RESEARCH ARTICLE

Variations in the antioxidant and free radical scavenging under induced heavy metal stress expressed as proline content in chickpea

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Abstract This study pertains to the effects of heavy metal salts viz., copper (Cu), manganese (Mn), lead (Pb) and zinc (Zn) on the chickpea accession ICC-4812. The salts were given as treatments to the chickpea seeds at various ascending levels of doses till proving toxic. The treatment of 24 h soaked and swollen seeds were then extended to 7 days duration from the date of treatment. Atomic absorption spectrophotometric analysis of bioassay tissue *Cicer*, showed maximum uptake of 9.41 mg/g and minimum of 1.65 mg/g tissue dry weight for Pb and Zn respectively. The study reveals that enhanced antioxidant responses are associated with substantial proline accumulation indicating induced stress. Ferric reducing antioxidant power assay measuring antioxidant activity was highest in the chickpea seedling treated with Zn, whereas, free radical scavenging activity was highest in the treatments with Mn. The total phenolic and flavonoid contents ranged between 0.24–0.97 and 0.27–1.00 mg/g of dry matter content respectively. Higher Pb and Zn doses seem to elicit higher proline levels therefore, suggesting an extreme condition of induced abiotic stress. Dose dependent protein oxidation coupled with DNA degradation was observed in all treatments, depicting genotoxicity. Unweighted pair-group method arithmetic average analysis presented similarity coefficients between the treatments.

Keywords Chickpea · Metal toxicity · Antioxidants · DNA damage · Multivariate analysis

Introduction

Xenobiotic interventions have created an acute imbalance of various earth sustaining domains. The phenomenon is not so recent however, is steady. In the name of human progress, it has now manifested into industrialisation and overzealous exploitation of natural resources and pollution (Fryzova et al. 2017). The resource degradation thus caused has led to and is further leading to certain systems approaching unsustainable saturation. These systems then become untenable for growth and development of vegetation and cropping. One such phenomenon in nature is accumulation of metallic salts in water and soil, as organic or inorganic, in quantities which become non-conductive for over as well as under cover vegetation or cropping. These metals also permeate into the food chain of both animals and human beings through food and water. Such major metals include As, Ag, Cd, Co, G, Fe, Pb and Zn etc. (Mildvan 1970; Gall et al. 2015; Jaiswal et al. 2018).

Presence of some of these metals in traces is fundamental in carrying out certain metabolic processes. They constitute enzyme cofactors or activators for the cell and also the tissue level metabolism of both plants and animals (Nagajyoti et al. 2010; Anjum et al. 2015). However, when threshold limits for such metals exceed in the edaphic environment due to various factors, these are then translocated by the plants through roots as water soluble salts. This can cause cellular dysfunction in crops as well as other plants. These subsequently become part of the food chain of both animals and humans and cause immense

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deterioration in their healthy growth and development (Akram et al. 2017).

Industrial dumps, mining activities, sludges and agro chemicals are the chief sources of soil and water contaminants. Bound heavy metal ions cease to be biodegradable hence, remain lodged in the soil and finally contaminate underground aquifers. This mineral overloaded water is then absorbed by roots and transferred into aerial parts of plants causing toxicity. The effect of heavy metal on various physiological, biochemical, cytological and molecular levels are already well elaborated (Malec et al. 2008; Maleva et al. 2009; Yusuf et al. 2012; Siddiqui 2013; Šiukšta et al. 2019). Human and animal ingestion of metals occur primarily through plants when used as food or fodder. Therefore, their excessive presence in these becomes a point of concern (Sharma et al. 2004; Rehman et al. 2019).

Chickpea is the third largest pulse crop, important as it is, for compensating high levels of food proteins, vitamins and carbohydrates (Chauhan et al. 2016). The chickpea seeds also have active components known to show antioxidant characteristics for free radical scavenging (Sahu et al. 2010; Gupta et al. 2017; Shahi et al. 2019). The present study therefore, undertakes to discuss the results wherein chickpea seedlings were subjected to various metal salt treatments under in vitro conditions at low and high doses. An abiotic stress was induced within the tissue by higher metal accumulation. Stress was expressed by an incremental tissue level of amino acid proline. It is an established fact that an increase in proline in the tissues indicates conditions of stress (Hayat et al. 2012; Ghori et al. 2019).

Studies on legume crop seeds vis-à-vis their physiological responses to metal ion availability and accumulation are meagre. Moreover, such studies available for the species of genus *Cicer* are almost negligible. As already reported by our laboratory, there are inherent components present in the chickpea seeds which are known to have health related antioxidant and free radical scavenging activities (Gupta et al. 2017; Bhagyawant et al. 2018). Further, the legumes are also known to withdraw excess metals from the soil (Abdelkrim et al. 2019; Zeng et al. 2019). This characteristic of chickpea, a common man legume, may be affected by metal ions and this study is therefore directed towards that objective. This should also provide a basis for future studies ascertaining the effect of excess metal ion accumulation by chickpea in nature and its effect on antioxidants and free radical scavengers.

Materials and methods

Plant material and growth conditions

Chickpea seeds of accession ICC-4812 provided by the Indian institute of Pulses Research (IIPR) Kanpur were used as plant material. Seeds were surface sterilized by a 3 min dip in 5% (v/v) sodium hypochlorite, washed and soaked in sterile non-ionic water for 24 h in an incubator ($30 \pm 2^\circ\text{C}$). Seeds were then floated over a layer of cotton in 75 mm diameter petriplates and stored at $25 \pm 2^\circ\text{C}$ under sterile dark conditions. The radical emergence was treated as germination.

Germinated seeds were transferred to sterile petriplates supplemented with ascending logarithmic concentrations of Himedia AR grade copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), manganese sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), lead nitrate ($\text{Pb}(\text{NO}_3)_2$) and zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$). Non-ionic sterile distilled water served as control. Each metal treatment solution was made at 50, 100, 150, 200 and 250 mg l^{-1} in 20 ml and then poured over the layered cotton of the respective petriplate. The treatment was terminated after 7 days, after which the seeds of respective treatments were washed with sterile non-ionic water and stored at -20°C Voltas deep freezer for subsequent analysis. The experimental sketch is represented in Fig. 1.

Fig. 1 Analytical work chart

Growth inhibitory rate (GIR)

Ten centimetre diameter sterile petriplates were lined with two layers of sterilised Whatman 3 filter paper discs. Five millilitre of each metal salt solution mentioned as above was poured in its respective petriplate till filter paper discs were completely wet. Surface sterilised 10 seeds of chickpea were placed in each petriplate and incubated in a biological oxygen demand (BOD) chamber (Remi, India) at 25 ± 2 °C maintaining at 15-h-light-9-h-dark cycle for 7 days (Li et al. 2005). Each treatment had 3 replicates and percent rate of root growth inhibition was calculated by the following relation

$$\text{Inhibitory rate \%} = ((y - x)/y) \times 100$$

where x is the average radical/root length and y is average root length of the control.

