The Effect of Nitric Oxide on Amaranthus Tricolor L. Under Cadmium Stress

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Abstract

This study aimed to appraise the crosstalk between sodium nitroprusside (SNP), as a source of nitric oxide (NO), and cadmium (Cd) toxicity on growth and physiological traits in *Amaranth tricolor* L. by using different multivariate statistical methods. The results showed that growth-related traits of *A. tricolor* were significantly reduced ($p<0.05$) under Cd stress. Contrarily, Cd treatments increased lipid peroxidation and reduced total protein content. Delving on the results of SNP application showed the suitability of its medium level (100 µM) on increasing the growth-related traits and also plant tolerance to Cd stress via lowering the lipid peroxidation and radical molecules production due to the higher activities of superoxide dismutase and catalase. Increasing the amount of Cd in roots and shoots, as the results of Cd treatment, reduced the growth and production of *A. tricolor* plants by high rates (over 50% in 60 mg kg^{-1} Cd level) indicating its susceptibility to high Cd toxicity. Contrarily, treating plants with NO showed no effect on shoot Cd content, while it significantly increased Cd allocation in the root, which might be attributable to the protective effect of NO on Cd toxicity by trapping Cd in the root. Subsequently, the application of a medium level of SNP (around 100 µM) is recommendable for *A. tricolor* plant to overcome the negative impacts of Cd toxicity.

Introduction

Industrialization and urbanization combined with some other humans' activities for harnessing the environment and obtaining their everlasting requirements have led to plenty of challenges regarding heavy metals toxicity. One of these challenges is the contamination of the soil, as a base and medium for crops, that affect crop productivity and food security (Rai et al. 2019). However, crops can use and/or withstand limited contents of different heavy metals, while a higher concentration of these compounds can critically damage the productivity of susceptible crops. This scenario would become even worse by the gradual accumulation of these heavy metals in animals' bodies and then transfer into the human body (Adegbeye et al. 2020). Among numerous heavy metals, because of the high mobility in soil and water and also high ability to form various types, cadmium (Cd) is one of the most important heavy metals involved in different ecosystems through industrial wastes, atmospheric deposition, sludge disposal, fertilizers, etc., (Assaad et al. 2020; Fadel et al. 2020). Additionally, according to Kumar et al. (2019), even a low concentration of Cd could be toxic to living organisms.

Most of the plant species are very sensitive to Cd and show phytotoxic symptoms such as growth retardation, chlorosis, necrosis, brown roots, and even death under low to moderate (5-30 mg kg^{-1}) content of Cd in the shoots (Kabata-Pendias and Mukherjee 2007). At the cellular level, Cd also has numerous negative effects, including membrane distortion, production of toxic metabolites, and reactive oxygen species (ROS) (Genchi et al. 2020). Excess ROS could be cleared by enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and nonenzymatic antioxidants, such as glutathione, proline, and phenolic compounds (Hussain et al. 2019; Marques et al. 2019)

Since nitric oxide (NO) is a diffusible gaseous compound, different sources can provide NO for plants and can be used as exogenous treatments such as sodium nitroprusside (SNP). NO can act as a cellular signaling agent in living organisms (Munawar et al. 2019), interact with phytohormones, and perform some biological functions containing plant growth and development, and lead to altered responses under stress conditions (Sharma et al. 2020). According to Sharma et al. (2020), NO can act as ROS scavengers through triggering the activation of antioxidants in plant cells. However, there are few studies regarding the crosstalk between SNP and plant tolerance to Cd toxicity and therefore it requires more research yet.

Amaranthus tricolor L. normally called edible amaranth (amaranth for short), is a species of Amaranthus genus which is unusually used as an ornamental plant and common leafy vegetable. It grows widely in Southeast Asia, Africa, arid and semiarid areas around the globe (Zhong et al. 2020). However, to the best of our knowledge, there is still no study related to effect of nitric oxide on Cd tolerance of amaranth plant. Therefore, this study aimed to appraise the effects of SNP as a source of nitric oxide on growth and physiological traits in amaranth against Cd toxicity by using different multivariate and advanced statistical methods. As a result, this research may deepen our understanding of the NO-mediated mechanisms to amelioration Cd toxicity in plants particularly with relation to the oxidative defense system.

Materials And Methods

Soil sample

To conduct this research, a sample of loamy-sand soil was collected from agricultural fields located in the College of Agricultural, Shahid Bahonar University, Kerman, Iran. Some of the physical and chemical properties of the soil sample including the soil texture by hydrometric method, organic matter content by wet oxidation method, pH in saturated mud by pH meter, electrical conductivity in the saturated extract by an electrical conductivity meter along with some important mineral elements were measured (Table 1). Before the application of treatments, the content of cadmium in the soil was also measured using Diethylene triamine pentaacetic acid (DTPA) solution according to the method of Lindsay and Norvell (1987) that is presented in Table 1.

Plant material and growth conditions

A greenhouses experiment was carried out in the College of Agriculture, Shahid Bahonar University, Kerman, Iran (geographical coordinates: 30.2520° N, 57.1055° E, altitude: 1,755 m). The condition of the greenhouse was fixed at an average temperature of 25-30 C, relative humidity of 60±2%, average daylight length of 14 hours, and light intensity of 400 microns per centimeter square per second. A three by three (3 × 3) factorial experiment based on a completely randomized design with three replications was used in 2019. The treatments were included three levels of cadmium (0, 30, and 60 mg kg^{-1}) from cadmium nitrate source and three levels of sodium nitroprusside (SNP: 0, 100, and 200 µM). To apply the cadmium

treatment, soil samples (sandy loam), before the cultivation of the plants, were spiked with solutions of Cd (NO3)2·4H2O, mixed well, and aged in the dark for two months.

Amaranthus tricolor L. seeds were prepared from Pakan Seed Company located in Isfahan province of Iran and were sanitized by sodium hypochlorite (5%) for 15 minutes and washed three times by distilled water. Seeds were sown in pots containing a mixture of cocopeat and perlite (1:1). At the four leaves stage seedlings were transferred to pots containing 3 kg propped sandy loam soil, and after 14 days of the transfer, the entire leaves surface was sprayed by desired concentrations of SNP using a hand sprayer. This process of treating plants with SNP was repeated two more times at intervals of 10 days from the first application. The control plants (with no application of SNP) were treated with distilled water and sprayed three times at the same time with SNP applications.

Growth parameters

About 80 days after the starting ofthe experiment, the plants were harvested and samples were washed with sterile distilled water, and then, samples were dried in an oven instrument at 70 °C for 48 hours; following, the dry weight of roots and shoots were calculated. The length of roots and shoots were also measured using a digital caliper instrument.

Carotenoid

Carotenoid content was measured following the method of Lichtenthaler (1987) with using 80% (w/v) acetone.

Total phenolic content

Total phenolic content was determined with Folin-Ciocalteu reagent using gallic acid as a standard phenolic compound and was expressed as $mg \cdot g^{-1}$ FW (Folin and Ciocalteu 1927)

Malondialdehyde (MDA) and Hydrogen peroxide (H_2O_2)

MDA content was measured by method Heath and Packer (1969) by using thiobarbituric acid in 10% trichloroacetic acid. An extinction coefficient equal to 155 mM^{-1} cm^{-1} was used to determine MDA concentration, and the results were calculated as nanomole per gram of fresh weight. H_2O_2 was also determined according to Velikova et al. (2000), which trichloroacetic acid, potassium phosphate buffer (pH 7.0), and 1 mL of 1 M potassium iodide were used for the determination of H_2O_2.

