minor reduction after 300 mM saline level, the 600 mM saline condition resulted in 73, 61, and 45% decline. Nonetheless, the plants treated with C1 application displayed improved terpenoids contents following both saline stress levels. The C2 application has otherwise results though (Fig. 5).

Flavonoids (rutin, narcissin, and quercentin) were also reduced by salinity stress. Whereas the C1 application enhanced their levels under both the studied salinity levels. Unlike C1, the C2 further reduced the flavonoids content by 38, 28, and 17% at moderate while 45, 13, and 22% at severe salinity stress compared to untreated plants, respectively.

Like sugars and their linked products, the organic acids including lactate, citrate, pyruvate, and butyrate were declined by both the applied saline stress levels. Nonetheless, the C1 application mitigated the saline stress by significantly improving the organic acid contents as compared to untreated plants, respectively. Interestingly, the C2 even substantially decreased the various metabolites (lactate, citrate, pyruvate, and butyrate) when the plants were grown under600mM saline stress, respectively (Fig. 5).

Amino acids such as proline, alanine, lysine, leucine, serine, and valine were also declined by the salinity levels. In contrast, the C1 application enhanced the amino acids contents irrespective of the severity of saline stress. Unlike C1, the C2 application further suppressed levels of amino acids in the flowers of *Tagestes eracta* L. plants at both salinity levels (Fig. 5).

Likewise amino acids and other metabolites, the fatty acids showed the same pattern of reduction under both the salinity levels. All the extracted fatty acids i.e. oleic, stearic, palmitic, and lenolic acid were slightly diminished by 300 mM saline stress, but this decline was 62, 38, 33, and 53% after 600 mM salinity stress, respectively. Contrarily, the C1 application enhanced the amino acids under both salinity levels. Unlike C1, the C2 application decreased the fatty acids content by 41, 7, 17, and 46% under moderate saline stress while (54, 40, 19, and 44%) under severe stress, respectively, thereby inducing the greater oxidative stress in *Tagestes eracta* L. plants (Fig. 5).

3.5.4. PCA analysis

The aim of this study was to investigate the impact of cobalt on morphological, physio-biochemical, nutritional, and metabolic responses in *Tagestes erecta* L. under salinity stress. To analyze the data, a Principal Component Analysis (PCA) was performed. The PCA results revealed that the first principal component (PC1) of leaf attributes accounted for 70% of the total variance, while PC2 accounted for 20%. Combined, these two components explained over 90% of the variance. The analysis showed that metabolites such as GSH, LA, valine, proline, pyruvate, sucrose, and serine were closely grouped together under the C1 treatment, as depicted in (Fig 6a). However, in the root metabolic analysis, PCA1 contributed 46% of the variance, while PCA2 contributed 33%. Together, these two components accounted for 79% of the cumulative variance. The PCA results indicated that under the C1 treatment, the metabolites (valine, proline, pyruvate, sucrose, and serine) exhibited a negative relationship with GlyI, GlyII, and MG (Fig 6b).

4. Discussion

4.1. Dose-dependent modulation of the morphological changes by cobalt under salinity stress

The current study set out to examine the vegetative growth, biochemical mechanisms, and metabolic response of African marigold (*Tagestes erecta* L.) as well as the possible benefits of cobalt (Co) application on marigold plant under salinity stress. The current research revealed that salinity stress substantially reduced the vegetative growth of marigold plants. Similar results were found in cowpea and basil plants under high salt concentrations (El-Taher et al., 2021; Rady et al., 2016; Swathy Lekshmi and Jayadev, 2017). Our results were in line with some of the previous studies on *Tagestes erecta, Olea europaea,* and *Nitraria retusa* undergoing drought stress (Boughalleb et al., 2012; Xu and Zhou, 2008). Specifically for our study, Co application improved the morphological traits of the African marigold irrespective of the severity of saline stress. Previous studies on Co application under abiotic stressors were found in accordance with the current results (Gad et al., 2020, 2018). Roots play a multifaceted role in plants such as providing anchorage for the plants , absorbing water and nutrients from soil, and forming symbiotic association with microbes in the rhizosphere (Acosta-Motos et al., 2017; Ma et al., 2022). Exposure to high saline conditions adversely affected anatomical structures, and morphological indices such as RFW, RDW, RL etc. The current results were in

