Research Article

Assessment of biochemical and physiological tolerance mechanism of the multipurpose paradise tree (*Simarouba amara* Aubl.) under zinc and copper stress

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Abstract

Simarouba amara Aubl., commonly known as paradise tree, is a multipurpose, evergreen, poly-gamodioecious, and oil yielding tree. The plant is famous for its seeds containing 55-65% oil, a potent source of biodiesel production and is being utilized in cosmetics, pharmaceuticals, and other industries. The study aimed to evaluate the physiological and biochemical changes that occur in *S. amara* seedlings under heavy metals stress. Two-month-old *S. amara* seedlings were exposed to different concentrations of zinc (Zn) and copper (Cu) (Zn and Cu: 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1}, 200 mg Kg^{-1}). The study indicated that both the heavy metals resulted in a significant decrease in leaf relative water content (LRWC), photosynthetic pigments and an increase in lipid peroxidation and antioxidant levels. Regarding lipid peroxidation, Cu proved to be more toxic to seedlings compared to Zn. However, in terms of LRWC and photosynthetic pigments, Zn showed higher toxic effects than Cu. Proline and cysteine content increased by 234% and 270%, respectively, due to Zn stress and 117% and 102%, respectively, due to Cu stress at 200 mg Kg^{-1}. Among antioxidant enzymes, a maximum increase in glutathione reductase (GR) activity was observed (600% due to Cu stress and 320% due to Zn stress) at 200 mg Kg^{-1}. At the same concentration, a minimum increase was seen in glutathione peroxidase (GPX) activity (60% under Cu stress) and catalase (CAT) activity (69% under Zn stress). The present study revealed that *S. amara* has a better antioxidant defensive mechanism against oxidative stress and can be used for its large scale cultivation on wastelands.

Keywords: *Simarouba amara*, Antioxidants, Wastelands, Lipid peroxidation

INTRODUCTION

Depleting fertile lands due to wastelands extension is one of the major burning problems in the current scenario. Wastelands are degraded lands that have deteriorated due to natural causes or lack of soil management. Wastelands are environmentally fragile regions with poor production (80% less biomass production) and are heavily impacted by soil erosion, stress, and adverse environmental conditions (Pandey and Singh, 2020).Wastelands remediation is essential and best solution for solving hunger and malnutrition problems. Restoration of plant cover is a more environmentally friendly approach to wasteland rehabilitation than other

physical or chemical methods (Bhattacharyya, 2022). Wastelands can be brought under vegetation cover through proper management techniques. In wastelands, plants face various abiotic stresses such as water stress (drought or flood), salt stress, heavy metal contamination stress, etc. Abiotic stresses are the major cause of limited crop production and are serious threats for agriculture sustainability. Most of the different types of stresses share common phenomenon of redox metabolism disturbances due to overproduction and accumulation of reactive oxygen species (ROS) such as superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), which lead to the induction of oxidative stress (Cavalcanti *et al*., 2007; Adem *et al*., 2014). Above threshold levels, ROS result in enhanced lipid peroxidation (both in cellular and organellar membranes), protein oxidation, nucleic acid damage, programmed cell death, etc. Thus, enhanced lipid peroxidation is an indicator of oxidative stress.

Heavy metals such as zinc (Zn) and copper (Cu) are essential for plant life but only in trace amounts. These metals become toxic for the plant at higher concentrations due to oxidative stress induction, causing various physiological and biochemical changes (Hussain *et al*., 2010). Zinc is involved in various biochemical processes, such as the production of chlorophyll pigments, enzyme activation, nucleotide and cytochrome synthesis, auxin metabolism and membrane integrity (Marschner, 1995). Zn induces iron and magnesium deficiency at higher concentrations, leading to chlorosis in young leaves. Copper functions as constituent of various enzymes that catalyze redox reactions in chloroplast and mitochondria and involved in electron flow (Hansch and Mendel, 2009). Copper toxicity leads to chlorosis, ion leakage due to plasma membrane permeability damage, etc. (Bouazizi *et al*., 2010).

To overcome the harmful effects under stress conditions (oxidative damage), plants have evolved antioxidant defense mechanism (both enzymatic and non-enzymatic) that allows rapid stress detection followed by modification in the plant physiology and metabolism to minimize the damage. Enzymatic antioxidants involve superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR), while proline and cysteine constitute non-enzymatic antioxidants (Nanda and Agrawal, 2016). Plants having higher levels of antioxidants are considered to be more resistant to oxidative damage.

Phytostabilization (reduction in the bioavailability of heavy metals in soil by establishing vegetation cover) and phytoextraction (extraction of heavy metals from soil and translocate to aerial parts of plants) are the two important strategies for remediation of heavy metal contaminated soils by using plants (Jacob *et al*., 2018, Mench *et al*., 2010). Identification of hyperaccumulator

plants is crucial for heavy metal phytoremediation. Over, 450 plant species have been identified as effective metal hyperaccumulators (Suman *et al*., 2018). Brassica species have been reported as effective phytoremediator (Bortoloti and Baron, 2022). Other crop plants such as *Cannabis sativa*, *Nicotiana tabacum*, *Zea mays*, etc. have been reported to remove heavy metals from contaminated soil through phytoextraction (Tlustos *et al*., 2006; Vangronsveld *et al*., 2009; Herzig *et al*., 2014). *Eleocharis acicularis*, *Aeolanthus biformifolius*, *Ipomoea alpine*, etc., are copper hyper accumulator species (Chaney *et al*., 2010, Mitch, 2002) and *Thlaspi caerulescens*, *Eleocharis acicularis*, *Thlaspi calaminare*, etc. are zinc hyper accumulator species (Sakakibara *et al*., 2011, Sheoran *et al*., 2009).

Simarouba amara Aubl. (Paradise tree) is a member of the family Simaroubaceae and indigenous to El Salvador in Central America. It is a versatile, evergreen, polygamodioecious, oil yielding tree in which seeds produce 65-75% oil, suitable for both edible and non-edible purposes. Its oil is a promising source of biodiesel and is being utilized by various cosmetic, pharmaceutical, beverages, fermentation, furniture industries, etc. Being suitable for growing even on wastelands, this plant was introduced in India in 1966 mainly for soil conservation. However, information about the performance of *S. amara* plants under soil contaminated with heavy metals is limited. In *S. amara*, no studies have been conducted to analyze the effect of heavy metals on the plants. Effect of water stress and its amelioration through foliar application of salicylic acid, putriscine, gamma amino butyric acid and abscisic acid have been investigated by Awate and Gaikwad (2014). Thus, the aim of the present study was to determine the effect of heavy metals (Zn and Cu) on *S. amara* plants. Since most crop plants' seedlings' growth stages are sensitive to various biotic or abiotic stresses, two-month-old seedlings were preferred for the stress study.