Protein and antioxidants enzymes

Bradford method (1976) was used in order to determine leaf protein content. CAT was assayed by the method of Aebi (1984), 0.5 ml of 0.2 M H2O2 in 10-mM phosphate buffer add to the enzyme extract. CAT activity was measured by the reduction in absorbance of H2O2 at 240 nm for 180s. CAT activity was expressed as units (μmol of H2O2 consumed per minute) per mg of protein. SOD activity was estimated

by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) by the enzyme. 2 mM riboflavin (0.1 mL) was added in 3 mL of the reaction mixture (13.33 mM methionine, 75 µM NBT, 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate, 0.1 mL enzyme extract) and placing the tubes under two 15 W fluorescent lamps for 15 min to start the reaction. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme. SOD activity was expressed as units per mg protein^{-1} min^{-1}. Peroxidase activity (POD) was determined by Hemeda and Klein (1990) method. One ml of reaction mixture contained 13 mM guaiacol, 5 mM H2O2, and 50 mM potassium phosphate buffer (pH 7). An increase in absorbance due to oxidation of guaiacol (extinction coefficient: 26.6 mM·cm-1) was monitored at 470 nm for a minute. Peroxidase activity was expressed as units (µmol guaiacol oxidized per minute) per mg protein. and polyphenol oxidase was measured by method of Arnon (1949).

Cadmium content

At the end of the experiment, the amount of cadmium in the root, shoot, and the soil samples were measured. To measure Cd in plant materials, the roots and shoots were separated, washed cleanly with deionized water, and dried at 62 °C for 72 h. About 50-100 mg of each sample was digested in 2 ml of a 4:1 mixture of HNO3 (65%) and HCL (37%), in Teflon bombs at 140°C for 7h and then their total volume was adjusted to 10ml with demineralized water. Concentrations of Cd were measured using flame atomic absorption spectrophotometry (Varian Spectra AA 220). Total uptake was calculated on a total plant dry weight basis as described by Villafort Carvalho et al. (2013). The amount of cadmium in shoots and roots was reported based on dry weight used for extraction as mg kg^{-1}. After measuring the amount of Cd in shoot and root, the following formula was used for estimating the ratio of translocation factors (TF) (Nahar et al. 2016):

$$\mathrm{TF} = \frac{\text{Cadmium content in shoot}}{\text{Cadmium content in root}}$$

Statistical analysis

The collected data were subjected to two-way ANOVA (analysis of variance) where cadmium and SNP applications were used as a source of variations (experimental factors). Alongside calculating ANOVA separately for all traits, multivariate analysis of variance (MANOVA) was carried out for considering the overall effects of experimental factors on measured traits all at once. Mean comparison according to the least significant difference (LSD) at 0.05 level of probability was carried out next to the ANOVA using “proc glm” of SAS 9.4 statistical software. Residuals of the ANOVA model were extracted for normality tests based on Shapiro–Wilk, and Q-Q-plot methods in SAS software using “proc univariate”. Bar plot in the Excel package (Office 2019) was used for plotting the corresponding figures of the treatments’ mean comparisons. Paired linear correlation based on the Pearson method was used in R 3.5 software for drawing a correlation plot using package “ggcorrplot”. To find out about the structural association of

traits and also the combinational influences of each applied treatments, biplot analysis based on the first two components (Dim1 and Dim2) extracted from principal component analysis alongside heatmap plot were applied using "factoextra" and "gplots" packages, respectively, in R 3.5 software.

Results

Analysis of variance was carried out for every measured trait i.e. shoot dry weight, root dry weight, shoot length, root length, the content of hydrogen peroxide, the content of malondialdehyde, the total content of protein, the content of carotenoid, the total content of phenol, catalase activity, superoxide dismutase activity, peroxidase activity, polyphenol oxidase activity, the content of cadmium in the shoot, the content of cadmium in the root, the content of cadmium in soil, and TF (data is not presented) of edible amaranth and the results showed a significant effect ($P<0.05$) of cadmium treatment on all mentioned traits. ANOVA results also showed a significant effect ($P<0.05$) of SNP treatment for all measured traits except the content cadmium content in the shoot. According to the ANOVA results, the interaction effect between cadmium and SNP did not show a statistically significant effect on shoot length, root length, malondialdehyde content, carotenoid content, total phenol content, and cadmium content in the shoot, while its effect on the rest of the measured traits was significant. MANOVA result indicated significant effects of cadmium, SNP, and cadmium by SNP interaction on all of the measured traits together at $p<0.01$ (Table 2). Following the analysis of variance, mean comparison based on the least significant difference ($p<0.05$) alongside some advanced multivariate statistical analyses (correlation plot, principal component analysis, biplot, and heatmap) were conducted and the following results were obtained.

Morphology and Growth traits

Results of mean comparison for shoot dry weight, root dry weight, shoot length, and root length of amaranth under the application of cadmium and SNP are presented in Fig. 1a, 1b, 1c, and 1d, respectively. Shoot dry weight responded negatively to cadmium treatment; in other words, the higher the applied levels of cadmium applied, the lower the dry weight of shoot reached. Under no cadmium application, treating the amaranth plants with either 0 (control) or 100 µM SNP resulted in a higher shoot dry weight than the highest level of SNP (200 µM). Under 30 and 60 mg kg^{-1} application of cadmium, no statistically significant difference was found between 0 and 200 µM SNP application, but applying 100 µM SNP resulted in significantly higher shoot dry weight (Fig. 1a).

The highest root dry weight was observed in plants treated with no cadmium application (control) under the application of either 100 µM SNP and 0 µM SNP (control). The lowest mean value for root dry weight was obtained in plants treated with the highest level of cadmium (60 mg kg^{-1}) and no application of SNP, which caused about a 60% decline in root dry weight compared to control, (Fig. 1b). A decreasing trend in response to cadmium levels was observed for root dry weight under all levels of SNP. Medium level (100 µM) application of SNP brought about higher shoot weight under eighter medium (30 mg kg^{-1}) or the highest (60 mg kg^{-1}) applied level of cadmium.

A decreasing trend in shoot length was found in response to the application of cadmium (Fig. 1c). However, the application of the eighter medium or the highest level of SNP resulted in higher shoot length than the control. The highest shoot length was observed in plants treated with both 100 or 200 µM and no cadmium application. No application of SNP and applying 60 mg kg^{-1} Cd resulted in the lowest shoot length, about a 65% decrease in comparison with the non-treatment plant.

The response of the root length of amaranth plants to cadmium application was changed by SNP application. The highest root length was shared among the plants treated with all three levels of SNP under no application of cadmium without any significant difference. Meanwhile, their difference with plants treated with 100 µM SNP under 60 mg kg^{-1} Cd was not statistically significant (Fig.1d). The lowest length of amaranth's root was recorded for the plants treated with the highest level of Cd under no application of SNP, which showed over 40% decrease compared to control treatment.

Correlation plot (Fig.2) showed high and significant associations between all pairs of growth traits. Regarding principal component analysis, scree plot (Fig. 3a) indicated that the first and second components (PC1 and PC2) explained over 85% of variability among all measured traits. Biplot verified the results of the correlation plot and showed obtuse angles between growth traits and low distances of these traits from one another (Fig. 3b). Similarly, heatmap showed that growth traits could be considered as one cluster alongside total protein content (Fig. 4). Also, treatments were clustered into three different groups: the first group contained all three levels of SNP under no application of Cd, the second and the third groups have similarly consisted of all three levels of SNP under medium and the highest levels of Cd, respectively. More consideration over the heatmap verified the negatives effect of Cd application on growth traits as the color became darker (dark red) by application of higher Cd concentration (Fig. 4). Under no Cd and medium Cd treatments, the application of 100 µM SNP showed lighter color than other treatments indicating positive effects of applying medium content of SNP on these growth-related features.