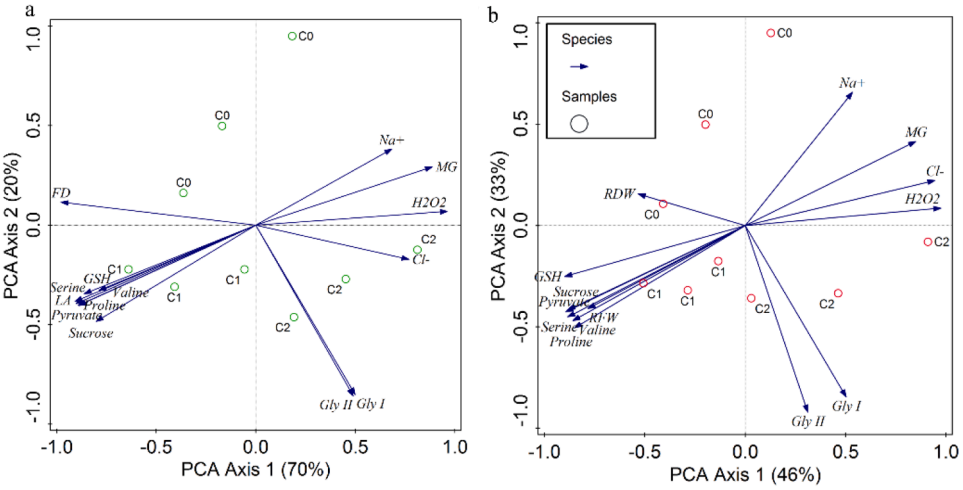

Fig. 6. PCA analysis of marigold flowers metabolites of leaves (a) and roots (b) under salinity stress and cobalt concentations. SS; salinity stress, C0; cobalt 0 mg/L (control treatment), C1; cobalt 10 mg/L, and C2; cobalt 20 mg/L.

agreement with the previous findings (Franco et al., 2011; Gómez-Bellot et al., 2013). Whereas C1-application considerably enhanced the marigold morphological indices by improving root morphological characteristics. These results were found in line with the previous findings on tomato, onion, cucumber, and maize plants under salinity stress (Gad et al., 2020, 2018, 2017; Gad and El–Metwally, 2015).

The reduced leaf area, another important typical stress response, in plants helps to conserve water in leaf tissues by minimizing evapotranspiration and allowing for stomatal closure (Suzuki et al., 2014). These physiological adaptations limits the accumulation of toxic ions in aerial parts of plants by retaining them in roots. Like roots and leaves, stem growth is also associated with the translocation of toxic ions from root to shoot. These studies were found to be consistent with the current results (Acosta-Motos et al., 2017; Isayenkov and Maathuis, 2019).. Previous studies suggested that reduced fresh and/or dry weights (FW and DW) are associated with leaf abscissions and the reduction in leaf number. The current study also revealed that reduced FFW, SFW, RFW, FDW, and RDW in marigold plants directly affecting the LN/plants. However, the increased SDW under severe salinity was linked to higher SDW and RDW in marigold plants, that served to improve the source/-sink relation in plants. These findings were found in congruence with the previous studies (Gad et al., 2017; Isayenkov and Maathuis, 2019; Riyazuddin et al., 2020).

4.2. Salt stress ion-dependent pathway (cytotoxicity)

Salinity reduces plant growth imposes cytotoxicity through ionic and osmotic imbalance, resulting in the higher accumulation of toxic Na^+ reduces water movement through the root with a decrease in hydraulic conductivity reduces overall root growth (Isayenkov and Maathuis, 2019). Excessive salt ions (Na^+ and Cl^-) causes essential nutrients imbalance such as Ca^{2+}, K^+, NO_3^- ratio (Hussain et al., 2022; Mahmud et al., 2017; Munns and Tester, 2008). Briefly, Na^+, a non-essential element for plant growth and development, enters the plants through both ion-dependent or ion-independent channels. However, Na^+ uptake depends on the species type, growth conditions, and salt ion levels in soil and leaf age. Previous studies on potato, rice, and tomato genotypes revealed that Na^+ concentration was significantly high in early maturing variety compared to late maturing genotypes of potato plants (Hakim et al., 2014; Manaa et al., 2011; Shahid et al., 2020). Due to the ionic similarity between Na^+ and K^+, plants are not able to distinguish between them resulting in the over-accumulation of Na^+ions in roots and leaves of plants which ultimately hamper the K^+ absorption in plants. The reduced K^+ level causes nutritional deficiency and restrict plant growth and development at both cellular and entire plant level (Rossi et al., 2015; Wang et al., 2013; de Bang et al., 2021).