MATERIALS AND METHODS

Plant material and stress treatments

Seeds of *S. amara* were procured from Dr. Panjabrao Deshmukh Krishi Vidyapeeth (PDKV) University, Akola, Maharashtra and University of Agriculture Sciences, Bangalore, India (Fig. 1A-C). Seeds were surface sterilized with 5% sodium hypochlorite solution for 20 minutes and then washed thoroughly with distilled water. Seeds were sown in clay pots containing autoclaved soil and were allowed to germinate under natural conditions of light, temperature and humidity. After 15-25 days of sowing, *S. amara* seeds started germination (Fig. 1D). Two-month-old uniform seedlings were transferred to other clay pots containing 4 kg of autoclaved sandy loam soil exposed to different concentra-

Fig. 1. *S. amara (A) trees growing at the Dr. Panjabrao Deshmukh Krishi Vidyapeeth (P.D.K.V.) University, Akola, Maharashtra, India, (B) fruits, (C) seeds, and (D) fifteen-day-old seedlings growing under controlled conditions.*

tions of Zn and Cu (10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1}, 200 mg Kg^{-1}). Before conducting experiments, aqueous solutions of $ZnSO_4$ and $CuSO_4$ were added to the soil in pots and pots were allowed to equilibrate for one month by undergoing repeated cycles of saturation with distilled water and air drying. In each pot, three seedlings (2-month-old) were planted, and pots were placed in greenhouse with 25±2°C and 55% relative humidity. Each of these treatments had three replicates and pots without the addition of Zn and Cu served as control. The leaves of plants were harvested after 21 days of stress treatment (Fig. 2) and stored at -80°C for further experiments. All the fine chemicals used in the study were procured from Merck, Life Science, India.

Morphology analysis and LRWC determination
Morphological traits of all the *S. amara* seedlings after 21 days of stress treatment were analyzed. Leaf relative water content was determined following the method of Turner (1981) and calculated by the equation:
LRWC (%)=[(fresh weight of the sample-dry weight of sample)/(turbid weight of the sample-dry weight of sample)] x 100.   …..Eq. 1

Heavy metal accumulation
S. amara seedlings exposed to different concentrations of Zn and Cu were washed properly with 0.1 M HNO_3 solution. Leaves from all the seedlings were taken and then oven dried at 60°C for 90 h. Dried leaves were digested with 4 mL of HNO_3/$HClO_4$ (3:1, v/v) solution and further dissolved in 10 mL of 0.1 N HNO_3 solution. Atomic absorption spectrophotometer was employed to measure metal content in the leaf samples.

Malondialdehyde (MDA) estimation
For calculating the MDA content, protocol given by Heath and Packer (1968) was employed.

Photosynthetic pigments
Leaf discs (100 mg) were soaked in 80% (v/v) acetone solution and kept in dark overnight. The absorbance of the acetone extracts was measured at 663, 645 and 470 nm. The concentrations of chlorophyll a and chlorophyll b were calculated using Arnon's equations (Arnon, 1949) and carotenoids content was measured following the formula given by Lichtenthaler and Wellburn (1983).

Non-enzymatic antioxidants
Proline estimation
Proline content was calculated following the protocol given by Bates *et al*. (1973).

Cysteine estimation
For calculating cysteine concentration, Gaitonde (1967) method was employed.
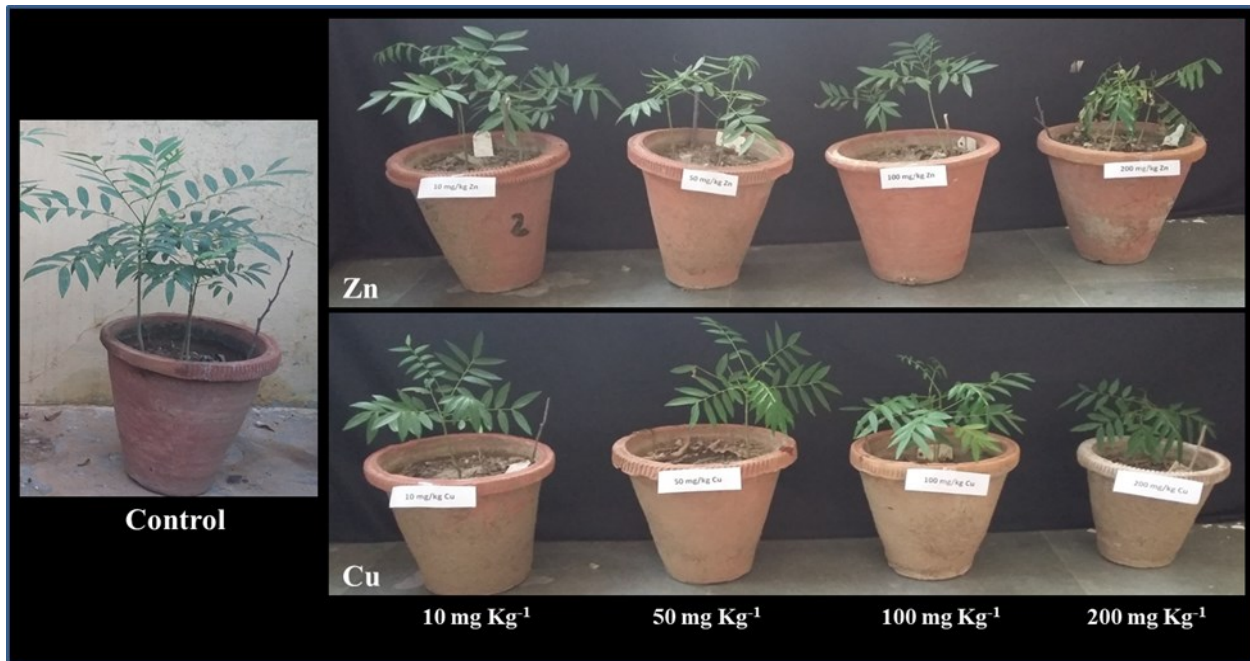
Fig. 2. *Effect of different concentrations (0, 10, 50, 100, and 200 mg Kg^{-1}) of Zn and Cu on two-month-old Simarouba amara plants, showing healthy plants under control condition and lower concentrations of Cu and Zn. Plants exposed to higher concentrations of Zn and Cu (200 mg Kg^{-1}) showing yellowing, curling, and wilting of leaves.*

Antioxidant enzyme assays

S. amara leaves (1 g) were ground to powder in liquid nitrogen using pre-chilled mortar and pestle. The powder was homogenized with 4 mL of chilled 0.2 M phosphate buffer (pH 7.8) containing 0.1 mM EDTA. The homogenate was centrifuged at 13,000 x g at 4ºC for 20 min. The supernatant was used for all the enzyme activity assays. Total soluble protein of the extract was measured following the Bradford assay (Bradford, 1976) employing bovine serum albumin (BSA) as the standard. All the assays were performed thrice.