Biochemical and antioxidant traits

Hydrogen peroxide showed an increasing trend in response to an increase in the level of Cd under all three levels of SNP (Fig. 5a). However, the application of SNP showed a decreasing impact on H_2O_2 through which the medium level showed the highest effectiveness and the lowest H_2O_2 content. Accordingly, the lowest level of H_2O_2 was achieved in no Cd and 100 µM SNP treatment, whereas, the highest H_2O_2 content was recorded for plants treated with 60 mg kg^{-1} Cd under no SNP. Similarly, MDA content was the highest for plants treated with 60 mg kg^{-1} Cd under either no application of SNP that showed no significant difference with plants treated with 60 mg kg^{-1} Cd under 200 µM SNP (Fig. 5b). However, the lowest content of MDA was shared among plants treated with no Cd under all three levels of SNP.

The results of the mean comparison for the interaction between Cd and SNP regarding carotenoid content are presented in Table 3. The results showed no significant differences among all three levels of

SNP under either no application or 60 mg kg^{-1} application of Cd. The medium concentration of Cd, however, showed higher content of carotenoid than the other two levels. Plants treated with 100 µM SNP under 30 mg kg^{-1} Cd obtained the highest carotenoid content.

Total protein content was negatively affected by Cd application where the higher concentration of Cd led to a lower concentration of total protein (Fig. 5c). Meanwhile, the application of SNP relatively induced higher total protein content in amaranth. Total phenol content increased by the positive slope in response to the application of Cd (Fig. 5d). Delve over the impacts of SNP application on phenol showed that increasing the concentration of SNP up to 100 µM effectively increased total phenol content, while higher SNP concentration showed negative impacts and dramatically decreased phenol content.

The results of the mean comparison for the enzymatic activity of catalase, SOD, POD, and polyphenol oxidase are presented in Fig. 6a, 6b, 6c, and 6d, respectively. Cd treatment led to higher activity for catalase, SOD, and POD; the higher the level of Cd applied, the higher the activities of these enzymes reached. Additionally, treating with SNP caused plants to increase their enzymatic activities for catalase, SOD, and POD; however, applying 100 µM had higher impacts on these enzymes than applying 200 µM. On the contrary, the application of SNP led to a decrease in the activity of polyphenol oxidase (Fig. 6d). Under all three levels of SNP, application of medium level of Cd (30 mg kg^{-1}) resulted in higher polyphenol oxidase activity, nonetheless, application of the highest level of Cd (60 mg kg^{-1}) led to lower activity of polyphenol oxidase in compare with control treatment.

Catalase, POD, and SOD showed positive and significant correlations with one another; polyphenol oxidase, on the other side, showed no significant association with them. Carotenoid and protein showed either negative or no significant correlation with enzymatic antioxidants. Aside from polyphenol oxidase, total phenol content had a positive correlation with antioxidants. The correlation of H_2O_2, MDA, enzymatic antioxidants, and total phenol contents paired with growth traits were negative and significant. The correlation of protein content paired with growth-related traits was significantly positive, but carotenoid content had no significant correlation with growth attributes (Fig. 2). Biplot verified the results of correlation analysis related to biochemical features. Accordingly, carotenoid content and polyphenol oxidase were placed on two arrows in two reverse directions indicating a high negative correlation between these two traits. Polyphenol oxidase and carotenoid contents showed angles relatively equal to 90˚ indicating no significant association with other attributes (Fig. 3b). In concordance with this result, carotenoid and polyphenol oxidase were placed in two different clusters in which there were no other traits contained based on heatmap analysis. SOD, catalase, and total phenol content were grouped as one cluster. Protein, however, was contained in the same cluster where the growth-related traits were included (Fig. 4).

Cadmium content

Treating amaranth plants with Cd and SNP led to an increase in the ratio of TF. The content of Cd in shoot and root of amaranth plants along with Cd content in were measured and the results of their mean

comparison are provided in Table 3. The lowest content of Cd for shoot, root, and soil was recorded for control plants without the application of Cd. Under control treatment, there were no statistically significant differences among SNP levels related to Cd content in shoot, root, and soil. The highest content of Cd in shoot, root, and soil was observed in plants treated with the highest level of Cd (60 mg kg^{-1}). Under each separate level of 30 and 60 mg kg^{-1} Cd application, there were no significant differences among all three levels of SNP regarding the content of Cd in shoot and root. On the other hand, the application of 100 µM under 60 mg kg^{-1} Cd treatment showed lower Cd content in soil comparing with control (no SNP application) and 200 µM SNP. The results of the mean comparison for TF showed that the highest TF ratio was obtained under the application of 60 mg kg^{-1} Cd and 200 µM SNP. The lowest content of TF was observed in the application of neither Cd nor SNP (Table 3).

The correlations between all paired of attributes regarding Cd content in shoot, root, and soil were positively significant; TF, on the other side, showed a low positive correlation with Cd in shoot and root and no correlation with Cd content in soil (Fig.2). Cd content in plants and soils showed negative correlations with growth traits and positive correlations with enzymatic antioxidants. Similar results were obtained in biplot analysis (Fig. 3b) in which Cd content in shoot, root, and soil showed a negative association with growth traits and positive association with CAT, SOD, and POD. Total phenol and protein contents showed a positive and negative association with Cd content related traits, respectively. Heatmap showed that Cd content in shoot, root, and soil, along with TF, H_2O_2, and MDA were grouped as one cluster (Fig. 4).

Discussion

Cadmium treatments significantly decreased all growth-related traits i.e. shoot dry weight, root dry weight, shoot length, and root length. The highest Cd level caused over 50% inhibition of plant growth. The results indicated that increasing the levels of SNP, as a nitric oxide source, up to 100 µM had a positive effect on shoot dry weight, root dry weight, and shoot length, but more increase in the concentration of SNP led to a decrease these traits. Root length, however, showed an increased response to a higher concentration of SNP, but there was not a statistically significant difference between 100 µM and 200 µM regarding root length. These results indicated that the application of SNP at the concentration of about 100 µM is recommendable for the cultivation of amaranth in Cd contaminated soils. Moreover, according to the results of heatmap and biplot, under no application of Cd, the application of 100 µM SNP showed a great association with growth-related traits (with a short distance) indicating the effectiveness of SNP on the productivity of this species even under no stress situations.

Based on a study by Benavides et al. (2005), Cd comminuted areas of agricultural fields surpassed the acceptable limit, about 7% of samples, and eventually, Cd ranked as one of the top seven mineral contaminants in agricultural areas. The limited level of soil Cd in soil that higher than that could be considered as contaminated soil and damage the agricultural production is generally around 1 mg kg^{-1} (Shanmugaraj et al. 2019), which is lower than the level of Cd applied in this research. The content of Cd

around the root area could be readily absorbed by plants' root, transport to xylem, and then reach leaves and other plant tissues and organelles (Bali et al. 2019). According to Ismael et al. (2019), the main negative impacts of Cd toxicity on plants is the impairment of stomatal activity and causing problems in the transpiration rate together with water imbalance and interfere in nutrients uptake. Lower transpiration rate, immobility of water molecules, and a lower rate of essential nutrients uptake by root leads to a reduction in photosynthesis and therefore lower plant growth and production. By the negative and damaging effects of Cd on growth and plant production in the current study, some other studies regarding *Amaranthus hypochondriacus* L. (Zou et al. 2020), *Amaranthus mangostanus* (Chi et al. 2019), and *Amaranthus spinosus* (Huang et al. 2019) plants have been also published.

