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

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**How to Cite**

**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$_2$O$_2$), 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, 2008). 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, Aelanthus bifomfolius, Ipomoea alpina, 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, putrescine, 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-
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±2ºC 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 -80ºC 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:

\[
\text{LRWC} (%) = \frac{\text{(fresh weight of the sample-dry weight of sample)}}{\text{(turbid weight of the sample-dry weight of sample)}} \times 100.
\]

**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 60ºC 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.
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 4°C 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, 464.04, 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\% increase.

### 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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Zn</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200 mg Kg(^{-1})</td>
<td>% ↑ or ↓</td>
<td>200 mg Kg(^{-1})</td>
</tr>
<tr>
<td>LRWC (%)</td>
<td>69.74</td>
<td>38.89</td>
<td>↓ 44.23</td>
</tr>
<tr>
<td>MDA content (µmol g(^{-1}) FW)</td>
<td>13.12</td>
<td>45.59</td>
<td>↑ 247.48</td>
</tr>
<tr>
<td>Chl. a content (mg g(^{-1}) FW)</td>
<td>1.43</td>
<td>0.74</td>
<td>↓ 48.25</td>
</tr>
<tr>
<td>Chl. b content (mg g(^{-1}) FW)</td>
<td>1.003</td>
<td>0.08</td>
<td>↓ 92.02</td>
</tr>
<tr>
<td>Carotenoids content (mg g(^{-1}) FW)</td>
<td>10.44</td>
<td>4.87</td>
<td>↓ 53.35</td>
</tr>
<tr>
<td>SOD activity (Unit mg(^{-1}) protein)</td>
<td>6.25</td>
<td>13.08</td>
<td>↑ 109.28</td>
</tr>
<tr>
<td>CAT activity (mmol H(_2)O(_2) min(^{-1}) mg(^{-1}) prot(^{-1}))</td>
<td>0.62</td>
<td>1.05</td>
<td>↑ 69.35</td>
</tr>
<tr>
<td>APX activity (mmol GSH min(^{-1}) mg(^{-1}) prot(^{-1}))</td>
<td>1.57</td>
<td>2.74</td>
<td>↑ 74.52</td>
</tr>
<tr>
<td>GPX activity (mmol tetrahydrobiopterin min(^{-1}) mg(^{-1}) prot(^{-1}))</td>
<td>0.05</td>
<td>0.09</td>
<td>↑ 80.00</td>
</tr>
<tr>
<td>GR activity (mmol NADPH min(^{-1}) mg(^{-1}) prot(^{-1}))</td>
<td>0.05</td>
<td>0.21</td>
<td>↑ 320.00</td>
</tr>
<tr>
<td>Proline content (µmol g(^{-1}) FW)</td>
<td>26.19</td>
<td>87.50</td>
<td>↑ 234.10</td>
</tr>
<tr>
<td>Cysteine content (µmol g(^{-1}) FW)</td>
<td>20.17</td>
<td>74.82</td>
<td>↑ 270.95</td>
</tr>
</tbody>
</table>
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\(^{-1}\) 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\(^{-1}\) protein\(^{-1}\), 13.23 Unit mg\(^{-1}\) protein\(^{-1}\), 12.9 Unit mg\(^{-1}\) protein\(^{-1}\) and 13.08 Unit mg\(^{-1}\) protein\(^{-1}\) respectively (Fig. 4A). SOD activity increased by 109% at 200 mg Kg\(^{-1}\) Zn concentration (Table 1). Similarly, pattern of increase in SOD activity was observed in case of Cu treated seedlings. SOD activities were found to be 15.23 Unit mg\(^{-1}\) protein\(^{-1}\), 15.67 Unit mg\(^{-1}\) protein\(^{-1}\), 15.68 Unit mg\(^{-1}\) protein\(^{-1}\) and 15.72 Unit mg\(^{-1}\) 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\(\text{H}_{2}\text{O}_{2}\)min\(^{-1}\) mg\(^{-1}\) 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\(\text{H}_{2}\text{O}_{2}\)min\(^{-1}\) mg\(^{-1}\) prot\(^{-1}\), 0.64 mmol\(\text{H}_{2}\text{O}_{2}\)min\(^{-1}\) mg\(^{-1}\) prot\(^{-1}\), 0.88 mmol\(\text{H}_{2}\text{O}_{2}\)min\(^{-1}\) mg\(^{-1}\) prot\(^{-1}\) and 1.05 mmol\(\text{H}_{2}\text{O}_{2}\)min\(^{-1}\) mg\(^{-1}\) 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\(\text{H}_{2}\text{O}_{2}\)min\(^{-1}\) mg\(^{-1}\) prot\(^{-1}\), 0.61 mmol\(\text{H}_{2}\text{O}_{2}\)min\(^{-1}\) mg\(^{-1}\) prot\(^{-1}\), 0.81 mmol\(\text{H}_{2}\text{O}_{2}\)min\(^{-1}\) mg\(^{-1}\) prot\(^{-1}\) and 1.33 mmol\(\text{H}_{2}\text{O}_{2}\)min\(^{-1}\) mg\(^{-1}\) 