Superoxide dismutase (SOD) (EC 1.15.1.1) assay

SOD was determined employing modified NBT method (Beyer and Fridovich, 1987).

Catalase (CAT) (EC 1.11.1.6) assay

CAT was measured by the procedure described by Aebi (1984).

Glutathione peroxidase (GPX) (EC 1.11.1.7) assay

GPX assay was conducted following the method of Thimmaiah (1999).

Ascorbate peroxidase (APX) (EC 1.11.1.11) assay

APX assay was conducted employing modified method of Nakano and Asada (1981).

Glutathione Reductase (GR) (EC 1.6.4.2) assay

GR was measured following the protocol of Schaedle and Bassham (1977).

Statistical analysis

All data in the present study was represented as mean ± standard deviation of the mean (SD) and was analyzed employing one-way ANOVA through SPSS version 21.0 software. The differences between treatments were detected using Duncan's multiple range tests (p<0.05).

RESULTS

Morphology analysis and leaf relative water content (LRWC) determination

No striking differences in the plant height were observed between the control and stress treated *S. amara* seedlings. However, under severe stress conditions, leaves of *S. amara* seedlings showed curling, chlorosis and senescence. Leaves of *S. amara* seedlings exposed to 200 mg Kg^{-1} concentration of Zn revealed maximum wilting. Copper treated seedlings seemed to be less affected and did not show significant changes compared to control (Fig. 2). Leaf relative water content (LRWC) of control seedlings was found to be 69.74% (Table 1)which was higher than *S. amara* seedlings exposed to different concentrations of Zn and Cu. Zn treated seedlings exhibited 61.86%, 59.88%, 54.40% and 38.89% LRWC at 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} concentrations respectively (Fig. 3A). Decrease in LRWC was found to be 44% at the highest Zn concentration (200 mg Kg^{-1}) (Table 1). The present study also observed a decrease

in LRWC due to Cu stress. At 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} of Cu concentrations, LRWC was found to be 67.10%, 58.53%, 56.04% and 52.01% respectively in *S. amara* seedlings (Fig. 3A). LRWC decreased by 25% at 200 mg Kg^{-1} Cu concentration (Table 1).

Heavy metal accumulation
Concentration of Zn in the leaves of *S. amara* seedlings was found to be 142.33, 401.06, 502.45, and 601.43 mg kg^{-1} DW exposed to 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} of Zn concentrations, respectively. While, accumulation of Cu in the leaves was 63.09, 279.09, 446.04, and 523.45 mg kg^{-1} DW exposed to 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} of Cu concentrations, respectively (Table 1).

MDA estimation
S. amara seedlings showed a significant (p<0.05) increase in MDA content when exposed to increasing concentration of Zn and Cu. Compared to the MDA content of control seedlings (13.12 µmol g-1 FW), the MDA content of stress-treated seedlings was higher. Seedlings exposed to 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} Zn concentrations, exhibited MDA content of 11.18 µmol g^{-1} FW, 23.44 µmol g^{-1} FW, 25.38 µmol g^{-1} FW and 45.59 µmol g^{-1} FW respectively (Fig. 3B). MDA content increased by 247% at maximum Zn concentration (200 mg Kg^{-1}) (Table 1). Cu stress also resulted in increase in MDA content in *S. amara* seedlings. MDA content was found to be 14.84 µmol g^{-1} FW, 21.72 µmol g^{-1} FW, 24.09 µmol g^{-1} FW and 68.60 µmol g^{-1} FW in *S. amara* seedlings exposed to 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} of Cu concentrations respectively (Fig. 3B). The

increase in MDA content at highest Cu concentration (200 mg Kg^{-1}) was 422% (Table 1).

Photosynthetic pigments
Both Zn and Cu stresses resulted in decrease in photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) in *S. amara* seedlings. Control seedlings exhibited 1.43 mg g^{-1} FW, 1.003 mg g^{-1} FW and 10.44 mg g^{-1} FW chlorophyll a, chlorophyll b and carotenoids contents respectively (Fig. 3C and D). Chlorophyll a content in *S. amara* seedlings was found to be 0.94 mg g^{-1} FW, 1.18 mg g^{-1} FW, 1.26 mg g^{-1} FW and 0.74 mg g^{-1} FW at 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} of Zn concentrations respectively (Fig. 3C). At similar zinc concentrations, chlorophyll b content was 0.84 mg g^{-1} FW, 0.85 mg g^{-1} FW, 0.26 mg g^{-1} FW and 0.08 mg g^{-1} FW and carotenoids content was 12.41 mg g^{-1} FW, 12.77 mg g^{-1} FW, 9.04 mg g^{-1} FW and 4.87 mg g^{-1} FW respectively (Fig. 3C). Percentage decrease in chlorophyll a, chlorophyll b and carotenoids content at 200 mg Kg^{-1} Zn concentration was 48%, 92% and 53% respectively (Table 1). Cu stress also resulted in decrease in photosynthetic pigments in *S. amara* seedlings. At 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} Cu concentrations, seedlings exhibited 1.01 mg g^{-1} FW, 1.02 mg g^{-1} FW, 1.26 mg g^{-1} FW and 0.75 mg g^{-1} FW chlorophyll a content, respectively (Fig. 3D). At same concentrations of Cu, chlorophyll b content was 0.71 mg g^{-1} FW, 0.84 mg g^{-1} FW, 0.73 mg g^{-1} FW and 0.57 mg g^{-1} FW and carotenoids content was 11.43 mg g^{-1} FW, 11.65 mg g^{-1} FW, 11.32 mg g^{-1} FW and 10.50 mg g^{-1} FW respectively (Fig. 3D). The decrease in chlorophyll a, and chlorophyll b content at maximum Cu concentration (200 mg g^{-1} FW) was 47% and 43%, respectively. Carotenoids content increased with 0.57%

Table 1. Effects of Zn and Cu stresses on different physiological and biochemical parameters in *S. amara* seedlings in terms of percentage increase (↑) or decrease (↓) in their activities and contents compared to control seedlings