Sodium nitroprusside act as a donor of NO in soil and plant materials. This compound has been widely used in several studies to appraise the regulatory acts of NO (Kaya et al. 2020). It has been reported that NO, which is available in gaseous form in nature, can stimulate several biochemical and physiological pathways in plants (Kaya et al. 2019). Based on various studies, NO can regulate some plant defenses against Cd stress and stimulates tolerance in plants (Kaya et al. 2020; Shanmugaraj et al. 2019; Subiramani et al. 2019; Zou et al. 2020). However, there is no report, to the best of our knowledge, regarding the effect of NO on *A. tricolor* under Cd toxicity. The investigation regarding the impacts of NO on heavy metal toxicity in different species, according to Zhu et al. (2020), is still a research interest. Zhu et al. (2020) reported that NO can reduce the damaging effects of other free radicals generated under Cd toxicity in tall fescue plants leading them to tolerant oxidative stress caused by Cd toxicity and stimulated plants to produce much mass in compare to control condition. Conversely, a high concentration of NO may lead to higher oxidative stress on account of its oxidant free radical form, such as what was observed in our study. Plants root, on the other hand, is the most stimulated organ of plants and therefore, the root length in the current study was not decreased by a high level of SNP treatment. In concordance with our results regarding mitigating effects of NO, Kaya et al. (2019) and Kaya et al. (2020) reported higher growth rate and dry matters for plants treated with NO sources than control treatment in wheat and pepper, respectively.

Lipid peroxidation under Cd toxicity was increased: under all SNP treatment levels, a higher concentration of Cd caused more production of MDA. Application of 200 µM SNP along with the control treatment showed a similar trend in response to Cd levels, but treating the plant with 100 µM SNP led to a significant lower MDA content than the other two SNP treatments. Similarly, the production of H_2O_2 dramatically increased in response to the higher concentration of Cd, but applying 100 µM SNP showed significantly less production in comparison with two other levels of SNP treatments. The high concentration of Cd in soil results in an uptake competition between nutritional elements such as iron, zinc, potassium, etc. Subsequently, the competition among nutritional elements leads to a low concentration of essential nutrients and nutritional shortage stress. Following that, nutritional stress causes oxidative stress on account of higher production of free radicals such as H_2O_2. Higher content of free radicals and oxidant compounds is bringing about damages to cell walls and lipid peroxidation that is measured by the content of MDA. Under oxidative stress conditions, NO has been found to have an

ability in adjusting several metabolic pathways by playing a crucial role as a signaling molecule in plants (Prakash et al., 2019). Some signaling molecules are mediating the relationship between tolerance-related genes and their expression through other regulatory genes and pathways. NO, at its accurate concentration, is capable to be a proper signaling molecule to regulate this relationship, however, its high concentration might lead to more oxidative stress in the cells owing to its radical form. (Fancy et al. 2017). Mitigation of Cd toxicity and decreasing lipid peroxidation via exogenous application of SNP was also reported by other studies (Kaya et al. 2019; Sharma et al. 2020; Zhu et al. 2020). Kaya et al. (2019) reported significantly higher production of H_2O_2 and MDA in wheat plants treated with Cd in comparison with the control treatment. Their results also indicated a significant effect of NO exogenous application on lowering the lipid peroxidation rate.

Total protein content showed a negative response to the application of Cd. Under low Cd toxicity (30 mg kg^{-1} Cd), the application of SNP at both concentrations showed to be a reason for increasing protein content comparing with control; however, under sever Cd toxicity (60 mg kg^{-1} Cd), there were not any differences among SNP treatment levels. On the other hand, total phenol content showed a positive inclination toward the higher level of Cd treatment. Application of 100 µM SNP led to more increase in phenol content under all Cd levels in comparison with control and 200 µM SNP application. Carotenoid content showed to be the highest in plants treated with 30 mg kg^{-1} Cd, and the application of SNP, especially at the medium level (100 µM) significantly increased the rate of carotenoid under low Cd toxicity (30 mg kg^{-1} Cd) condition. One of the main reasons for decreasing the content of protein under Cd stress might be due to the interference caused by Cd in photosynthetic related pathways such as Calvin cycle and hindering the enzymatic activity of those who are active in polypeptide production and decomposition (Ismael et al. 2019). Nutritional element imbalance uptake, on the other hand, is leading to low availability of elements crucial for some peptides and protein formation. Additionally, the oxidative stress occurring as a result of Cd toxicity can bring about various damages to cell compartments and compounds such as DNA, RNA, and different types of proteins (Riasat et al. 2019). In concordance with our results, Huang et al. (2019) reported lower protein content in *Amaranthus spinosus* plants treated with Cd. Meanwhile, Subiramani et al. (2019), in agreement with our results, corroborated the influence of SNP on inducing higher contents of total phenols and carotenoid. Carotenoid, aside from its role in photosynthesis pathways and photo-absorption activity, has been distinguished as a non-enzymatic antioxidants act as a shield against high damages of free radical produced under oxidative stress condition. Therefore, applying medium level (around 100 µM) of SNP could be an appropriate strategy to maintain plant production through different regulation effects especially increasing the availability of carotenoid.

Catalase, SOD, and POD activities as enzymatic antioxidants were induced as the concentration of Cd increased. Also, the medium level of SNP had the greatest effect on inducing higher activity of these enzymes. On the other hand, polyphenol oxidase increased in response to the application of 30 mg kg^{-1} Cd while decreased in response to the higher Cd level (60 mg kg^{-1}). As was mentioned earlier, Cd toxicity causes oxidative stress leading to the higher generation of detrimental reactive oxygen species (ROS)

which subsequently damages the plant tissues (Hossain et al. 2015). Plants develop various mechanisms to cope with the damaging effects of high ROS contents mainly by inducing higher activities and also a greater level of various antioxidants (Saed-Moucheshi et al. 2014). Accordingly, higher activity of enzymatic antioxidants means higher tolerance against stress conditions. Since the medium level application of SNP caused the highest activity in mentioned enzymatic antioxidants, it could be a reason for higher tolerance and higher growth (shoot and root dry weights alongside shoot and root length) of amaranth plants. Rizwan et al. (2018) reported a positive role of NO against heavy metal toxicity in rice seedlings through regulation and the induction of antioxidant defense systems and reduction of oxidative damages by free radicals.