Atomic absorption spectrophotometric (AAS) analysis

The treated seeds were dried in a forced draught oven at 100 ± 2 °C and grinded to obtain homogenous dry powder of each treatment. One gram powder was digested with 50 ml HNO_3 and H_2O_2 (3:1) mix on a hot plate at 100 °C. Each digest was filtered using Whatman filter paper No. 42 under suction in a buchner funnel and made to 100 ml final volume by sterile non-ionic water. The clear digested samples were subjected to quantitative analysis of metal elements with an atomic absorption spectrophotometer (Shimadzu, AA-6300, USA).

Estimation of total protein and proline content

Hundred milligram of seed powder was vortexed using 1 ml of 20 mM Tris–HCl buffer (pH 7.5). The resulting homogenates were centrifuged at 10,000 rpm for 20 min at cooling conditions (Remi C-24, India) and supernatants were collected. The total seed protein content of supernatant was determined by the method of Bradford (1976) using bovine serum albumin (BSA) fraction IV for preparing the calibration curve. UV–Visible spectrophotometer (Systronics 2203, India) was used for all other estimations wherever photometry was involved.

Extraction and estimation of proline was carried out by following the method of Bates et al. (1973) and modified accordingly for the seedling tissue as per the requirements of quantity of reagents. The final absorbance of the toluene layer was measured at 520 nm.

Estimation of total phenolic content (TPC) and total flavonoid content (TFC)

A modified Folin–Ciocalteu (FC) colorimetric method of Swain and Hillis (1959) was used to estimate TPC. Seed/seedling homogenates were centrifuged, supernatant vacuum dried and finally taken up in sterile distilled water. From this, TPC estimation was performed at 650 nm against a reagent blank and gallate as standard at ascending series concentrations.

TFC of methanolic extract was estimated following Khoo et al. (2013). One millilitre from each metal treated seed extracts was added to 1 ml of 2% aluminium chloride (AlCl_3), incubated for 10 min and absorbance measured at 415 nm using a calibration curve made from quercetin with methanolic AlCl_3 as blank.

Antioxidant activity

DPPH radical-scavenging activity

Radical scavenging activity (RSA) of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined using the method of Bondet et al. (1997). One millilitre of methanolic extract (50 mg in 1 ml methanol) was added in 3 ml of 0.1 mM DPPH. After vigorous mixing, it was allowed to stand in dark at room temperature for 30 min and its absorbance was measured at 517 nm and the radical scavenging activity was calculated by the relation:

$$\text{Percent (\%)} \text{ scavenging activity} = ((A_c - A_s)/A_c) \times 100$$

where A_c is absorbance of control and A_s is absorbance of sample respectively.

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation decolorisation assay

The method to ascertain radical scavenging was that of Arnao et al. (2001) with slight modification. The method exploits the characteristic of ascorbate to scavenge the free radicals. Ascorbate is employed as a standard antioxidant in a dose response curve with an assay to scavenge 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS^+) radical cation. However, the present samples were directly applied to the ABTS solution than using ascorbate assay and the outcomes explained as percentage of scavenging activity as per following equation:

$$\text{Percent (\%)} \text{ scavenging activity} = ((A_c - A_s)/A_c) \times 100$$

where A_c is absorbance of control and A_s is absorbance of sample respectively.

Ferric reducing antioxidant power (FRAP) assay

One gram of seeds from each treatment was ground with 10 ml of methanol and centrifuged at 10,000 rpm for 20 min. The antioxidants presence was measured by FRAP assay of Benzie and Strain (1996). FRAP reagent was prepared fresh and consisted of 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-Tripyridyl-s-Triazine (TPTZ) in 40 mM HCl and 20 mM FeCl₃ in a ratio of 10:1:1 (v/v/v). About 200 µl of each extract was mixed with 1.3 ml of the FRAP reagent and after an incubation of 30 min at 37 °C, absorption was read at 600 nm. The antioxidant status was expressed as µM FeSO₄ l⁻¹ ± SD.

Assessment of DNA damage

The method for evaluation of DNA damage was employed wherein it was associated with assessing induced DNA damage using Fenton's reaction (Xiao et al. 2014). Slight modifications were done in this method. A total of 15 µl volume contained in a microcentrifuge tube included 0.5 µg of calf thymus DNA, 3 µl of 50 mM phosphate buffer (pH 7.4), 3 µl of 2 mM FeSO₄ and 2 µl of each purified seed extract at different concentrations (50–250 mg ml⁻¹) was added. Four microlitre of 30% H₂O₂ was added and the mixture was finally subjected to incubation at 37 °C for 1 h. Electrophoresis of the mixture was done post incubation over 1% agarose gel to visualise under UV-transilluminator if the treatments induced any DNA damage.

Protein oxidation assay

Salt treated seedling proteins (as extracted for protein estimation) were investigated for their protective ability against H₂O₂/Fe⁺³/ascorbic acid attack (Kizil et al. 2011) with slight modifications. Briefly, BSA (1 mg ml⁻¹) was used as a marker protein dissolved in 20 mM potassium phosphate buffer (pH 7.4). To the control protein and treated seedling extracts, 50 µM FeCl₃, 1 mM H₂O₂ and 100 µM ascorbic acid were added. As the reaction mixture was incubated in the presence or absence of tested sample extracts at different concentration range between 50–1000 mg ml⁻¹ in a final volume of 0.5 ml. After incubation for 3 h at 37 °C, the post reaction mixture was subjected to electrophoresis in 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Prior to electrophoresis, samples were mixed with equal volumes of sample buffer consisting of Tris HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% sucrose and 0.002% bromophenol blue. After 5 min boiling, 5 µl of each sample was electrophoresed on SDS-PAGE in running buffer (25 mM Tris pH 8.3, 190 mM

glycine and 0.1% SDS) at maximum voltage and a constant current of 25 mA for a mini gel, using a power supply. Gels were stained with 0.15% coomassie brilliant blue R-250 for 2 h, destained and digitally photographed.

Statistical analysis

Data is expressed as mean ± standard deviation (SD). Statistical analysis is done using the Graph pad prism version 5 software. The differences in mean are procured using the Duncan multiple-range tests for means set at 95% confidence limit ($p < 0.05$). Principal Component Analysis (PCA) was performed using PAST software (Hammer et al. 2001) to examine the phytochemical profile of different metal treated chickpea seedlings at increasing concentrations. Correlation coefficients (r) were calculated using Microsoft Excel 2010. Mean values were used to create correlation matrix from standardized PC scores. Dendrogram were created using UPGMA feature of NTSYS-pc software Version 2.2.