Parameters	Control	Zn		Cu	
		200 mg Kg^{-1}	% ↑ or ↓	200 mg Kg^{-1}	% ↑ or ↓
LRWC (%)	69.74	38.89	↓ 44.23	52.01	↓ 25.42
MDA content (µmol g^{-1} FW)	13.12	45.59	↑ 247.48	68.60	↑ 422.86
Chl. a content (mg g^{-1} FW)	1.43	0.74	↓ 48.25	0.75	↓ 47.55
Chl. b content (mg g^{-1} FW)	1.003	0.08	↓ 92.02	0.57	↓ 43.17
Carotenoids content (mg g^{-1} FW)	10.44	4.87	↓ 53.35	10.50	↑ 0.57
SOD activity (Unit mg$_{protein}^{-1}$)	6.25	13.08	↑ 109.28	15.72	↑ 151.52
CAT activity (mmolH$_2$O$_2$min^{-1} mg$_{prot}^{-1}$)	0.62	1.05	↑ 69.35	1.33	↑ 114.52
APX activity (mmol$_{Vit.C}$min^{-1} mg$_{prot}^{-1}$)	1.57	2.74	↑ 74.52	2.66	↑ 69.43
GPX activity (mmol$_{tetraguaiacol}$min^{-1} mg$_{prot}^{-1}$)	0.05	0.09	↑ 80.00	0.08	↑ 60.00
GR activity (mmol$_{NADPH}$min^{-1} mg$_{prot}^{-1}$)	0.05	0.21	↑ 320.00	0.35	↑ 600.00
Proline content (µmol g^{-1} FW)	26.19	87.50	↑ 234.10	56.85	↑ 117.07
Cysteine content (µmol g^{-1} FW)	20.17	74.82	↑ 270.95	40.80	↑ 102.28

at 200 mg Kg^{-1} Cu concentration (Table 1).

Non-enzymatic antioxidants
Proline estimation
Proline content was found to be significantly (p<0.05) increased in *S. amara* seedlings exposed to different concentrations of both the heavy metals (Zn and Cu). Control seedlings exhibited 26.19 μmol g^{-1} FW proline content (Fig. 3E). At 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} Zn concentrations, seedlings exhibited proline content of 31.40 μmol g^{-1} FW, 37.88 μmol g^{-1} FW, 70.88 μmol g^{-1} FW and 87.50 μmol g^{-1} FW respectively. (Fig. 3E). Proline content increased significantly (p<0.05) by 234% at 200 mg Kg^{-1} Zn (Table 1). Similarly, *S. amara* seedlings exposed to different Cu concentrations also resulted in increase in proline concentration. Proline content was found to be 31.04 μmol g^{-1} FW, 33.55 μmol g^{-1} FW, 34.91 μmol g^{-1} FW and 56.85 μmol g^{-1} FW in *S. amara* seedlings exposed to 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1}, 200 mg Kg^{-1} of Cu concentrations respectively (Fig. 3E). The increase in proline content at maximum Cu concentration (200 mg Kg^{-1}) was 117% (Table 1).

Cysteine estimation
Cysteine content in control *S. amara* seedlings was found to be 20.17 μmol g^{-1} FW which was lesser than seedlings exposed to different concentrations of Zn and Cu. At 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} Zn concentrations, *S. amara* seedlings exhibited proline content of 21.61 μmol g^{-1} FW, 44.11 μmol g^{-1} FW, 46.70 μmol g^{-1} FW and 74.82 μmol g^{-1} FW respectively (Fig. 3F). Cysteine content increased by 270% at 200 mg Kg^{-1} Zn concentration (Table 1). Increase in cysteine content was also observed in *S. amara* seedlings exposed to Cu stress. Cysteine content was found to be 29.02 μmol g^{-1} FW, 33.12 μmol g^{-1} FW, 35.71 μmol g^{-1} FW and 40.80 μmol g^{-1} FW in *S. amara* seedlings treated with 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1}, 200 mg Kg^{-1} Cu concentrations respectively (Fig. 3F). Percentage increase in cysteine content at maximum Cu concentration (200 mg Kg^{-1}) was 102% (Table 1).

Antioxidant enzyme assays
SOD assay
In *S. amara* seedlings, both Zn and Cu stresses resulted in increase in SOD activity. Control seedlings exhibited 6.25 Unit mg$_{protein}$$^{-1}$ SOD activity and with increase in stress concentrations, SOD activity increased significantly (p<0.05). At 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} concentrations of Zn, seedlings exhibited SOD activities of 12.97 Unit mg$_{protein}$$^{-1}$, 13.23 Unit mg$_{protein}$$^{-1}$, 12.9 Unit mg$_{protein}$$^{-1}$ and 13.08 Unit mg$_{protein}$$^{-1}$ respectively (Fig. 4A). SOD activity increased by 109% at 200 mg Kg^{-1} Zn concentration (Table 1). Simi-

lar pattern of increase in SOD activity was observed in case of Cu treated seedlings. SOD activities were found to be 15.23 Unit mg$_{protein}$$^{-1}$, 15.67 Unit mg$_{protein}$$^{-1}$, 15.68 Unit mg$_{protein}$$^{-1}$ and 15.72 Unit mg$_{protein}$$^{-1}$ in *S. amara* seedlings exposed to 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} Cu concentrations respectively (Fig. 4A). SOD activity increased by 151% at maximum Cu concentration (200 mg Kg^{-1}) (Table 1).

CAT assay
A significant (p<0.05) increase in CAT activity was observed, when *S. amara* seedlings were exposed to different concentrations of Zn and Cu.CAT activity of control seedlings was found to be 0.62 mmol$_{H2O2}$min^{-1} mg$_{prot}$$^{-1}$ and it increased significantly (p<0.05) due to both of the stresses (Fig. 4B).At 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} concentrations of Zn, seedlings exhibited CAT activities of 0.58 mmol$_{H2O2}$min^{-1} mg$_{prot}$$^{-1}$, 0.64 mmol$_{H2O2}$min^{-1} mg$_{prot}$$^{-1}$, 0.88 mmol$_{H2O2}$min^{-1} mg$_{prot}$$^{-1}$ and 1.05 mmol$_{H2O2}$min^{-1} mg$_{prot}$$^{-1}$ respectively (Fig. 4B). CAT activity increased by 69% at maximum Zn concentration (200 mg Kg^{-1}) (Table 1). Similarly, CAT activity also increased significantly (p<0.05) in *S. amara* seedlings, exposed to Cu stress.CAT activities were found to be 0.55 mmol$_{H2O2}$min^{-1} mg$_{prot}$$^{-1}$, 0.61 mmol$_{H2O2}$min^{-1} mg$_{prot}$$^{-1}$, 0.81 mmol$_{H2O2}$min^{-1} mg$_{prot}$$^{-1}$ and 1.33 mmol$_{H2O2}$min^{-1} mg$_{prot}$$^{-1}$ in seedlings exposed to 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} concentrations of Cu respectively (Fig. 4B). At maximum concentration of Cu (200 mg Kg^{-1}), CAT activity increased by 114% (Table 1).