Cd content in shoot increased dramatically as the result of Cd treatment; nevertheless, the differences in Cd content among all three levels of SNP treatment were not significant. Similarly, the measured amount of Cd in the root of amaranth plants showed an increase in the amount of Cd as a feedback response to the higher level application of Cd. Unlike shoot, SNP treatment increased Cd root content and accumulated more Cd under the application of 100 µM SNP than the highest SNP level (200 µM) approving the aptness of medium SNP level to donate more tolerance to amaranth plants against Cd toxicity by trapping Cd in the root. TF ratio, which indicates the ratio of Cd in shoot per root, was mostly affected by cadmium root and it was higher in plants treated with Cd, and SNP application, especially at the medium level, caused an increase in its ration. This result indicates that one of probably the key reasons for the effectiveness of SNP in making amaranth plants more tolerant to Cd toxicity and it helps plants to accumulate Cd molecules in the root, through which, plants can regulate the osmotic potential of root cells and uptake more water and/or required elements. In accordance with these results, Zhoe et al. (2016) showed NO enhanced Cd content in root and decreased Cd content in the shoot of *Typha angustifolia*. On the contrary, some studies such as Subiramani et al. (2019) reported lower content of Cd in either root or shoot of the *Canscora diffusa* plants treated with SNP. Our result indicated that Cd treatment increased dramatically Cd content in both tissues of *A. tricolor*, however, based on dramatic growth reduction under the high level of Cd exposure, this species may not be tolerant enough to high Cd toxicity. Contrary to our result, Watanabe et al. (2009) revealed that *Amaranthus tricolor* L. accumulate a high amount of Cd without toxic effect on growth. In addition, Chi et al. (2019) reported the accumulation of Cd in *Amaranthus mangostanus* L. plants which might be a verification of the ability in Amaranthaceae family to lessen the Cd toxicity of the soil. Moreover, under the Cd toxicity in the current study, the application of medium level of SNP (100 µM) showed less content of Cd in the soil than its highest level demonstrating its suitability for being applied as the treatment in amaranth plants cultivated in Cd contaminated areas.

Conclusion

Cd treatments caused a reduction in growth-related traits i.e. shoot and root dry weight and also shoot and root length and under severe Cd toxicity. *A. tricolor* showed over 50% reduction in growth and production under Cd toxicity. Also, Cd treatments increased lipid peroxidation, enzymatic antioxidant activity, total phenol, and carotenoid contents, but it reduced the amount of total protein in the edible

amaranth plants. Delve over the results regarding SNP application showed the suitability of its medium level (100 μM) on increasing the growth-related traits. This level of SNP was also able to increase the activity of superoxide dismutase and catalase leading to lower lipid peroxidation. The suitability of SNP application is probably due to the ability of NO in playing a role as an active signaling molecule mediating the expression of stress-responsive genes. Additionally, the available sodium in SNP might act as a balancing agent regarding the uptake of essential nutrients and adjusting the negative effect of Cd that causes a competition related to nutritional elements uptake. Reduced plant growth by increasing Cd content in roots and shoots indicates that *A. tricolor* is sensitive to high cadmium toxicity. In addition, shoot Cd content was not affected by NO, while the Cd allocation was increased in the root, which can demonstrate the protective impact of NO on Cd toxicity by trapping Cd in the root. Finally, the application of medium levels of SNP (about 100 μM in based on the results of the current study) is recommendable to increase the tolerance of *A. tricolor* to Cd toxicity in contaminated fields.

Abbreviations

Cd-soil: cadmium content in soil, Cd-rt: cadmium content in shoot, TF; translocation factors, T-phen: total phenol, Cart: carotenoid content,. Rl: root length, Shl: shoot length, RDW: root dry weight. SDW: shoot dry weight, H2O2: hydrogen peroxidase, MDA: malondialdehyde SOD: superoxide dismutase, CAT: catalyze, POD: peroxidase. SNP: sodium nitroprusside.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

There is no competing interest or conflict of interest in this study.

Authors' contributions

In this manuscript, FB, carried out the experiment and performed main parts in the data gathering, analyzing, and writing the manuscript. MA, cooperated in performing the experiment and data gathering

along with the manuscript writing. VRS, and MM, edited the manuscript scientifically. The grammatical corrections and structural editing of the final manuscript were carried out by FB and VRS.

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Consent to participate

All authors consented to the publication laws

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Tables

Table 1. Physical and chemical properties of soil sample prior to application of treatments

Mn	Cd	Zn	Cu	Fe	K	P	Ec	pH	Texture
(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(dS m^{-1})		
1.1	0.4	0.53	0.48	2.4	209	11.1	2.8	8	Sandy-loam

Table 2. Multivariate analysis of variance for cadmium, SNP and their interaction regarding measured traits.

source	df	statistic	F value	Pr(>F)	significance
Cadmium (Cd)	2	1.9961	56.922	0.000635	***
SNP	2	1.9912	25.222	0.00313	**
Cd × SNP	4	3.8609	6.168	0.000104	***

** and *** indicate significance levels at 0.01 and 0.001 of probability, respectively.

Table 3. Mean comparison of cadmium by SNP for content of cadmium in root and soil, TF, and carotenoid content.

Cadmium (mg. kg^{-1})	SNP (µM)	Carotenoid (mg. g^{-1} FW)	TF	Cd in shoot (mg.kg DW)	Cd in root (mg. Kg^{-1} DW)	Cd in soil after harvest (mg.kg^{-1})
0	0	0.10±0.01c	0.26±0.03g	0.36±0.00c	1.25±0.12e	0e
0	100	0.10±0.02c	0.29±0.00f	0.34±0.02c	1.3±0.08e	0e
0	200	0.09±0.00c	0.3±0.04ef	0.41±0.06c	1.25±0.07e	0e
30	0	0.13±0.00ab	0.32±0.00e	40.6±2.01b	90.32±5.31d	10.9±0.81d
30	100	0.15±0.00a	0.36±0.03cd	40.87±2.87b	138.4±7.25c	8.01±0.17ed
30	200	0.11±0.00bc	0.35±0.04d	35.18±3.59b	98.65±3.89d	9.47±1.22d
60	0	0.10±0.00c	0.39±0.01b	63.93±8.76a	164.32±8.77b	21.18±3.19b
60	100	0.10±0.00c	0.45±0.00a	67.27±8.67a	185.62±8.67a	16±0.81c
60	200	0.10±0.00c	0.36±0.01cd	62.11±9.07a	175.77±9.07ab	24.55±0.41a

In each column, means with the same letter(s) are not significantly different (LSD5%). Data are means± SD of three independent replication (n=3).