Like Na^+, Cl^- also produced negative effects on plant growth and development as it moves along a passive transport system in plants. The excessive Cl^-causes stressed plant to become succulent, which lowers the nitrate reductase (NR) activity; in turn, reduces nitrogen assimilation which drastically reduce the protein synthesis, lowering enzymatic metabolism, decrease the photosynthesis, and overall growth(Gowayed et al., 2017; Tavakoli et al., 2019). Furthermore, salt-tolerant species could retain a higher concentration of K^+ associated with the higher biomass production in plants (Gowayed et al., 2017; Shahid et al., 2020). Calcium (Ca^{2+}), another important element plays an important role in various physiological and biochemical processes such as stomatal regulation, Na^+/Ca^{2+} interaction, molecular signaling, and activation of defense system in plants under abiotic stresses (de Bang et al., 2021; Song et al., 2020). The excessive Na^+ accumulation reduces Ca^{2+} availability to plants resulted in growth inhibition (Shahid et al., 2020). Additionally, Ca^{2+}/Na^+ ratio is crucial in ameliorating the effects of osmotic stress in plants. These evidences are in agreement with the current study (Seifikalhor et al., 2019; Tanveer et al., 2020). On the other hand, Co at low concentration (C1 application) improved osmotic potential in both the above-ground and below-ground parts of salt-stressed plants, decreased the decreased the toxic ion accumulation, buildup of harmful ions, and enhanced K^+ and Ca^{2+} concentrations, which ultimately improved marigold growth. Another recent study reported similar observations with our current results (Alhammad et al., 2023).

4.3. Reactive oxygen species and methylglyoxal production and their scavenging mechanism

Salinity stress also induces another secondary stress response i.e. ROS (O_2^-, H_2O_2, and OH^-) and MG production which leads to oxidative damage in various cellular components by oxidizing lipid, protein and DNA adversely affect vital cellular functions in plants (Dorion et al., 2021). MG is the byproducts of plants' carbohydrate metabolism (Hossain et al. 2021; Saleem et al. 2023), however its' over-accumulation is associated with abiotic stresses such as salinity, whereas reduced O_2leads to production of toxic O_2^-, H_2O_2, and OH^-. Furthermore, the subtle changes in MG levels mediate both dose-dependent toxic and signaling effects in different plant species; the currents results were found inconsistent with these previous studies (Li, 2020; Parvin et al., 2019). Another ROS generation pathway involves Ca^{2+}-regulated Respirator Burst Oxidase Homologs (RBOHs) (Acosta-Motos et al., 2017; Turgut Yiğit et al., 2020). Because of their highly reactive nature they are known to causes several damages to plants at both cellular and molecular levels, while some previous studies revealed that ROS also possess stress signaling effects in response to abiotic stress (Janků et al., 2019; Mansoor et al., 2022; Hossain et al. 2021; Saleem et al. 2023). Over production of ROS and MG in marigold roots and leaves was observed, and C2 application further increased ROS and MG which might be due to the high cobalt toxicity in both roots and the aerial parts of marigold plant. Early senescence reduced morphological growth of roots and leaves might be due to the combined ROS- and MG-induced oxidative stress in marigold plants. In contrast, C1 application substantially reduced the oxidative stress by lowering the ROS and MG levels and increasing antioxidants in both roots and leaves. The ROS-scavenging antioxidants defense system including SOD, CAT, POD, GSH, AsA, GR, GPX, GST, MDHAR, DHAR, and DHA widely studied in different plants under drought (Jiang et al., 2022), salinity (Kaya et al., 2023), cold (Asghar et al., 2022), and heavy metals stress(Kaya et al., 2022) in different plants, respectively. These studies revealed that antioxidants play a crucial role in ROS-scavenging in plants under abiotic stress. Though, only a few studies on the non-essential micro-elements such as Co application as stress protectants confirmed the findings of current study (Brengi et al., 2022; Gad et al., 2020, 2018, 2017; Gad and El–Metwally, 2015).