APX assay
Control *S. amara* seedlings exhibited 1.57 mmol$_{Vit.c}$min^{-1} mg$_{prot}$$^{-1}$ of APX activity and it increased significantly (p<0.05) in *S. amara* seedlings exposed to different concentrations of Zn and Cu. (Fig. 4C). At 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} of Zn concentrations, seedlings exhibited APX activities of 2.10 mmol$_{Vit.c}$min^{-1} mg$_{prot}$$^{-1}$, 2.25 mmol$_{Vit.c}$min^{-1} mg$_{prot}$$^{-1}$, 2.37 mmol$_{Vit.c}$min^{-1} mg$_{prot}$$^{-1}$ and 2.74 mmol$_{Vit.c}$min^{-1} mg$_{prot}$$^{-1}$ respectively (Fig. 4C). APX activity increased by 74% at maximum Zn concentration (200 mg Kg^{-1}) (Table 1). Cu stress also resulted in a significant (p<0.05) increase in APX activity in *S. amara* seedlings. APX activities were found to be 2.29 mmol$_{Vit.c}$min^{-1} mg$_{prot}$$^{-1}$, 2.40 mmol$_{Vit.c}$min^{-1} mg$_{prot}$$^{-1}$, 2.54 mmol$_{Vit.c}$min^{-1} mg$_{prot}$$^{-1}$ and 2.66 mmol$_{Vit.c}$min^{-1} mg$_{prot}$$^{-1}$ in *S. amara* seedlings exposed to 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} Cu concentrations respectively (Fig. 4C). APX activity increased by 69% at 200 mg Kg^{-1} Cu concentration (Table 1).

GPX assay
GPX activity of control *S. amara* seedlings was found to be 0.05 mmol$_{tetraguaiacol}$min^{-1} mg$_{prot}$$^{-1}$ ((Fig. 4D). Com-

pared to control seedlings, GPX activity was found higher in *S. amara* seedlings exposed to both Zn and Cu stresses. GPX activity increased significantly (p<0.05) with increase in concentrations of each stress treatment. At 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} of Zn concentrations, *S. amara* seedlings exhibited APX activities of 0.06 mmol$_{tetraguaiacol}$min^{-1} mg$_{prot}$$^{-1}$, 0.07 mmol$_{tetraguaiacol}$min^{-1} mg$_{prot}$$^{-1}$, 0.08 mmol$_{tetraguaiacol}$min^{-1} mg$_{prot}$$^{-1}$ and 0.09 mmol$_{tetraguaiacol}$min^{-1} mg$_{prot}$$^{-1}$ respectively (Fig. 4D). GPX activity increased by 80% at 200 mg Kg^{-1} Zn concentration (Table 1). Significant (p<0.05) increase in GPX activity was also observed in *S. amara* seedlings exposed to Cu stress. GPX activities were found to be 0.06 mmol$_{tetraguaiacol}$min^{-1} mg$_{prot}$$^{-1}$, 0.06 mmol$_{tetraguaiacol}$min^{-1} mg$_{prot}$$^{-1}$, 0.07 mmol$_{tetraguaiacol}$min^{-1} mg$_{prot}$$^{-1}$and 0.08 mmol$_{tetraguaiacol}$min^{-1} mg$_{prot}$$^{-1}$in *S. amara* seedlings exposed to 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100

mg Kg^{-1} and 200 mg Kg^{-1} Cu concentrations respectively (Fig. 4D). GPX activity increased by 60% at maximum Cu concentration, i.e. at 200 mg Kg^{-1} (Table 1).

GR assay
GR activity significantly (p<0.05) increased in *S. amara* seedlings exposed to Zn and Cu stresses. Control seedlings exhibited 0.05 mmol$_{NADPH}$min^{-1} mg$_{prot}$$^{-1}$ of GR activity, which significantly increased with increase in concentrations of both Zn and Cu stresses (Fig. 4E). At 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} of Zn concentrations, seedlings exhibited GR activities of 0.12 mmol$_{NADPH}$min^{-1} mg$_{prot}$$^{-1}$, 0.13 mmol$_{NADPH}$min^{-1} mg$_{prot}$$^{-1}$, 0.14 mmol$_{NADPH}$min^{-1} mg$_{prot}$$^{-1}$ and 0.21 mmol$_{NADPH}$min^{-1} mg$_{prot}$$^{-1}$ respectively (Fig. 4E). GR activity increased by 320% at 200 mg Kg^{-1} Zn concentration (Table 1). GR activity also significantly (p<0.05)
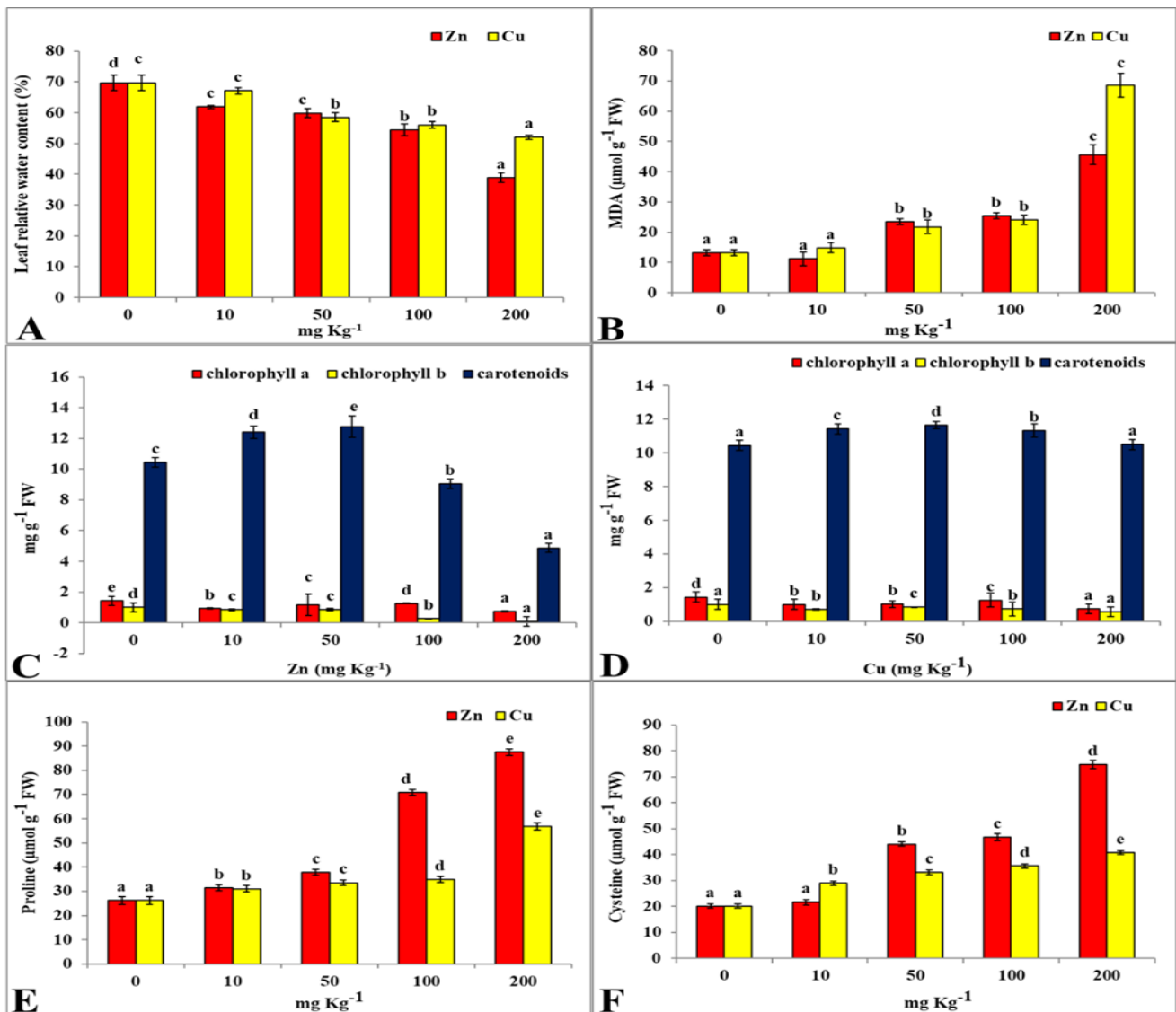