Results and discussion

GIR and AAS

Under heavy metal stress, seed germination and seedling establishment are important for plant growth (Rizwan et al. 2018; Seneviratne et al. 2018). Irrespective of the metal ion available, radical length expressed as percent of control showed retardation at all levels of treatment (Fig. 3a, Table 1); the inhibition sequence order being Zn < Cu < Mn < Pb (Fig. 2). Metal uptake for each metal ion seems to start immediately, once available in the medium with almost linear increase in tissue accumulation with increased dose (Fig. 3b). Level of each metal in the physiologically active seedling tissue showed nearly sigmoid relationship with growth inhibition percentage. Pb given at lowest concentration showed nearly 16-fold higher tissue accumulation than the control at minimal dose and reaching more than 50-fold at highest dose of 250 mg l⁻¹ dose.

At lower treatment doses, Zn and Mn accumulation in the tissues was nearly threefold while Pb and Cu intake was around sixteen and 14-folds respectively higher when compared to their controls. Probably, Pb is the cause of phytotoxicity by changing the cell membrane permeability, elevated water and lipid soluble antioxidants and increase in ROS generation (Ahmad et al. 2015, 2016; Kumar et al. 2017; Kohli et al. 2017). Jiang et al. (2010) suggested that elevation of stressful conditions due to heavy metal treatments use an efficient antioxidative defense mechanism, thus balancing ROS production and metal removal.

Table 1 Radical elongation test, metal tissue accumulation, total phenolics, flavonoids, total protein, proline and antioxidant values of metal treated chickpea seeds

Sample	Concentration (mg l ⁻¹)	GIR (%)	AAS (mg g ⁻¹ dry weight)	Protein (mg ml ⁻¹)	Proline (μM g ⁻¹)	TPC (mg g ⁻¹)	TFC (mg g ⁻¹)	RSA (%)	FRAP values (μM FeSO ₄ l ⁻¹)	ABTS (%)
Control	–	0	0.35 ± 0.05	28.44 ± 1.89	8.60 ± 1.22	0.68 ± 0.01	0.18 ± 0.01	6.39 ± 0.11	147 ± 6.89	53.16 ± 1.21
Zinc	50	6.76 ± 1.60	1.50 ± 0.04	20.00 ± 0.89	11.27 ± 1.98	0.50 ± 0.05	0.12 ± 0.01	3.48 ± 0.14	119 ± 7.54	61.05 ± 1.98
	100	12.16 ± 1.80	1.55 ± 0.10	19.85 ± 4.71	13.39 ± 3.40	0.54 ± 0.03	0.16 ± 0.02	4.79 ± 0.14	106 ± 8.26	61.53 ± 3.40
	150	30.41 ± 1.30	1.65 ± 0.03	19.56 ± 4.68	15.71 ± 1.21	0.62 ± 0.01	0.22 ± 0.07	8.53 ± 0.13	98 ± 9.04	63.61 ± 1.21
	200	70.27 ± 1.40	1.63 ± 0.02	19.26 ± 2.45	18.42 ± 2.73	0.63 ± 0.04	0.23 ± 0.05	9.79 ± 0.13	84.5 ± 6.08	69.61 ± 2.73
Copper	250	75.68 ± 0.70	1.61 ± 0.03	14.67 ± 1.94	21.21 ± 1.18	0.89 ± 0.12	0.28 ± 0.18	10.31 ± 0.13	69 ± 12.00	70.87 ± 1.18
	50	10.81 ± 2.00	2.12 ± 0.04	24.89 ± 2.67	10.29 ± 1.06	0.24 ± 0.01	0.20 ± 0.02	6.05 ± 0.14	124 ± 6.76	53.91 ± 1.81
	100	14.19 ± 2.90	2.14 ± 0.14	23.70 ± 3.73	12.28 ± 1.72	0.35 ± 0.01	0.21 ± 0.01	6.70 ± 0.14	108 ± 7.50	62.45 ± 0.95
	150	46.62 ± 2.30	2.33 ± 0.17	23.11 ± 6.01	12.50 ± 1.46	0.35 ± 0.02	0.24 ± 0.01	6.74 ± 0.14	72.5 ± 6.76	63.11 ± 1.64
Manganese	200	52.03 ± 1.50	2.37 ± 0.14	20.00 ± 2.91	16.20 ± 0.71	0.61 ± 0.06	0.25 ± 0.01	7.14 ± 0.14	52.5 ± 3.00	65.68 ± 1.19
	250	83.11 ± 0.70	2.35 ± 0.10	10.52 ± 2.28	24.03 ± 1.10	0.79 ± 0.08	0.27 ± 0.04	8.53 ± 0.14	37 ± 6.06	66.76 ± 1.25
	50	13.51 ± 1.20	1.92 ± 0.09	26.37 ± 7.47	8.73 ± 1.73	0.62 ± 0.10	0.09 ± 0.01	4.44 ± 0.14	112.5 ± 12.00	59.95 ± 1.73
	100	24.32 ± 1.90	2.26 ± 0.09	16.30 ± 3.28	9.81 ± 3.57	0.64 ± 0.16	0.17 ± 0.02	6.27 ± 0.14	92.5 ± 13.52	64.54 ± 3.57
Lead	150	31.76 ± 1.60	2.72 ± 0.08	15.85 ± 2.00	12.39 ± 2.42	0.67 ± 0.02	0.23 ± 0.02	8.09 ± 0.14	78 ± 6.00	64.6 ± 2.42
	200	47.97 ± 1.50	2.72 ± 0.12	15.78 ± 0.22	13.66 ± 1.58	0.82 ± 0.02	0.23 ± 0.03	9.05 ± 0.13	51.5 ± 12.12	67.35 ± 1.58
	250	78.38 ± 1.61	2.73 ± 0.02	13.04 ± 3.78	18.29 ± 1.83	0.93 ± 0.09	0.41 ± 0.07	10.27 ± 0.13	42 ± 6.00	71.12 ± 1.83
	50	19.59 ± 2.10	1.51 ± 0.04	21.04 ± 4.32	10.12 ± 1.25	0.25 ± 0.02	0.17 ± 0.03	2.39 ± 0.14	109.5 ± 4.50	53.3 ± 1.25
Lead	100	33.78 ± 1.70	2.35 ± 0.08	19.70 ± 4.92	12.71 ± 1.29	0.44 ± 0.03	0.21 ± 0.03	2.94 ± 0.14	91.5 ± 7.50	58.37 ± 1.29
	150	39.19 ± 1.10	2.50 ± 0.09	19.11 ± 0.89	16.86 ± 1.84	0.67 ± 0.13	0.27 ± 0.06	3.66 ± 0.14	84.5 ± 6.06	68.61 ± 1.84
	200	58.11 ± 0.90	2.77 ± 0.02	13.78 ± 0.89	21.37 ± 1.13	0.87 ± 0.01	0.34 ± 0.07	4.53 ± 0.14	69.5 ± 8.26	69.58 ± 1.13
	250	65.54 ± 1.80	4.71 ± 0.11	12.30 ± 0.93	21.79 ± 2.15	0.97 ± 0.04	0.53 ± 0.02	4.92 ± 0.14	58.5 ± 7.50	71.97 ± 2.15