Furthermore, MG-detoxification mechanism consists of two metallo enzymes named Gly I and Gly II which convert toxic MG to nontoxic pyruvate using GSH as co-factor. In this 2 step MG-detoxification mechanism, Gly I and Gly II uses GSH as cofactor and convert toxic MG into nontoxic and mobilized into pyruvate (Dorion et al., 2021; Li, 2020; Mustafiz et al., 2010). The current results revealed that marigold plants under salinity experienced MG-induced oxidative stress; however, the toxicity symptoms were more visible under combined salinity and high Co stress. Moreover, to the best of our knowledge, no evidences were found on the use of Co as stress-protectant under salinity and MG-detoxification in marigold plants. Though, C1 reduced MG and elevated Gly I and Gly II under both moderate and severe salt stress indicating that Co had significant effects on MG-detoxification like ROS-scavenging. Nevertheless, there is still a lot to understand, especially a specific molecular pathway used by Co and the kinetic movement of Co ions in plants to detoxify MG-induced oxidative stress. The aforementioned evidences revealed that among other antioxidants, GSH pool play a central role in both ROS- and MG-induced oxidative stress in plants under environmental changes (Dorion et al., 2021). Nevertheless, there is currently a research gap in GSH redox state and GSH pool size depletion in ROS- and MG-induced oxidative damage in plants under

various environmental stressors.

4.4. Metabolic response under interactive effect of saline stress and cobalt

Soils with high salinity have deleterious impacts on the plants' metabolic profile resulting in alterations in growth and development at both cellular and entire plant levels (Munns and Gilliham, 2015; Wu et al., 2023). Sugars are the versatile metabolites taking part in regulation of various biochemical and molecular functions such as energy transport, source/sink relationship, osmoregulation and metabolite synthesis (Hennion et al., 2019). Besides this, sucrose synthesis also play crucial role in many metabolic activities including protein storage, cellulose production, and starch accumulation in plants (Niron and Türet, 2022). Furthermore, changes in the metabolism of glucose and sucrose may influence the absorption of Na^{+} in the roots and shoots of plants, resulting in an imbalance of Ca^{2+} and K^{+} ions. This ionic imbalance induces changes in stressed plants' chlorophyll levels, which in turn affect the tricarboxylic acid cycle (TCA cycle) (Niron et al., 2020). Additionally, the metabolism of carbohydrates is linked to the cytosolic glycolytic cycle, which produces pyruvate from reactive MG and powers the Krebs cycle (Dorion et al., 2021).The current study likewise observed similar outcomes, with sugar concentrations in marigold roots, leaves, and flowers drastically decreasing under salt stress. This reduction was likely caused by over-accumulation of toxic salt ions and a substantial reduction in essential nutrients. Although, no studies were found on plants' metabolic response under salinity stress and its mitigation by Co application, however, Co improved nutrient uptake and enhanced proline content in salt-stressed cucumber plant is the indication that carbohydrate metabolism might be the main reason behind this scenario (Gad et al., 2018).

Additionally, flavonoids and carotenoids are associated with plant stress tolerance, ROS scavenging and antioxidant regulation under salinity stress. Furthermore, these metabolites act as coloring compounds in flowers and leaves (Hossain et al., 2022). Similar to sugars, carotenoids and flavonoids also experienced adverse effects under salinity stress, these results ties well with the previous findings (Ali et al., 2009; Khandaker et al., 2008). However, no published studies were found in the literature pertaining to the use of Co under salinity stress and the associated metabolomics of the test species. This study demonstrated that Co application (in a very low concentration) significantly improved the marigold metabolic profile under salinity stress as described by the PCA analysis shown in (Fig. 6). Terpenoids are simple, natural, and undefined plant products often accompanying carbohydrates. Additionally, terpenes in interactions with various transcription factors are associated with apoptosis, cell cycle, and DNA reform due to oxidative imbalance under different abiotic stresses, i.e., salinity stress (Miettinen et al., 2018; Thimmappa et al., 2014). However, current results were go beyond the previous findings, where terpenoids level significantly increased under salinity stress (El-Badri et al., 2021). *Tagestes* sp. has been reported to have a wide range of phytochemicals such as carbohydrates, fatty acids, proteins, organic acids, flavonoids, carotenoids and terpenoids (Shahrbabaki et al., 2013). The orange flower contains a high content of carotenoids including auroxanthin and flavoxanthin (John and Jan, 2017; Zhang et al., 2020). In our current study, Co (300 mM) application considerably improved the marigold's metabolic profile. To the best of our knowledge no studies were found elaborating the Co-induced molecular pathway involved in plants' aromatic compounds production and/or reduction under abiotic stress.