Fig. 3. *(A) Mean leaf relative water content (%), (B) MDA content, (C, D) photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids), (E) proline, and (F) cysteine contents in leaves of 2-month-old S. amara seedlings after 3 weeks in soil inoculated with different Zn and Cu concentrations. Values are means ±SD (n = 9). Bars with the different letters represent significant differences between treatments at p < 0.05*

increased in *S. amara* seedlings exposed to different Cu concentrations. GR activity was found to be 0.1 $mmol_{NADPH}min^{-1}$ mg_{prot}^{-1}, 0.14 $mmol_{NADPH}min^{-1}$ mg_{prot}^{-1}, 0.23 $mmol_{NADPH}min^{-1}$ mg_{prot}^{-1} and 0.35 $mmol_{NADPH}min^{-1}$ mg_{prot}^{-1} in seedlings exposed to 10 mg Kg^{-1}, 50 mg Kg^{-1}, 100 mg Kg^{-1} and 200 mg Kg^{-1} concentrations of Cu respectively (Fig. 4E). GR activity increased by 600% at maximum Cu concentration, i.e., at 200 mg Kg^{-1} (Table 1).

DISCUSSION

With the advancement of industrialization, addition of non-biodegradable heavy metals in the soil has been increased rapidly, posing serious threats to the environment. These heavy metals enter into food chain and cause severe harms to human health through biomagnification. Phytoremediation is a technique which employs plants for the removal of heavy metals from the soil. In plants, detoxification mechanism involves avoidance and tolerance. Avoidance is the first line of

defense at extracellular level, which restricts the uptake of heavy metals and their movement into plant tissues through root cells. Tolerance is the second line of defense at intracellular level which include various mechanisms such as inactivation, chelation, and compartmentalization of heavy metal ions (Yan *et al.*, 2020). Organic acids (acetic acid, citric acid, oxalic acid, malic acid, etc.), amino acids (proline), metallothioneins, etc. are the organic compounds/ ligands which are involved in chelation of heavy metal ions by making ligand-heavy metal complexes. Such complexes are later on transported from the cytoplasm into the vacuoles for storage (without toxicity). When heavy metals are present in excess, these strategies fail. Accumulation of metal ions in the plant cytoplasm results in the generation of reactive oxygen species (ROS), resulting in oxidative stress. Oxidative stress affects the various cellular processes and results in DNA damage and oxidation of proteins (DalCorso *et al.*, 2019). In defense, plants activate their antioxidant defensive system and
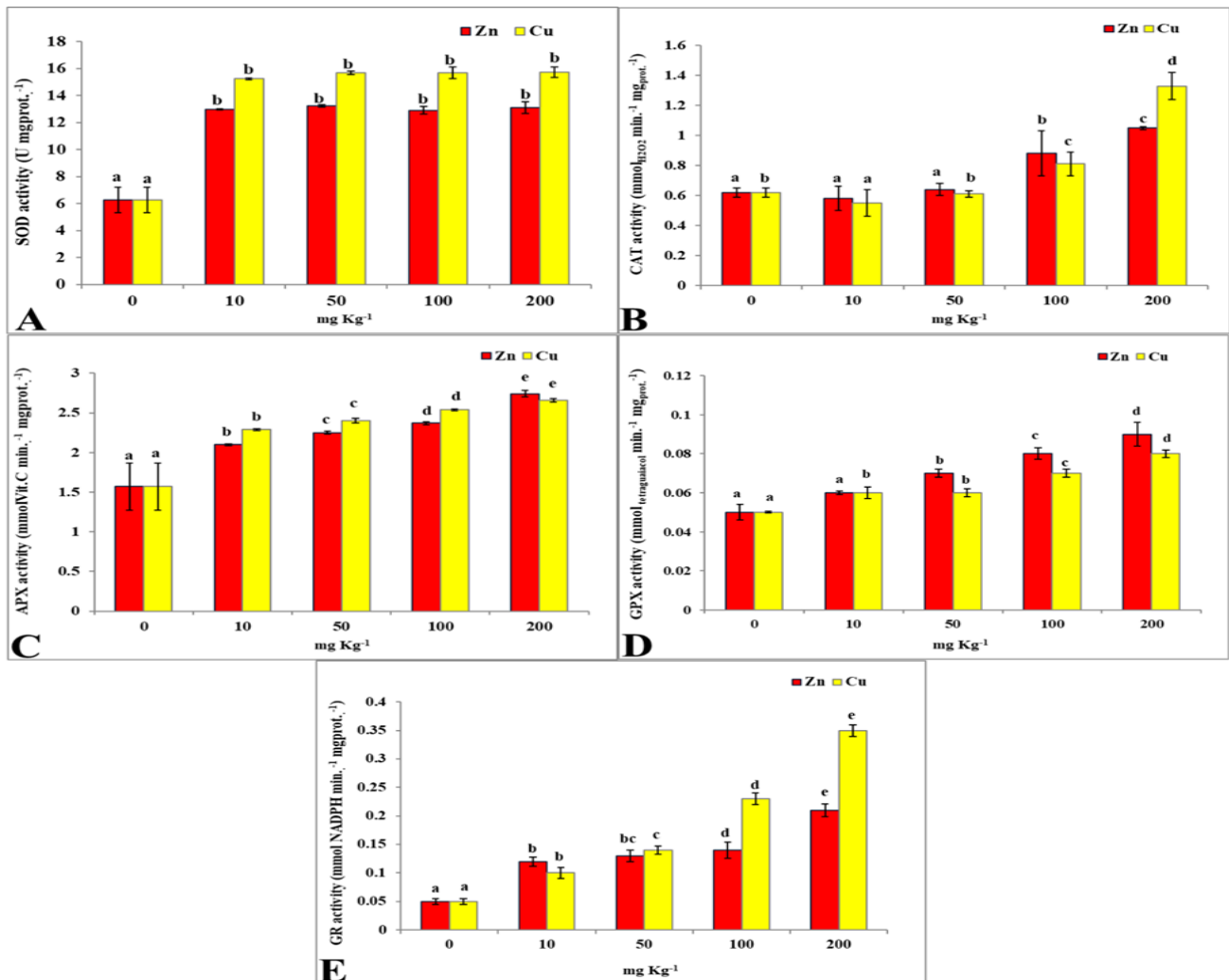