Values are mean ± SD, n = 3

GIR growth inhibitory rate, AAS atomic absorption spectroscopy, TPC total phenolic content, TFC total flavonoid content, RSA radical scavenging activity, FRAP ferrous reducing antioxidant power, ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

Except Pb at lower dose treatments, tissue uptake of each metal ion seems to show steady increase. Further increase in concentrations show hardly any further additional uptake and accumulation. This only shows that the inflection of these metals into tissues seems to be quick once these are available and it may be basically a onetime uptake and remains unaffected despite increasing availability. This seems to be an interesting observation in the present study with chickpea seedlings under in vitro conditions. Pb being an exception, all other metals showed steady but a slower accumulation despite increasing treatment concentrations. It is now known, rather established that under conditions of stress, both biotic and/or abiotic, the plant tissues respond by various mechanisms to either ward off the stress or delay adversity. This is achieved by increasing the cell levels of substances of low molecularity (Wang et al. 2003; Hasanuzzaman et al. 2012; Khan et al. 2018) and one of these being free amino acid proline (Hare and Cress 1997; Saif and Khan 2018). Logically, therefore the basis for ascertaining the induction of stress, if any, proline levels in tissues has been employed as a biomarker.

Protein and proline content

The total protein concentration was significantly decreased correspondingly with increase in the metal concentration

Fig. 2 Seven day morphological response of germinated chickpea seeds to metal salts at various ascending doses

Fig. 3 **a** Effect of metal concentration on radical length showing negative growth once provided with metal ions inhibition presented as percent of control, **b** metal accumulation by chickpea seedling at different dose levels of each metal after floating for 7 days, **c** total protein content of seedling tissue dry weight, **d** rise in content of tissue proline with respective metal ion suggesting abiotic stress induction treatments, **e** total phenolic content of chickpea seedling tissue in response to metal treatments, **f** total tissue flavonoid content of metal ion treated chickpea seedlings, **g** radical scavenging activity in chickpea seedling tissue, **h** radical cation assay of metal ions treated chickpea seedling tissue, **i** antioxidant potential in chickpea seedling tissue after metal treatment

(Fig. 3c). Pertinently more than normal accumulation of proline by the cell is employed as indicator of stress as per Hare and Cress (1997). The proline accumulation is therefore, generally observed in plants exposed to biotic and abiotic stresses and is assumed to be a protective mechanism (Shahzad et al. 2018; Ghorri et al. 2019). Moreover, published reports suggest proline accumulation being regulated by the hormonal signalling pathway (Herrera-Vásquez et al. 2015). Present data revealed that proline content gets increased substantially in chickpea seeds when they are exposed to induced abiotic metal stress (Table 1). The increased proline accumulation in response to metal stress here is specifically employed only to ascertain induction of stress conditions in the chickpea

seedling which may be due to an enhancing expression of proline related genes (La et al. 2019). In this study, data showed that seeds exposed to metal stress substantially leads to nearly three fold increase in proline content which was almost conforming to that of La et al. (2019). Earlier too, similar observations have been reported (Yadav et al. 2011; Wani et al. 2019).

The Fig. 3d seems to show that the free proline level is significantly higher than normal compared to controls at higher doses of metal treatments. This is irrespective of whether physiological metal ions Zn, Cu and Mn or a non-physiological ion Pb is provided. Insignificant increase in the free proline is shown at low concentration in all metals at 50 mg l⁻¹ dose. However, the proline content at 100 mg l⁻¹ treatment of Zn and Pb shows some significant increase whereas at 150 mg l⁻¹, all metal ions do induce proline accumulation thereby indicating stress. It may therefore, be pertinent to assume that at and after 100 mg l⁻¹ treatments, the tissue accumulation of each metal indicating that the tissue is under abiotic stress.

Total phenolics and flavonoids

Induction of stress in whatever form, as is known to be associated with some tissue level physiological responses viz., additional synthesis cum accumulation of phenolic acids, flavonoids, which are cell defensive mechanism/s to avoid or ward off any lethal effects due to stress (Manquian-Cerda et al. 2018; Davies et al. 2018). These and other synthates acting as antioxidant substances during metabolism are well described and documented in various systems including not only plants but also certain variety of seeds and seedlings (Chatterjee et al. 2004; Ahmad et al. 2015). The knowledge of metal ion related uptake by the germinating seeds, specifically by leguminous seeds such as chickpea are meagre and fragmented. The present study show that with the increasing metal concentration and tissue metal accumulation (Table 1), the TPC and TFC remain lower than that of the control and reaching to control or just a little higher to it as concentration of accumulated metal increase in the seed (Fig. 3e, f). The low toxicity of individual metals at low concentrations probably can be due to production of many primary and secondary metabolites other than TPC. Such observation is already substantiated by others also (Sabatini et al. 2009; Belghith et al. 2016; Strejckova et al. 2019).

Antioxidant activity

Subsequently free radical scavenging either through DPPH or ABTS assay also depicts similar trends (Fig. 3g, i). It justifies an inference that irrespective of the metal ion, whenever a chickpea seed succumbs to metal stress, it

responds by reduced total phenol and flavonoid synthesis which otherwise, as is an established norm should have been higher than controls (Kováčik et al. 2009). Therefore, particularly free radical scavenging activity too show almost similar suppression either and/or assayed by DPPH or ABTS (Table 1). Interestingly the ferric to ferrous assay as an antioxidant activity is always lower than the control and each metal treatment induce further steady reduction in antioxidant activity with increasing dose and accumulation of metal (Fig. 3h). This therefore, can be reported as an interesting observation and may need further investigation. According to Zou et al. (2016), due to the different mechanisms underlying between evaluation assays, the values or variation trends obtained by different assays differ widely, even for the same amino acid sequence and composition.

Inhibition of oxidative DNA damage

The inhibition of oxidative DNA damage was evaluated using extracted protein from control and metal treated chickpea seedling using calf thymus DNA. The assay investigated the protection effects of chickpea proteins on Fenton's reagent induced DNA damage. The metal treated seed proteins subjected to inhibit DNA degradation was examined by agarose gel electrophoresis. DNA damage inhibition by these proteins at low and high concentrations is displayed in Fig. 4. Fenton's reagent is known to cause oxidative breaks in DNA strands yielding DNA fragments (Shih and Hu 1996). Proteins of metal treated seed were unable to inhibit DNA damage even when checked in dose dependent manner. However, protein from control seeds did restrict DNA damage better compared to metal treated seeds. This though being the preliminary observation, yet seems somewhat different wherein elevated tissue metals are inhibiting the synthesis of proteins which can ward off DNA oxidative damage due to abiotic stress.