Figures

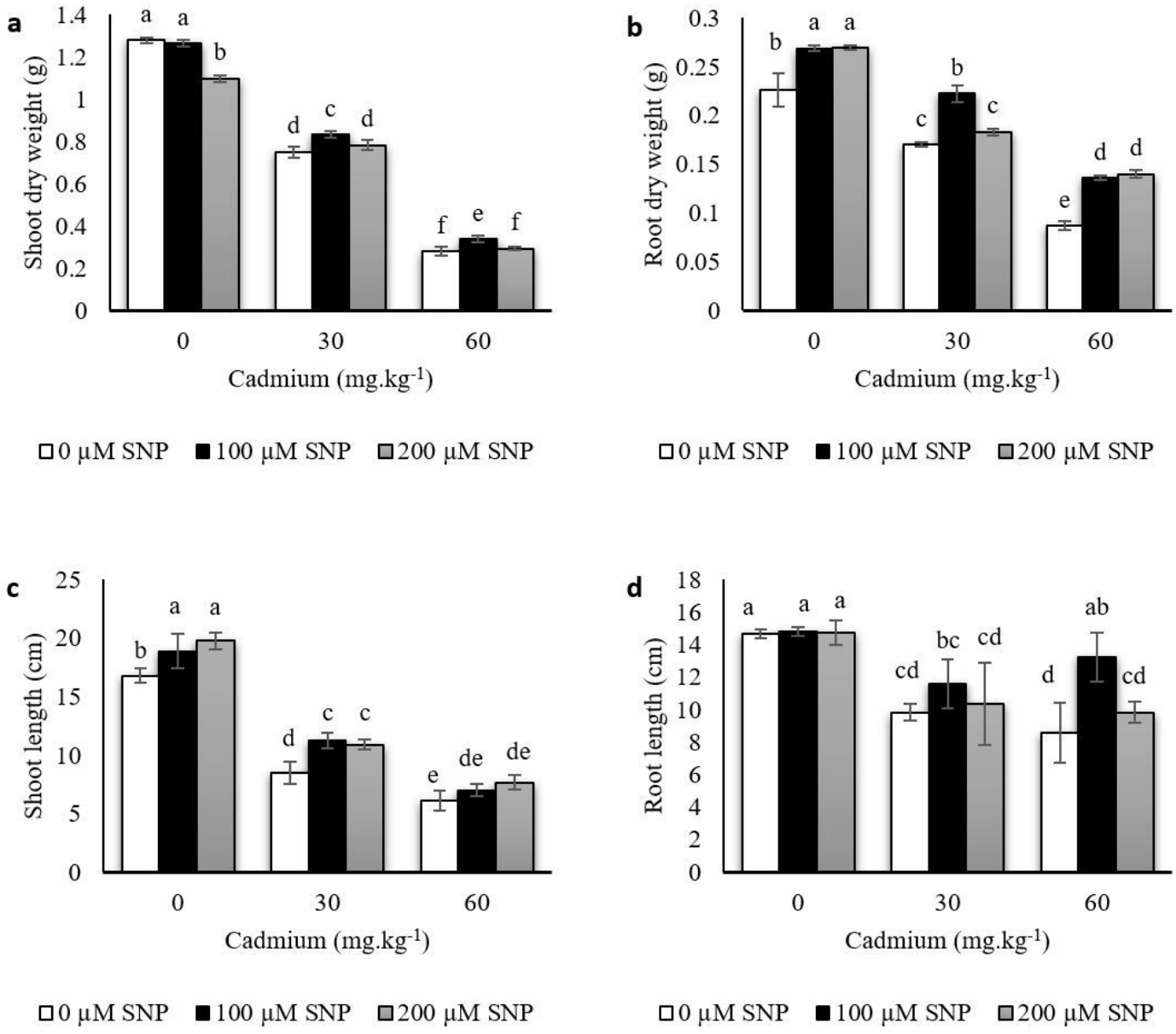

Figure 1

Mean comparison between cadmium by SNP interaction regarding shoot dry weight (a), root dry weight (b), shoot length (c), and root length (d) in A. tricolor. Data are means± SD of three independent replication (n=3).

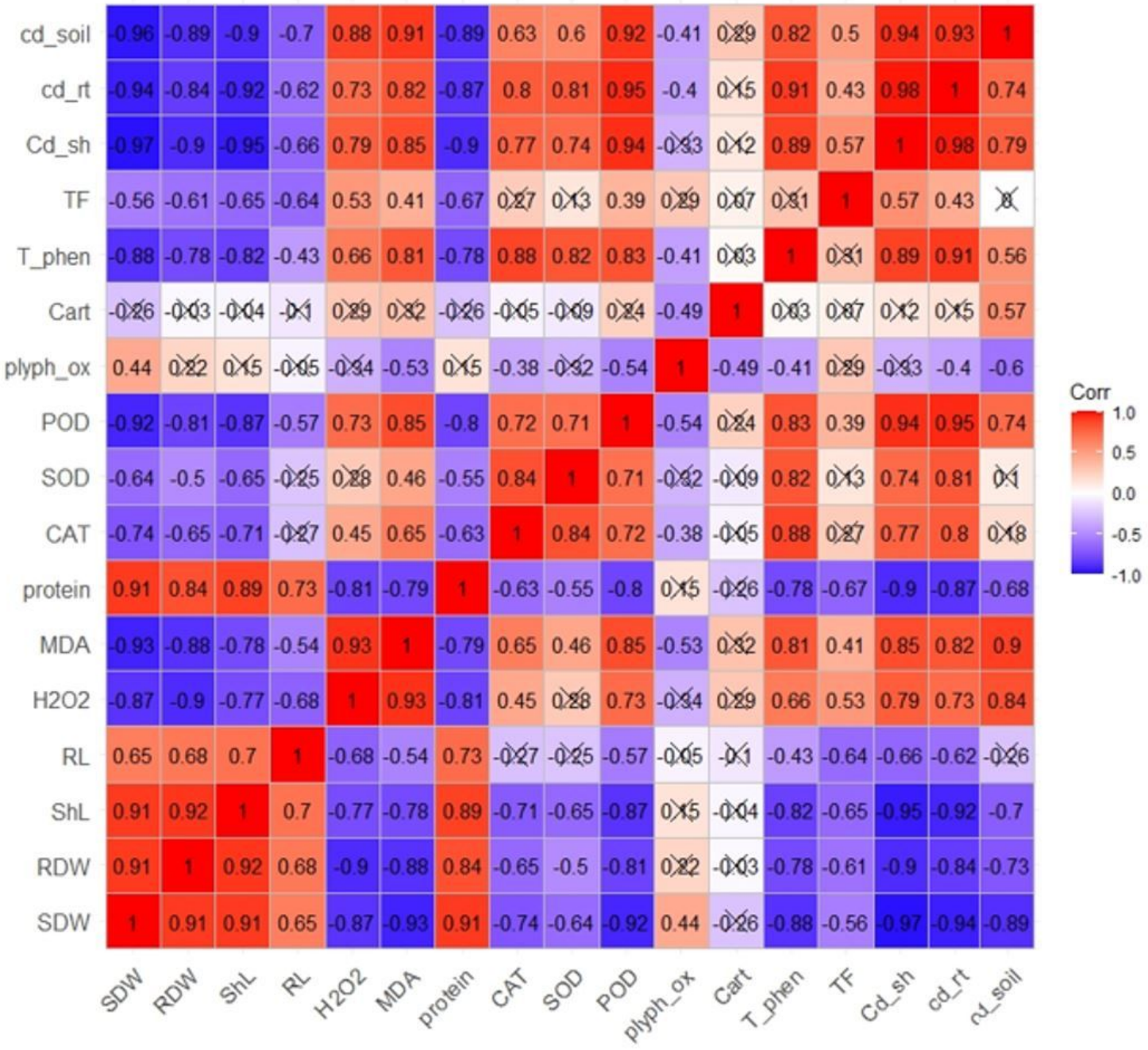

Figure 2

Correlation plot showing the association between measured traits under cadmium and SNP treatment.