Amino acids play a crucial role in plants' metabolism under salinity stress. They function as election donor in electron transport chain (ECT) and act as osmoregulators in plants subjected to salinity stress (El-Badri et al., 2021). Furthermore, amino acids are the primary factor stimulating lignin biosynthesis (Zhou et al., 2022), which strengthens the cell wall. Certain amino acids such as cysteine, glutamate, and glycine are the essential components of GSH biosynthesis, a key component of ROS- and MG-detoxification mechanisms (Preuss et al., 2014). Contrary to these studies, the current results showed that amino acid concentration significantly reduced under salinity stress, while C1 application substantially improved the amino acid level which might be due to ROS- and MG-induced oxidative stress and higher formation and consumption of GSH. However, more studies are needed to see the clear picture of this phenomenon. Furthermore, reduced morphological growth in marigold plants might be due to alteration in lignin metabolism under salt and high Co stress or their combined effect, Zhou et al. (2022), discovered similar results in *Citrus sinensis* under boron deficiency. Under salinity stress, fatty acids level, a major component of glycerol, drastically changed in marigold roots, leaves and flowers. These results were found in accordance with the previous findings (Benjamin et al., 2019; Kaur et al., 2015). In contrast, the current studies revealed that C1 application played a crucial role elucidating the hazardous effects of salinity on marigold metabolic profile and improved the sugar, amino acids, fatty acids, carotenoids, flavonoids, terpenoids, and organic acids. Whereas Co at higher concentrations (C2 application) restricted marigold's overall growth and development under all the salinity stress. However, there is a lot to understand regarding the kinetic movement of Co ions in plants (inter and intra-cellular), molecular pathways involved in Co functioning in plants under different environmental stresses.

5. Conclusion

The imposition of high salinity had negative effects on the physiology of the African marigold plants by accumulating excessive toxic salt ions within the whole-plant. Concomitantly, the over-accumulation of reactive species such as ROS and MG were observed in the tissues. To cope with higher levels of ROS and MG, plants activated two types of mechanisms i.e. ROS- and MG-detoxification antioxidants defense system such as the AsA-GSH cycle and Glyoxalase system consisting of Gly I and Gly II. Specifically, both defense mechanisms, harnessed the GSH pool as a cofactor in facilitating the detoxification of ROS and MG. At present, there is still a gap in understanding the GSH redox status and GSH pool size depletion in ROS- and MG-induced oxidative stress in plants under abiotic stresses especially salinity. Furthermore, MG is a relatively new concept in plant stress physiology. This trend in understanding plant resilience has emerged in the last few years, but there is still a lot to study; for example, the MG's effects during signalling and the metabolic profiles of plants remained unclear. This study revealed that MG, under moderate stress, delivered some signaling effects on the marigold plants; the MG exerted some cytotoxicity with the combined stress of salinity and high Co application. From the practical perspective, the C1 (Co at 10 mg/L) application delivered positive economic results by improving the morpho-anatomical and physiology of the marigold plants confirming the role of Co as a micro-fertilizer for marigold cut-flowers growing under unfavourable salinity conditions.

CRediT authorship contribution statement

Nadiyah M. Alabdallah: Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Khansa Saleem:** Writing – original draft, Software, Methodology, Data curation. **Aisha Saud Al-Shammari:** Software, Methodology, Formal analysis. **Saleha S. AlZahrani:** Software, Data curation. **Hafiz Hassan Javed:** Methodology, Data curation. **Ali Raza:** Software, Data curation. **Muhammad Ahsan Asghar:** Writing – review & editing, Visualization. **Jean Wan Hong Yong:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interests.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100507.

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