Studies of Imtiaz et al. (2016) in chickpea genotypes exposed to vanadium (V) revealed that DNA damage can be triggered due to exposure of higher V in dose dependent manner. The results here are in line with earlier reports of metal toxicity on chickpea seeds. However, some of the plant cells/genotypes exhibit mechanistic efficiency to counter higher and lower concentrations of metal toxicity. Few of the earlier reports showed that carrot cells offer resistance against metal stress with enhanced efficiency to manage metal toxicity (Ojima and Ohira 1983). Exposure of chickpea seedlings to four metals which seem to lead alterations in DNA integrity needs therefore, further investigation employing molecular techniques.

Fig. 4 DNA protective activity of control and treated seeds with metals of low and high concentration

Protein oxidation assay

To monitor protein damage qualitatively after metal treatments, the protein samples were subjected to SDS-PAGE. Electrophoretic patterns of BSA after 3 h incubation with $\text{Fe}^{3+}/\text{H}_2\text{O}_2$ /ascorbic acid system in the presence or absence of different concentrations of metal treated seeds is shown in Fig. 5. Compared to control seed proteins, the damaging effect of free radicals was visible in all treated chickpea proteins. This may be due to the oxidative protein damage, resulted by the modification/alteration of proteins either directly or indirectly by generation of free radicals (Stadtman and Levin 2000; Kizil et al. 2010). Therefore, lowest dose of 50 mg l^{-1} gave better protection from oxidation than highest 250 mg l^{-1} dose. The protein oxidation assay of metal treated seeds resulted in BSA degradation as a consequence of treatment. The study reveals a protective activity as dose dependent. The seeds treated with low metal doses demonstrated more protective activity viz., preventing oxidative protein damage. Hence, the protein carbonyl formation during glycoxidation is prevented at lower metal doses and enhanced at higher ones. This study demonstrated that heavy metals can

decrease total protein contents, total antioxidants level and induce DNA damage. Being a marker of genotoxicity, protein oxidation coupled with DNA degradation was observed in all the treated seeds with different metals in a dose dependent manner (Kizil et al. 2009, 2010). The findings of this study revealed that metal stress at subsequent level alters the physiology and biochemical responses of chickpea. This is in line with the experiments conducted by Bashi et al. (2012) and Kada et al. (2017). Stress higher than threshold level leads to genotypic damage eventually leading to intermittent metabolism supported by proline accumulation, antioxidant levels and truncated growth of seedling.

Relationship of phytochemicals with antioxidant capacity

Pearson's correlation analysis of the antioxidative responses and biochemical variation due to induced metal stress in chickpea seeds is shown in the Table 2. The positive significant correlations at the 99% confidence level were reported between, GIR-Protein ($r = 0.742$), GIR-FRAP ($r = 0.884$), TPC-TFC ($r = 0.646$), TPC-Proline

Fig. 5 Protein oxidation assay of metal treated seeds of low and high concentrations

Table 2 Pearson's correlation analysis of various analysed parameters as inter correlations due to induced metal stress in chickpea seedlings by zinc, copper, manganese and lead at various levels of significance

	GIR	AAS	TPC	TFC	Protein	Proline	RSA	ABTS	FRAP
GIR	1								
AAS	− 0.256	1							
TPC	− 0.722	0.355**	1						
TFC	− 0.719	0.711*	0.646*	1					
Protein	0.742*	− 0.370	− 0.815	− 0.668	1				
Proline	− 0.873	0.368**	0.733*	0.744*	− 0.766	1			
RSA	− 0.607	− 0.386	0.452**	0.264**	− 0.379	0.415**	1		
ABTS	− 0.789	0.316**	0.884*	0.692*	− 0.715	0.779*	0.552**	1	
FRAP	0.884*	− 0.284	− 0.731	− 0.678	0.769*	− 0.730	− 0.550	− 0.748	1

p* (< 0.005); *p* (< 0.001)

($r = 0.733$), TPC-ABTS ($r = 0.884$), TFC-Proline ($r = 0.744$), TFC-ABTS ($r = 0.692$), Protein-FRAP ($r = 0.769$) and Proline-ABTS ($r = 0.779$), while at the 95% confidence level, significant correlation were observed between TPC-RSA ($r = 0.452$), TFC-RSA ($r = 0.264$), Proline-RSA ($r = 0.415$) and ABTS-RSA ($r = 0.552$). The negative and significant correlations were found among the protein and increased metal concentration. This type of a Pearson's correlation analysis of antioxidants as shown in Table 2 may be a maiden attempt. This analysis might be used to determine correlations between different physiological parameters with reference to metal treatment effects vis-à-vis treatments.

The association among the antioxidative responses and biochemical variation due to stress of 4 different metals in chickpea seeds are illustrated by PCA, presented in Fig. 6a–d respectively. In the Fig. 6a, PC1 explained 88.21% of the variance and PC2 explained 8.11% of the variance. The Cu at 50 and 100 mg l^{−1} treatment observations are quite similar, thus forming the single group located at the down-left quadrant and being positively associated with GIR and FRAP and negatively related to TPC, RSA and proline with increasing Cu doses. Ranking in opposite direction scores Cu (250 mg l^{−1}) directly correlated to TPC, RSA and Proline. The observations at Cu 200 mg l^{−1} are positively related with TFC, ABTS and negatively related with protein content.

In Fig. 6b, PC1 accounted for 90.2% of the total variance and PC2 accounting 6.36% of the variance. Mn at 50 mg l^{−1} treated seeds are located towards the upper-left of quadrant and positively correlated with FRAP and protein and negatively related to TPC, TFC, ABTS and proline. Mn 100 mg l^{−1} and Mn 150 mg l^{−1} score the same group; scores being associated positively with GIR and negatively with RSA. In the down-right quadrant scores, Mn (250 mg l^{−1}) is positively linked to RSA. The Mn

250 mg l^{−1} is however, positively correlated with TPC, TFC, ABTS and proline and negatively with protein.

In Fig. 6c, PC1 explained 92.19% of the variance and PC2 explained 5.16% of the total variance. Together, Pb at 50 and 100 mg l^{−1} treatment seeds form a single group located at the down-left quadrant, score seems positively associated with FRAP and negatively related to TPC, RSA. Pb at 150 mg l^{−1} score occupied the space in the upper-left of the quadrant hence significant positive correlation with GIR and protein and negative with ABTS, TPC and Proline. Pb 200 and 250 mg l^{−1} ranked in the opposite direction and related to RSA, TFC but related negatively to FRAP.

In Fig. 6d, PC1 accounted total variance 86.7% and PC2, 9.92% of the variance. Zn at 50 mg l^{−1} score seems positively correlated to ABTS, Proline and negatively with TPC, TFC, FRAP and GIR. Zn at 100 and 250 mg l^{−1} scores are quite similar, producing the single group together located at the down-right of quadrant, being positively associated with TPC, GIR and negatively with ABTS. Zn 150 mg l^{−1} is positively correlated with TFC and negatively with protein whereas Zn at 200 mg l^{−1} show positive association with ABTS and negative with RSA.