Fig. 4. *Effects of different concentrations of Zn and Cu on SOD (A), CAT (B), APX (C), GPX (D), and GR (E) activities in leaves of 2-month-old S. amara seedlings exposed for three weeks in soil. Values are means ±SD (n = 9). Bars with the different letters represent significant differences between treatments at p < 0.05.*

produce antioxidant enzymes such as SOD, CAT, GR, etc. and non-enzymatic antioxidants such as glutathione, flavonoids, tocopherols, etc.

In plants, (LRWC represents the balance between the supply of water to leaves and rate of transpiration (Lugojan and Ciulca, 2011). It indicates the water status under stress situations (Kaya *et al.*, 2007). In the present study, LRWC was found to be significantly (p<0.05) decreased in *S. amara* seedlings exposed to both Zn and Cu stresses. However, the percentage decrease in LRWC was higher in *S. amara* seedlings exposed to Zn stress (44%) than seedlings exposed to Cu stress (25%) at their respective maximum concentrations, i.e. 200 mg Kg^{-1} (Table 1). Morphological analysis of *S. amara* plants exposed to both stresses also revealed the highest wilting symptoms at maximum Zn concentration. Metals are said to alter plant water relations (Barcelo and Poschenrieder, 1990). A reduction in LRWC could be due to loss of turgor or lipid peroxidation, leading to plant cell membrane damage (Pandey and Gautam, 2009). Zn salts are said to induce osmotic stress, and above toxic levels, it leads to chlorosis and necrotic lesions on plants leaves (Lucini and Bernardo, 2015). Similar to our results, Mukhopadhyay and Mondal (2015) observed RWC of 78.50 mg in *Camellia sinensis* (L.) O. Kuntze cv. T-78 plants under controlled conditions and 73.38 mg under the maximum concentration of Zn (30 μM). A decrease in leaf relative water content due to Zn stress has also been observed by Tavallali *et al.* (2009). Cu stress can also lead to osmotic stress (Sirhindi*et al.,* 2015) and a decrease in LRWC due to it has been observed by Singh *et al.* (2007).

Lipid peroxidation due to free radicals is an indicator of oxidative stress. The MDA formation is the last product of lipid peroxidation and an increase in its concentration is a sign of cell wall damage by ROS production (Quariti *et al.*, 1997; Thounaojam*et al.*, 2012). In the present study, high lipid peroxidation was observed and MDA content significantly (p<0.05) increased in response to both stresses, clearly indicating ROS induced oxidative damage. Compared to control plants, MDA content increased by 422% and 247% in *S. amara* seedlings exposed to maximum concentration of Cu and Zn respectively, i.e. at 200 mg Kg^{-1} concentration (Table 1). The present results are similar to other studies in which lipid peroxidation was increased due to increased Zn and Cu stresses concentrations. Dey *et al.* (2015) observed increase in MDA content in *Camellia sinensis* (L.) O. Kuntze with increase in Cu concentrations (50 μM, 200 μM, 300 μM, 400 μM, 500 μM, 600 μM). Similarly, increase in MDA content due to Cu stress has also been found in different studies (Yurekli and Porgali, 2006; Zhao *et al.*, 2010; Hejazi-Mehrizi*et al.*, 2011; Chen *et al.*, 2015; Sirhindi*et al.*, 2015). Mari-

chali *et al*. (2016) analyzed effects of Zn treatments on seeds, leaves, stems and roots of *Nigella sativa* L. and observed an increase in MDA content in each case. However, contrary to our results, a decrease in MDA content due to Zn stress has also been observed in different studies (D'souza and Devaraj, 2012; Peng *et al*., 2015; Badoni *et al*., 2016). In corroboration with present results of higher lipid peroxidation due to Cu stress than Zn stress, Nanda and Agrawal (2016) observed increase in MDA content up to 3.33 folds and 2.5 folds in *Cassia angustifolia* plants exposed to Cu and Zn stresses, respectively at 200 mg Kg^{-1} concentration.

Osmotic stresses, ionic stress, and reactive oxygen species (ROS) may suppress photosynthesis (Parvaiz and Satyawati, 2008; Ashraf and Harris, 2013). In the present study, photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) decreased with an increase in the concentration of both Zn and Cu stress (Table 1). Copper toxicity decreases photosynthetic pigments and inhibits various physiological processes such as electron transport (Demirevska-Kepova*et al*., 2004). Decrease in photosynthetic pigments under Cu stress has also been observed by Fidalgo*et al*. (2013). Photosynthesis impairment and chlorosis of leaves can also be due to excess amount of Zn in soil (Cambrolle*et al.,* 2012). A similar result of a decrease in photosynthetic pigments with an increase in Zn stress has been observed by Jayasri and Suthindhiran (2016).