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\(\text{V}_{5}\text{lC}\)min\(^{-1}\) mg\(^{-1}\) 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\(^{-1}\) concentrations of Zn concentrations, seedlings exhibited APX activities of 2.10 mmol\(\text{V}_{5}\text{lC}\)min\(^{-1}\) mg\(^{-1}\) prot\(^{-1}\), 2.25 mmol\(\text{V}_{5}\text{lC}\)min\(^{-1}\) mg\(^{-1}\) prot\(^{-1}\), 2.37 mmol\(\text{V}_{5}\text{lC}\)min\(^{-1}\) mg\(^{-1}\) prot\(^{-1}\) and 2.74 mmol\(\text{V}_{5}\text{lC}\)min\(^{-1}\) mg\(^{-1}\) 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\(\text{V}_{5}\text{lC}\)min\(^{-1}\) mg\(^{-1}\) prot\(^{-1}\), 2.40 mmol\(\text{V}_{5}\text{lC}\)min\(^{-1}\) mg\(^{-1}\) prot\(^{-1}\), 2.54 mmol\(\text{V}_{5}\text{lC}\)min\(^{-1}\) mg\(^{-1}\) prot\(^{-1}\) and 2.66 mmol\(\text{V}_{5}\text{lC}\)min\(^{-1}\) mg\(^{-1}\) 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\(\text{tetraguaiacol}\)min\(^{-1}\) mg\(^{-1}\) 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$_{tetra}$guaiacol min$^{-1}$ mg$_{prot}^{-1}$, 0.07 mmol$_{tetra}$guaiacol min$^{-1}$ mg$_{prot}^{-1}$, 0.08 mmol$_{tetra}$guaiacol min$^{-1}$ mg$_{prot}^{-1}$ and 0.09 mmol$_{tetra}$guaiacol 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$_{tetra}$guaiacol min$^{-1}$ mg$_{prot}^{-1}$, 0.06 mmol$_{tetra}$guaiacol min$^{-1}$ mg$_{prot}^{-1}$, 0.07 mmol$_{tetra}$guaiacol min$^{-1}$ mg$_{prot}^{-1}$ and 0.08 mmol$_{tetra}$guaiacol 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)
increased in S. amara seedlings exposed to different Cu concentrations. GR activity was found to be 0.1 mmol NADPH min$^{-1}$ mg$^{-1}$ prot, 0.14 mmol NADPH min$^{-1}$ mg$^{-1}$ prot, 0.23 mmol NADPH min$^{-1}$ mg$^{-1}$ prot, and 0.35 mmol NADPH min$^{-1}$ mg$^{-1}$ prot 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.](image-url)
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 (Srirhindiet 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; Thounaojamet 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-Mehrzi et al., 2011; Chen et al., 2015; Srirhindiet 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 (Parvaz 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-Kepovaet al., 2004). Decrease in photosynthetic pigments under Cu stress has also been observed by Fidalgoet al. (2013). Photosynthesis impairment and chlorosis of leaves can also be due to excess amount of Zn in soil (Cambrolleet al., 2012). A similar result of a decrease in photosynthetic pigments with an increase in Zn stress has been observed by Jayarsi 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-
Cysteine 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 present 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\textsubscript{2}\textsuperscript{-}) into molecular oxygen (O\textsubscript{2}) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{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\textsuperscript{-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\textsubscript{2}O\textsubscript{2} detoxification (Hossain et al., 2007). CAT is present in peroxisomes, mitochondria and glyoxysomes, and it dismutates H\textsubscript{2}O\textsubscript{2} into water and molecular oxygen. Ascorbate peroxidases (APX) are the most important peroxidases that catalyze the H\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{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\textsuperscript{-1} concentration, respectively (Table 1). Similar to our results, Cu stress resulted in increased CAT activity in Triticum aestivum cv. Hasaawi (Agrawal, 2016) observed an increase of 1.44 folds and 600% 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\textsuperscript{-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.

ACKNOWLEDGEMENTS

The authors are grateful to the Department of Life Sciences, School of Basic Sciences and Research, Sharda University, Greater Noida, India.

Conflict of interest

The authors declare that they have no conflict of interest.

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487