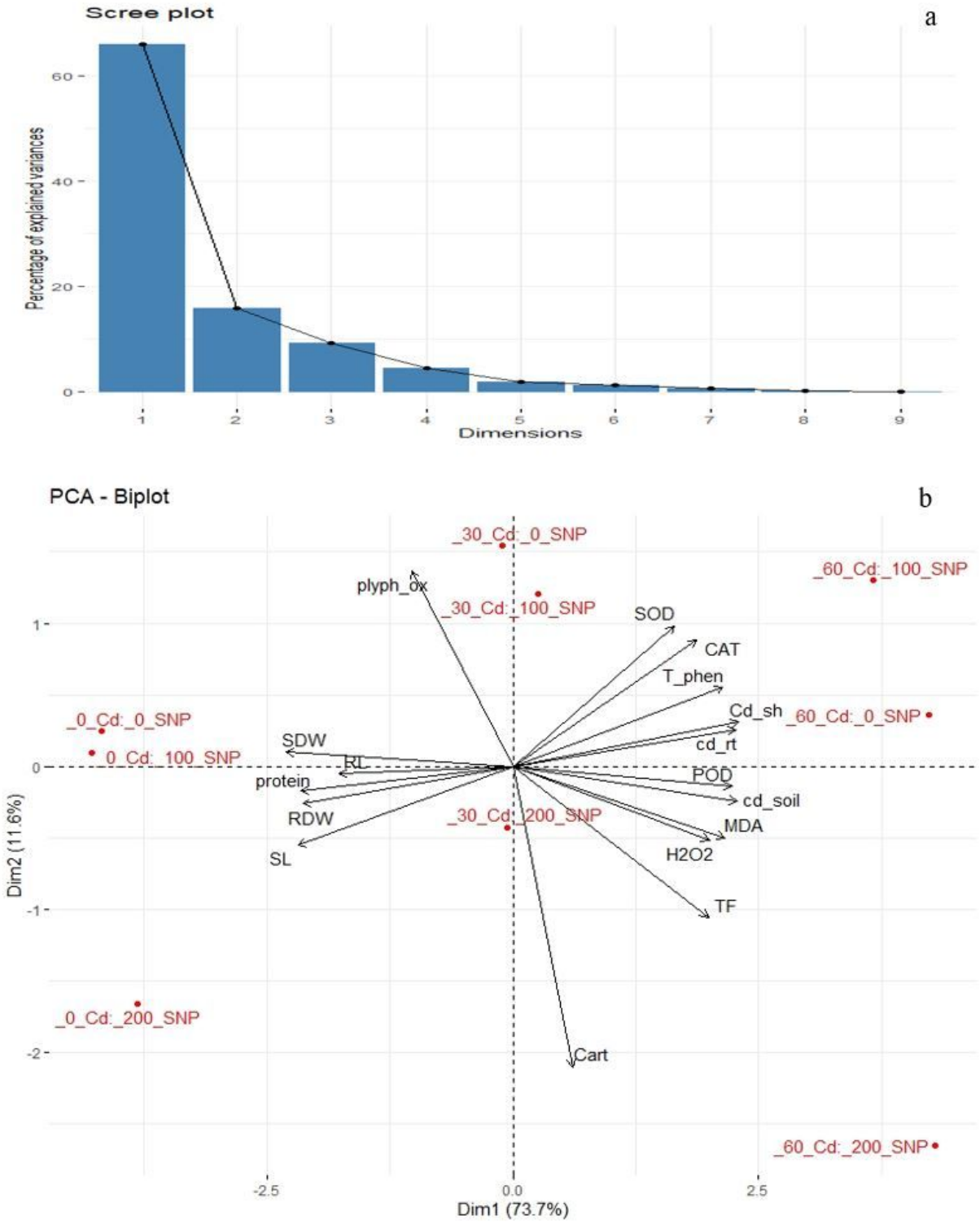

Figure 3

Scree plot (a) and biplot (b) showing the explained variation by each component and relationship among treatments in line with measured traits according to principal component analysis, respectively.

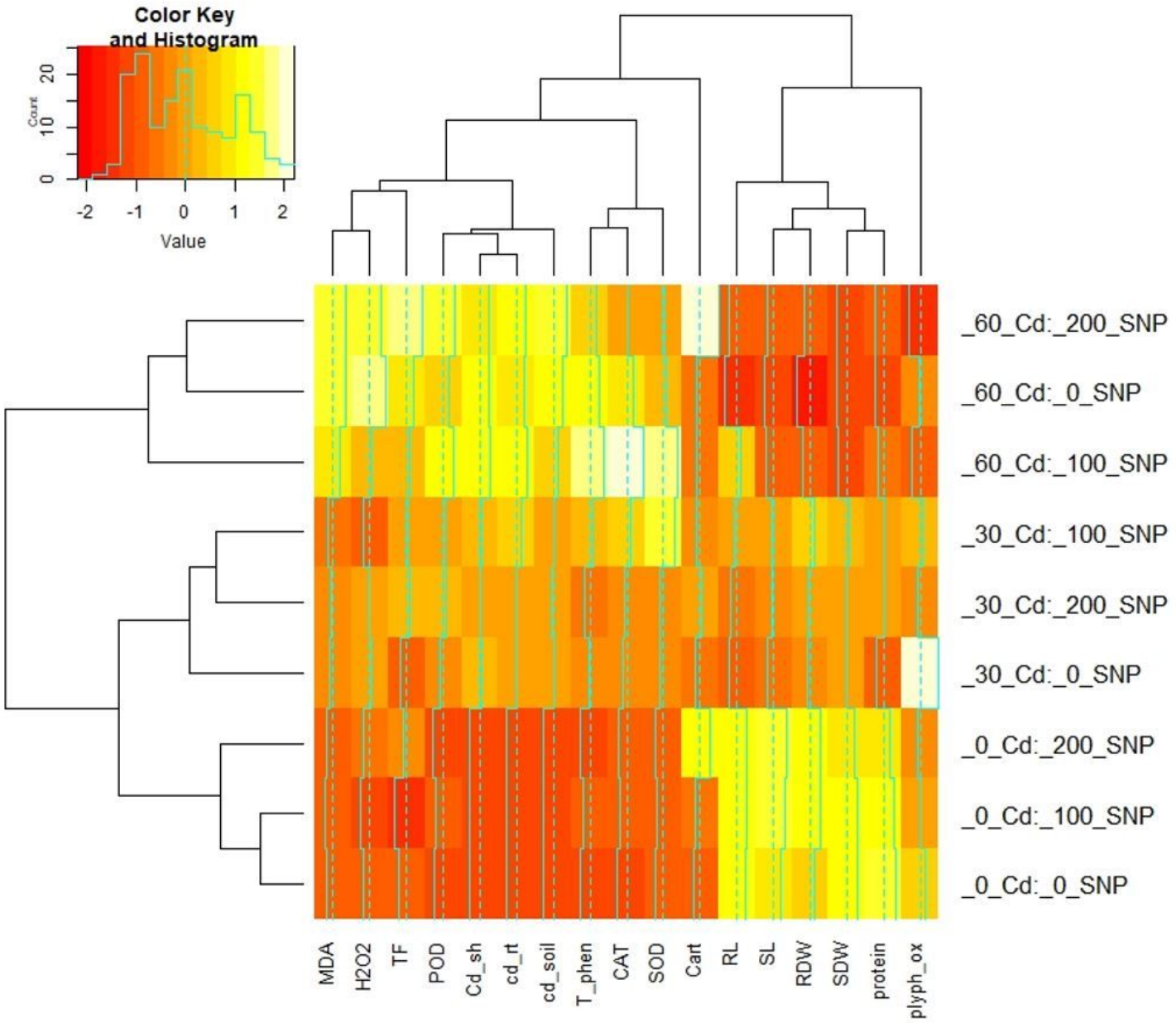

Figure 4

Heatmap showing clustering for treatments, measured traits, and their interaction effects.