Cluster analysis

The different antioxidative responses and biochemical variation in chickpea seeds regarding induced metal stress is illustrated in the dendrogram (Fig. 7a–d). In Cu treatment (Fig. 7a), the chickpea seeds response in relation to GIR, FRAP and protein are clustered into a single group. The copper metal and TFC directly make correlation with ABTS, hence a common group with TPC, proline and RSA. Mn treated seeds (Fig. 7b), are subdivided into two groups; first being comprised of GIR, FRAP and protein

Fig. 6 Principal components (PC), PC1 and PC2 based on concentrations of metal treatment

and the second as, TFC, proline, RSA. ABTS is seen to be related to each and other sub-groups, however, Mn was mended out from the group. Also for Pb treated seeds (Fig. 7c), the GIR, FRAP and protein are clustered together into a group. The TPC, RSA, proline are found to be clustered in a group with ABTS. Pb and TFC appeared more distant, which indicate a major difference with respect to the other antioxidative and biochemical responses. Finally, Zn treated seed (Fig. 7d) responses clustered with the group comprising parameters protein with GIR, FRAP. Zn metal was grouped as ousted. Finally the TPC is grouped with TFC, proline, RSA and ABTS.

The combined analytical results for all the metal induced stress indicated a considerable variability between the antioxidative response parameters and varied biochemical responses in thereof the chickpea seeds.

Conclusions

In summary, this study revealed that inflection of these metals into tissue is swift and one time uptake under in vitro conditions. High free proline level is observed irrespective of metal type at high concentrations. Stress

Fig. 7 Dendrogram of metal treated chickpea seeds on the basis of distance coefficient

induction resulted in additional synthesis cum accumulation of phenolic acids and flavonoids. Exposure of chickpea seedlings to four metals lead to alterations in DNA integrity. The findings of this study revealed that metal stress at subsequent level alters the physiology and biochemical responses of chickpea.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Abdelkrim S, Jebara SH, Saadani O, Chiboub M, Abid G, Mannai K, Jebara M (2019) Heavy metal accumulation in *Lathyrus sativus* growing in contaminated soils and identification of symbiotic resistant bacteria. Arch Microbiol 201(1):107–121. <https://doi.org/10.1007/s00203-018-1581-4>
- Ahmad P, Sarwat M, Bhat NA, Wani MR, Kazi AG, Lam-Son PT (2015) Alleviation of cadmium toxicity in *Brassica juncea* L. (Czern. & Coss.) by calcium application involves various physiological and biochemical strategies. PLoS ONE 10:e0114571. <https://doi.org/10.1371/journal.pone.0114571>
- Ahmad P, Abd Allah EF, Hashem A, Sarwat M, Gucel S (2016) Exogenous application of selenium mitigates cadmium toxicity in *Brassica juncea* L. (Czern. & Cross.) by up-regulating antioxidative system and secondary metabolites. J Plant Growth Regul 35(4):936–950. <https://doi.org/10.1007/s00344-016-9592-3>
- Akram R, Hashmi MZ, Nasim W (2017) Role of antibiotics in climate change. In: Hashmi M, Strezov V, Varma A (eds) Antibiotics and antibiotics resistance genes in soils, vol 51. Soil biology. Springer, Cham, pp 97–104. https://doi.org/10.1007/978-3-319-66260-2_6
- Anjum NA, Singh HP, Khan MIR, Masood A, Per TS, Negi A, Batish DR, Khan NA, Duarte AC, Pereira E, Ahmad I (2015) Too much is bad—an appraisal of phytotoxicity of elevated plant-beneficial heavy metal ions. Environ Sci Pollut Res 22(5):3361–3382. <https://doi.org/10.1007/s11356-014-3849-9>
- Arnao M, Cano A, Acosta M (2001) The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chem 73(2):239–244. [https://doi.org/10.1016/S0308-8146\(00\)00324-1](https://doi.org/10.1016/S0308-8146(00)00324-1)
- Bashi DS, Bazzaz BSF, Sahebkar A, Karimkhani MM, Ahmadi A (2012) Investigation of optimal extraction, antioxidant, and antimicrobial activities of *Achillea biebersteinii* and *A. wilhelmssii*. Pharma Biol 50(9):1168–1176. <https://doi.org/10.3109/13880209.2012.662235>
- Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water stress studies. Plant Soil 39(1):205–207. <https://doi.org/10.1007/BF00018060>