Plants have antioxidant mechanisms (enzymatic and non-enzymatic) to scavenge ROS side effects. Proline and cysteine are non-enzymatic antioxidants which accumulate in many plant species in response to environmental stresses. In the present study, the accumulation of both of these non-enzymatic antioxidants increased significantly (p<0.05) in *S. amara* seedlings exposed to both Zn and Cu treatments. An increase in proline and cysteine levels in the study revealed their protective role under stress conditions. Proline is an alpha amino acid which play an important role in scavenging ROS (Kishor *et al*., 2005). Proline is also an osmolyte, metal chelator, signaling molecule that stabilizes membranes, proteins and crucial for maintaining energy status and redox balance (Szabados and Savoure, 2010). The present study revealed percentage increase of 234% and 117% in proline content at 200 mg Kg^{-1} of Zn and Cu concentrations, respectively (Table 1). The present results agree with the study conducted by Nanda and Agrawal (2016), where they found an increase in proline content up to 9.42 folds and 11.20 folds at 200 mg Kg^{-1} of Zn and Cu concentrations, respectively. Azooz *et al*. (2012) and Chen *et al*. (2015) also observed an increase in proline content with an increase in Cu stress. In the present study, cys-

teine content increased significantly (p<0.05) in *S. amara* seedlings exposed to Zn and Cu stresses. Cysteine is an amino acid with a thiol side chain that is susceptible to oxidation to produce disulfide derivatives. It plays a crucial role in intracellular protection against oxidative damage. The role of cysteine in conferring protection against ROS damage has also been observed in different studies by different workers (Romero *et al.*, 2001; Fediuc *et al.*, 2005; Vijendra *et al.*, 2016).

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR) are known to play crucial role in the removal of reactive oxygen species (ROS). In the current study, the activities of all the antioxidant enzymes significantly (p<0.05) increased in response to both Zn and Cu stresses. SOD is a major scavenger (first line of defense) against ROS and occurs in three forms, Cu/ZnSOD, MnSOD or FeSOD (Bowler *et al.*, 1994). It catalyzes the dismutation of superoxide radical ($O_2^{.-}$) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (Scandalios, 1993). In the present study, SOD activity increased by 151% and 109% in *S. amara* seedlings exposed to Cu and Zn stress respectively (at 200 mg Kg^{-1}) (Table 1). Similar to our results, Zhao *et al.* (2010) observed increased SOD activity in *F. arundinacea* roots with increasing Cu concentration. Cu stress also resulted in an increase in SOD activity in other studies (Sirhindi *et al.*, 2015; Dey *et al.*, 2015).

CAT and peroxidases enzymes (APX and GPX) play an important role in H_2O_2 detoxification (Hossain *et al.*, 2007). CAT is present in peroxisomes, mitochondria and glyoxysomes, and it dismutates H_2O_2 into water and molecular oxygen. Ascorbate peroxidases (APX) are the most important peroxidases that catalyze the H_2O_2 reduction to water by employing the reducing power of ascorbate (Noctor and Foyer, 1998). Guaiacol peroxidases (GPX) are important heme-containing groups of peroxidases, which are present in cell walls, vacuole, apoplast, cytosol and extracellular medium, and oxidize guaiacol (o-methoxyphenol) at the cost of H_2O_2 (Sharma *et al.*, 2012). In the present study, activities of all these three enzymes increased significantly (p<0.05) in response to both stresses. Compared to control, CAT activity increased by 69% and 114% in response to maximum stress concentrations of Zn and Cu at 200 mg Kg^{-1} concentration, respectively (Table 1). Similar to our results, *Cu stress resulted in increased CAT activity in Triticum aestivum* cv. Hasaawi was conducted by Azooz *et al.* (2012). However, they observed significant changes in the CAT activity up to 10 mM of Cu concentration, whereas at higher Cu concentrations (20 mM, 40 mM, 80 mM, 100 mM), CAT activity significant increased. In the present study, APX activity also increased by 74% and 69% in response to maximum

stress concentrations of Zn and Cu (200 mg Kg^{-1}), respectively (Table 1). Similar increase in APX activity due to Zn stress has also been observed by Tavallali *et al.* (2010). They observed significant increase in APX activity in *Pistacia vera* L. with increasing Zn concentrations, i.e. 5, 10, 20 mg Zn Kg $soil^{-1}$. Regarding Cu stress, Dey *et al.* (2015) observed a significant increase in APX activity in 3-months-old *Camellia sinensis* (L.) O. Kuntze cultivars, S3A3 and TS-491, with increased Cu concentrations (50 μM, 200 μM, 300 μM, 400 μM, 500 μM, 600 μM). Azooz *et al.* (2012) observed non-significant increase in APX activity in *Triticum aestivum* cv. Hassawi upto 10 mM Cu treatment but at higher Cu concentrations (20 mM, 40 mM, 80 mM, 100 mM), APX activity significantly increased. In the present study, % increase of 80% and 60% in GPX activity was observed at 200 mg Kg^{-1} concentration of Zn and Cu, respectively (Table 1). In corroboration to our results, Badoni *et al.* (2016) observed an increase in GPX activity in *Jatropha curcas* L. at all Zn concentrations (500, 1000, 1500, 2000 mg Kg^{-1}) compared to control, but the maximum increase of 3.4 folds in GPX activity was observed at 2000 mg Kg^{-1} Zn concentration. Prakash *et al.* (2007) observed % increase of 21%, 34% and 46% in GPX activity in *Cicer arietinum* L. under Cu stress at 50, 100 and 200 ppm concentrations, respectively.

GR plays a critical role in scavenging ROS through the ascorbate-glutathione cycle, which removes ROS and is In the present study, GR activity increased by 320% and 600% in *S. amara* seedlings exposed to maximum concentrations of Zn and Cu (200 mg Kg^{-1}), respectively (Table 1).The increase in GR activity clearly indicates its role in mitigating ROS effects in *S. amara* seedlings. The present results are in corroboration with other studies in which GR activity significantly increased in response to Zn and Cu stresses. Nanda and Agrawal (2016) observed an increase of 1.44 folds and 1.75 folds in GR activity in *Cassia angustifolia* Vahl seedlings at maximum concentrations of Zn and Cu, respectively, i.e. 200 mg L^{-1}.

Conclusion

In conclusion, the present study revealed increased lipid peroxidation, reduced leaf relative water content, and photosynthetic pigments due to Zn and Cu stress toxicity at higher concentrations in *S. amara* seedlings. However, in response to both stresses, the activities of all antioxidant enzymes (SOD, CAT, APX, GPX and GR) significantly increased. Similarly, an increase in concentrations of both non-enzymatic oxidants (proline and cysteine) was observed. Significant enhancement in both antioxidant enzymatic activities and non-enzymatic antioxidant concentrations revealed a signifi-

cant role of antioxidant defense system in overcoming oxidative stress. The present study could be very helpful in making strategies for *S. amara* large scale cultivation on wastelands.

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

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