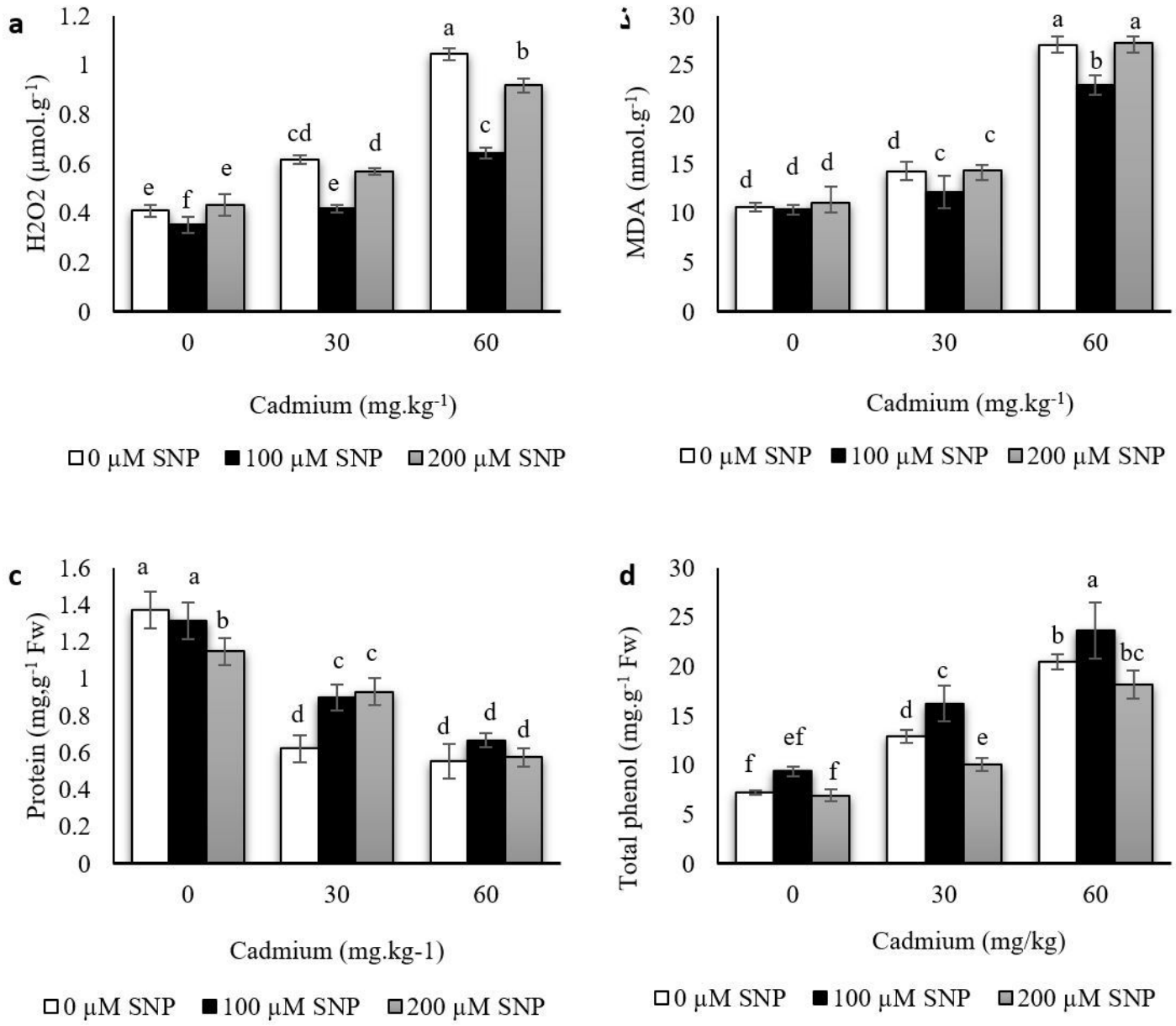

Figure 5

Mean comparison between cadmium by SNP interaction regarding the content of hydrogen peroxidase (a), malondialdehyde (b), total protein (c), and phenol (d) in A. tricolor. Data are means± SD of three independent replication (n=3).

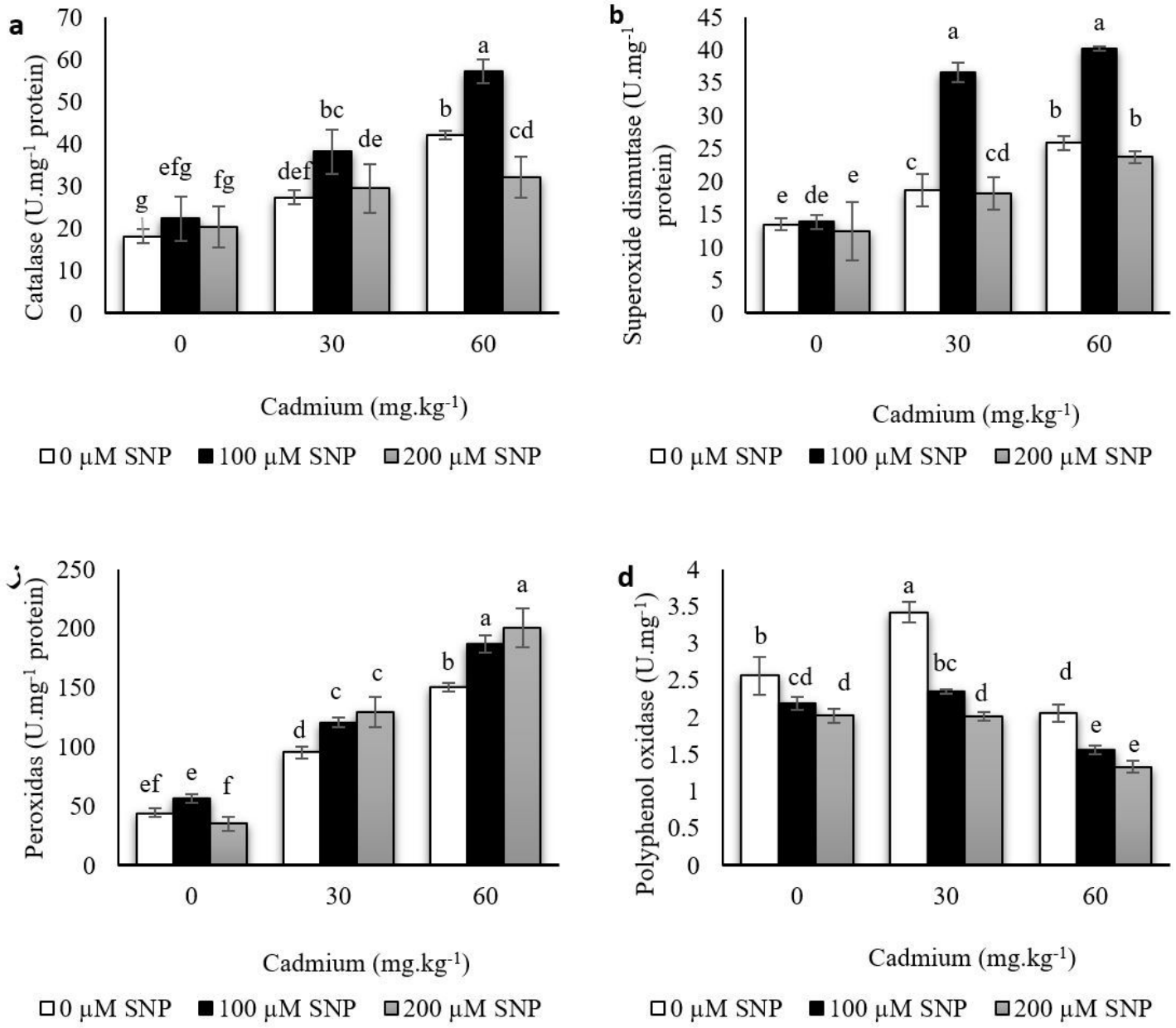

Figure 6

Mean comparison between cadmium by SNP interaction regarding the activities of catalase (a), superoxide dismutase (b), peroxidase (c), and polyphenol (d) in A. tricolor. Data are means± SD of three independent replication (n=3).