- Belghith T, Athmouni K, Bellassoued K, El Feki A, Ayadi H (2016) Physiological and biochemical response of *Dunaliella salina* to cadmium pollution. *J Appl Phycol* 28(2):991–999. <https://doi.org/10.1007/s10811-015-0630-5>
- Benzie IFF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of “Antioxidant Power”: the FRAP Assay. *Anal Biochem* 239(1):70–76. <https://doi.org/10.1006/abio.1996.0292>
- Bhagyaawant SS, Gautam AK, Narvekar DT, Gupta N, Bhadkaria A, Srivastava N, Upadhyaya HD (2018) Biochemical diversity evaluation in chickpea accessions employing mini-core collection. *Physiol Mol Biol Plants* 24(6):1165–1183. <https://doi.org/10.1007/s12298-018-0579-3>
- Bondet V, Brand-Williams W, Berset C (1997) Kinetics and mechanisms of antioxidant activity using the DPPH free radical method. *Lebensm Wiss Technol* 30(6):609–615. <https://doi.org/10.1006/fstl.1997.0240>
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72(1–2):248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
- Chatterjee C, Dube BK, Sinha P, Srivastava P (2004) Detrimental effects of lead phytotoxicity on growth, yield, and metabolism of rice. *Commun Soil Sci Plant Anal* 35(1–2):255–265. <https://doi.org/10.1081/CSS-120027648>
- Chauhan JS, Singh BB, Gupta S (2016) Enhancing pulses production in India through improving seed and variety replacement rates. *Indian J Genet Plant Breed* 76(4):410–419. <https://doi.org/10.5958/0975-6906.2016.00060.2>
- Davies KM, Albert NW, Zhou Y, Schwinn KE (2018) Functions of flavonoid and betalain pigments in abiotic stress tolerance in plants. *Annu Plant Rev* 1(1):1–41. <https://doi.org/10.1002/9781119312994.apr0604>
- Fryzova R, Pohanka M, Martinkova P, Cihlarova H, Brtnicky M, Hladky J, Kynicky J (2017) Oxidative stress and heavy metals in plants. *Rev Environ Contam Toxicol* 245:129–156. https://doi.org/10.1007/398_2017_7
- Gall JE, Boyd RS, Rajakaruna N (2015) Transfer of heavy metals through terrestrial food webs: a review. *Environ Monit Assess* 187(4):201. <https://doi.org/10.1007/s10661-015-4436-3>
- Ghori NH, Ghori T, Hayat MQ, Imadi SR, Gul A, Altay V, Ozturk M (2019) Heavy metal stress and responses in plants. *Int J Environ Sci Technol*. <https://doi.org/10.1007/s13762-019-02215-8>
- Gupta N, Shrivastava N, Bhagyaawant SS (2017) Multivariate analysis based on nutritional value, antinutritional profile and antioxidant capacity of forty chickpea genotypes grown in India. *J Nutr Food Sci* 7(3):600. <https://doi.org/10.4172/2155-9600.1000600>
- Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package for education and data analysis. *Palaeontol Electron* 4(1):9
- Hare PD, Cress W (1997) Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regul* 21(2):79–102. <https://doi.org/10.1023/A:1005703923347>
- Hasanuzzaman M, Hossain MA, da Silva JAT, Fujita M (2012) Plant response and tolerance to abiotic oxidative stress: antioxidant defense is a key factor. In: Venkateswarlu B, Shanker A, Shanker C, Maheswari M (eds) *Crop stress and its management: perspectives and strategies*. Springer, Dordrecht. https://doi.org/10.1007/978-94-007-2220-0_8
- Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A (2012) Role of proline under changing environments. *Plant Signal Behav* 7(11):1456–1466. <https://doi.org/10.4161/psb.21949>
- Herrera-Vásquez A, Salinas P, Holuigue L (2015) Salicylic acid and reactive oxygen species interplay in the transcriptional control of defense genes expression. *Front Plant Sci* 19(6):171. <https://doi.org/10.3389/fpls.2015.00171>
- Imtiaz M, Mushtaq MA, Rizwan MS, Arif MS, Yousaf B, Ashraf M, Shuanglian X, Rizwan M, Mehmood S, Tu S (2016) Comparison of antioxidant enzyme activities and DNA damage in chickpea (*Cicer arietinum* L.) genotypes exposed to vanadium. *Environ Sci Pollut Res* 23(19):19787–19796. <https://doi.org/10.1007/s11356-016-7192-1>
- Jaiswal A, Verma A, Jaiswal P (2018) Detrimental effects of heavy metals in soil, plants, and aquatic ecosystems and in humans. *J Environ Path Toxicol Oncol* 37(3):183–197. <https://doi.org/10.1615/JEnvironPatholToxicolOncol.2018025348>
- Jiang HW, Liu MJ, Chen IC, Huang CH, Chao LY, Hsieh HL (2010) A glutathione S-transferase regulated by light and hormones participates in the modulation of Arabidopsis seedling development. *Plant Physiol* 154:1646–1658. <https://doi.org/10.1104/pp.110.159152>
- Kada S, Bouriche H, Senator A, Demirtaş I, Özen T, Toptancı BC, Kızıl G, Kızıl M (2017) Protective activity of *Hertia cheirifolia* extracts against DNA damage, lipid peroxidation and protein oxidation. *Pharm Biol* 55(1):330–337. <https://doi.org/10.1080/13880209.2016.1261907>
- Khan F, Hussain S, Tanveer M, Khan S, Hussain HA, Iqbal B, Geng M (2018) Coordinated effects of lead toxicity and nutrient deprivation on growth, oxidative status, and elemental composition of primed and non-primed rice seedlings. *Environ Sci Pollut Res* 25(21):21185–21194. <https://doi.org/10.1007/s11356-018-2262-1>
- Khoo HE, Azlan A, Ismail A, Abas F (2013) Response surface methodology optimization for extraction of phenolics and antioxidant capacity in defatted dabai parts. *Sains Malays* 42(7):949–954
- Kizil M, Kizil G, Yavuz M, ÇeKen B (2009) Protective ability of ethanol extracts of *Hypericum scabroides* Robson & Poulter and *Hypericum triquetrifolium* Turra against protein oxidation and DNA damage. *Food Sci Biotechnol* 18(1):130–136
- Kizil M, Kizil G, Yavuz M, ÇeKen B (2010) Protective activity of ethanol extract of three achillea species against lipid peroxidation, protein oxidation and DNA damage in vitro. *Acta Aliment* 39(4):457–470. <https://doi.org/10.1556/AAlim.39.2010.4.7>
- Kizil G, Kizil M, Ceken B, Yavuz M, Demir H (2011) Protective activity of ethanol extracts of *Hypericum scabrum* L. and *Hypericum retusum* Aucher against the protein oxidation and DNA damage. *Int J Food Prop* 14(4):926–940. <https://doi.org/10.1080/10942910903491181>
- Kohli SK, Handa H, Sharma A, Gautam V, Arora S, Bhardwaj R, Alyemeni MN, Wijaya L, Ahmad P (2017) Combined effect of 24-epibrassinolide and salicylic acid mitigates lead (Pb) toxicity by modulating various metabolites in *Brassica juncea* L. seedlings. *Protoplasma* 255(1):11–24. <https://doi.org/10.1007/s00709-017-1124-x>
- Kováčik J, Klejdus B, Hedbavny J, Štork F, Bačkor M (2009) Comparison of cadmium and copper effect on phenolic metabolism, mineral nutrients and stress-related parameters in *Matricaria chamomilla* plants. *Plant Soil* 320:231–242. <https://doi.org/10.1007/s11104-009-9889-0>
- Kumar B, Kumari S, Flores LC (2017) Plant mediated detoxification of mercury and lead. *Arab J Chem* 10:S2335–S2342. <https://doi.org/10.1016/j.arabjc.2013.08.010>
- La VH, Lee BR, Islam MT, Park SH, Jung H, Bae DW, Kim TH (2019) Characterization of salicylic acid-mediated modulation of the drought stress responses: reactive oxygen species, proline, and redox state in *Brassica napus*. *Environ Exp Bot* 157:1–10. <https://doi.org/10.1016/j.envexpbot.2018.09.013>

- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685. <https://doi.org/10.1038/227680a0>
- Li W, Mao R, Liu X (2005) Effects of stress duration and non-toxic ions on heavy metals toxicity to *Arabidopsis* seed germination and seedling growth. *Ying Yong Sheng Tai Xue Bao* 16:1943–1947
- Malec P, Waloszek KA, Prasad MNV, Strzałka K (2008) Zinc reversal of Cd-induced energy transfer changes in photosystem II of *Ceratophyllum demersum* L. as observed by whole-leaf 77K fluorescence. *Plant Stress* 2:121–126
- Maleva MG, Nekrasova GF, Malec P, Prasad MNV, Strzałka K (2009) Ecophysiological tolerance of *Elodea canadensis* to nickel exposure. *Chemosphere* 77:393–398. <https://doi.org/10.1016/j.chemosphere.2009.07.024>
- Manquán-Cerda K, Cruces E, Escudey M, Zúñiga G, Calderón R (2018) Interactive effects of aluminum and cadmium on phenolic compounds, antioxidant enzyme activity and oxidative stress in blueberry (*Vaccinium corymbosum* L.) plantlets cultivated *in vitro*. *Ecotoxicol Environ Saf* 150:320–326. <https://doi.org/10.1016/j.ecoenv.2017.12.050>
- Mildvan AS (1970) Metal in enzymes catalysis. In: Boyer PD (ed) *The enzymes*. Academic Press, London, pp 445–536
- Nagajyoti PC, Lee KD, Sreekanth TVM (2010) Heavy metals, occurrence and toxicity for plants: a review. *Environ Chem Lett* 8:199–216. <https://doi.org/10.1007/s10311-010-0297-8>
- Ojima K, Ohira K (1983) Characterization of aluminium and manganese tolerant cell lines selected from carrot cell cultures. *Plant Cell Physiol* 24(5):789–797. <https://doi.org/10.1093/oxfordjournals.pcp.a076579>
- Rehman M, Maqbool Z, Peng D, Liu L (2019) Morpho-physiological traits, antioxidant capacity and phytoextraction of copper by ramie (*Boehmeria nivea* L.) grown as fodder in copper-contaminated soil. *Environ Sci Pollut Res*. <https://doi.org/10.1007/s11356-018-4015-6>
- Rizwan M, Ali S, Ali B, Adrees M, Arshad M, Hussain A, Rehman MZ, Waris AA (2018) Zinc and iron oxide nanoparticles improved the plant growth and reduced the oxidative stress and cadmium concentration in wheat. *Chemosphere* 214:269–277. <https://doi.org/10.1016/j.chemosphere.2018.09.120>
- Sabatini SE, Juarez AB, Eppis MR, Bianchi L, Luquet CM, de Molina MDR (2009) Oxidative stress and antioxidant defenses in two green microalgae exposed to copper. *Ecotoxicol Environ Saf* 72(4):1200–1206. <https://doi.org/10.1016/j.ecoenv.2009.01.003>
- Sahu KK, Biswas D, Naithani SC (2010) *Aluminum stress on germinating seeds of Cicer arietinum*. Lambert Academic Publishing House, Saarbrücken
- Saif S, Khan MS (2018) Assessment of toxic impact of metals on proline, antioxidant enzymes, and biological characteristics of *Pseudomonas aeruginosa* inoculated *Cicer arietinum* grown in chromium and nickel-stressed sandy clay loam soils. *Environ Monit Assess* 190:290. <https://doi.org/10.1007/s10661-018-6652-0>
- Seneviratne M, Rajakaruna N, Rizwan M, Madawala HMSP, Ok YS, Vithanage M (2018) The effects of heavy metal-induced oxidative stress on seed germination and seedling development: a critical review. *Environ Geochem Health*. <https://doi.org/10.1007/s10653-017-0005-8>
- Shahi S, Kumar R, Srivastava M (2019) Antioxidant enzyme and biochemical behaviour of chickpea cv. bg-256 and csg-8962 under salt stress conditions. *Appl Biol Res* 21(1):74–80. <https://doi.org/10.5958/0974-4517.2019.00009.0>
- Shahzad B, Tanveer M, Che Z, Rehman A, Cheema SA, Sharma A, Song H, ur Rehman S, Zhaorong D (2018) Role of 24-epibrassinolide (EBL) in mediating heavy metal and pesticide induced oxidative stress in plants: a review. *Ecotoxicol Environ Saf* 147:935–944. <https://doi.org/10.1016/j.ecoenv.2017.09.066>
- Sharma RK, Madhoolika A, Marshall FM (2004) Effects of waste water irrigation on heavy metal accumulation in soil and plants. Paper presented at a National Seminar, Bangalore University, Bangalore, Abstract No. 7, p 8
- Shih MK, Hu ML (1996) UVA-potentiated damage to calf thymus DNA by Fenton reaction system and protection by paraaminobenzoic acid. *Photochem Photobiol* 63(3):286–291. <https://doi.org/10.1111/j.1751-1097.1996.tb03027.x>
- Siddiqui S (2013) Exposure of Cu and Mn to *Cicer arietinum* L. Var. BGD-72 seeds induces morphological and biochemical changes in the plant. *South Asian J Exp Biol* 3:31–36
- Šiukšta R, Bondzinskaitė S, Kleizaitė V, Žvingila D, Taraškevičius R, Mockeliūnas L, Stapulionytė A, Mak K, Česnienė T (2019) Response of *Tradescantia* plants to oxidative stress induced by heavy metal pollution of soils from industrial areas. *Environ Sci Pollut Res* 26(1):44–61. <https://doi.org/10.1007/s11356-018-3224-3>
- Stadtman ER, Levin RL (2000) Protein oxidation. *Ann N Y Acad Sci* 899:191–208
- Strejkova A, Dvorak M, Klejdus B, Krystofova O, Hedbavny J, Adam V, Huska D (2019) The strong reaction of simple phenolic acids during oxidative stress caused by nickel, cadmium and copper in the microalga *Scenedesmus quadricauda*. *New Biotechnol* 48:66–75. <https://doi.org/10.1016/j.nbt.2018.07.004>
- Swain T, Hillis WE (1959) Phenolic constituents of *Prunus domestica* L.—Quantitative analysis of phenolic constituents. *J Sci Food Agric* 10:63–68. <https://doi.org/10.1002/jsfa.2740100110>
- Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* 218(1):1–14. <https://doi.org/10.1007/s00425-003-1105-5>
- Wani AS, Ahmad A, Hayat S, Tahir I (2019) Epibrassinolide and proline alleviate the photosynthetic and yield inhibition under salt stress by acting on antioxidant system in mustard. *Plant Physiol Biochem* 135:385–394. <https://doi.org/10.1016/j.plaphy.2019.01.002>
- Xiao Y, Xing G, Rui X, Li W, Chen X, Jiang M, Dong M (2014) Enhancement of the antioxidant capacity of chickpeas by solid state fermentation with *Cordyceps militaris* SN-18. *J Funct Foods* 10:210–222. <https://doi.org/10.1016/j.jff.2014.06.008>
- Yadav S, Irfan M, Ahmad A, Hayat S (2011) Causes of salinity and plant manifestations to salt stress: a review. *J Environ Biol* 32(5):667–685
- Yusuf M, Fariduddin Q, Varshney P, Ahmad A (2012) Salicylic acid minimizes nickel and/or salinity-induced toxicity in Indian mustard (*Brassica juncea*) through an improved antioxidant system. *Environ Sci Pollut Res* 19(1):8–18. <https://doi.org/10.1007/s11356-011-0531-3>
- Zeng L, Linc X, Zhoua F, Qin J, Li H (2019) Biochar and crushed straw additions affect cadmium absorption in cassava peanut intercropping system. *Ecotoxicol Environ Saf* 167:520–530. <https://doi.org/10.1016/j.ecoenv.2018.10.003>
- Zou TB, He TP, Li HB, Tang HW, Xia EQ (2016) The structure–activity relationship of the antioxidant peptides from natural proteins. *Molecules* 21(1):72. <https://doi.org/10.3390/molecules21010072>

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