Environmental stresses such as, extreme temperature, herbicides, drought, salinity or high light intensity accelerate generation of reactive oxygen species. The toxic radicals viz. O$_2^•$, H$_2$O$_2$, O$_2^-$, and (OH) are inevitable by-products of plant metabolism. The reactive oxygen radicals cause lipid peroxidation, and DNA mutation. Protection against oxidative damage is offered by an antioxidative system, which has both, enzymatic and non-enzymatic components. Plant cells accelerate generation of reactive oxygen species inevitable by-products of plant metabolism. The antioxidative system, which has both, enzymatic and non-enzymatic components. Plant cells utilize the ascorbate-glutathione pathway to reduce the accumulation of oxygen free radicals. Superoxide dismutase reacts with superoxide radicals to produce O$_2$ and H$_2$O$_2$. Hydrogen peroxide is broken down by peroxidases, particularly ascorbate peroxidase, which utilizes ascorbate as a substrate. Ascorbate is regenerated by glutathione and glutathione reductase. Low molecular weight compounds such as ascorbate, glutathione, α-tocopherol and carotenoids also play an important role as components of the antioxidative defense system of plants.

Plant culture—Zea mays L. var. kanaujia was grown in sand culture under glass house conditions with nutrient solution containing (mM): 4, Ca(NO$_3$)$_2$; 4, KNO$_3$; 2, MgSO$_4$·7H$_2$O; 1.33, Na$_2$HPO$_4$; 0.1, NaCl; 0.1, Fe-EDTA; (μM) 10, MnSO$_4$; 1, CuSO$_4$; 33, H$_3$BO$_3$; 0.2, Na$_2$MoO$_4$; 0.1, CoSO$_4$ and 0.1, NiSO$_4$. Zinc was supplied as ZnSO$_4$ at three levels, 0.1, 10, and 20 μM to represent low, adequate (control) and high supply of Zn as revealed in an earlier study. Thirty days after sowing (DAS) the top three fully expanded leaves of plants were analyzed for Zn, chlorophyll, ascorbate and malondialdehyde concentration and activities of superoxide dismutase (EC 1.15.1.1), ascorbate peroxidase (EC 1.11.1.11) and glutathione reductase (EC 1.6.4.2).

Leaf tissue Zn and chlorophyll—Zinc concentration was determined by Atomic Absorption Spectrophotometer (Perkin Elmer A Analyst 300) after HNO$_3$:HClO$_4$ (10:1) digestion of oven dry (70°C) leaf material. Chlorophyll (a+b) was extracted in 80% acetone and its concentration was measured at 645 and 663 nm (Perkin Elmer UV/VIS Lambda Bio 20 spectrophotometer).

Ascorbate, dehydroascorbate—Ascorbate (AS) in leaf tissue was extracted in 10% trichloroacetic acid TCA. The assay is based on reduction of Fe$^{3+}$ to Fe$^{2+}$ by ascorbic acid and Fe$^{2+}$ forming a complex with α,α'-bipyridyl, producing a pink color with abs max at 525 nm. Dehydroascorbate (DHAS) was determined by reduction of DHAS and estimating total AS. Calibration curve for ascorbate was prepared, using L-ascorbic acid (Sigma).

Lipid peroxidation—Lipid peroxidation was measured in terms of malondialdehyde (MDA) content. Fresh leaf material was homogenized with 0.1% TCA and centrifuged at 10,000 g for 5 min. The supernatant was treated with 0.5% thiobarbituric acid (TBA) in 20% TCA and the mixture was incubated in boiling water for 30 min. MDA content was estimated spectrophotometrically by reading absorbance at 532 nm and adjusting for non-specific absorbance at 600 nm, by using the extinction coefficient of 155 mmol cm$^{-1}$. 

Effect of zinc on antioxidant response in maize (Zea mays L.) leaves

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Maize (Zea mays L. cv kanaujia) plants grown with Zn [10 (control), 0.1 (low) and 20 μM (high)], were investigated for concentration of antioxidants and activities of antioxidative enzymes in leaves. Young leaves of low Zn plants developed whitish-necrotic spots. Leaves of both low and high Zn plants showed decrease in chlorophyll concentration and accumulation of lipid peroxides, ascorbate and dehydroascorbate, associated with a decrease in the activity of ascorbate peroxidase and superoxide dismutase. Low and high Zn, however, showed diverse effect on glutathione reductase. While low Zn increased the activity of glutathione reductase, high Zn decreased its activity. Zinc effect on antioxidative constituents suggested Zn involvement in sustaining the antioxidative defense system in maize leaves.
Enzymes—For assay of superoxide dismutase (SOD) and glutathione reductase (GR) activities, fresh leaf tissue (1g) was ground in potassium phosphate buffer (50 mM; pH 7) containing EDTA (1 mM) and insoluble PVP (2%). The homogenate was centrifuged for 10 min at 15,000 g and the supernatant was used as enzyme extract. For ascorbate peroxidase (APX) assay, 1mM of ascorbate was added in the above extraction buffer prior to grinding. All steps for extraction of enzymes were carried out at 4°C.

Superoxide dismutase was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT)\(^\text{12}\). One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition in reduction of NBT at 560 nm. Cu/Zn SOD was determined by inhibiting the activity by KCN (3 mM) and subtracting from total SOD. Ascorbate peroxidase was assayed by following spectrophotometrically the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM cm\(^{-1}\)) in a reaction mixture containing 0.5 mM ascorbic acid, 0.2 mM H\(_2\)O\(_2\), 1 mM EDTA, 50 mM phosphate buffer (pH 7.0) and appropriate volume of enzyme extract\(^\text{9}\). Glutathione reductase was determined by measuring the oxidation of 0.2 mM NADPH at 340 nm after addition of 0.5 mM of oxidized glutathione to 100 mM potassium phosphate buffer (pH 7.5) and 2 mM EDTA\(^\text{13}\). Enzyme activity was calculated using the extinction coefficient for NADPH (6.22 m \cdot M \cdot cm\(^{-1}\)).

Protein—Soluble protein in the enzyme extracts was measured by Coomassie Brilliant Blue G250 protein binding method of Bradford\(^\text{14}\) using bovine serum albumin (Sigma) as a standard.

About 25 DAS, growth of plants, supplied with low (0.1 \(\mu\)M) and high (20 \(\mu\)M) Zn, slowed down and their leaves developed visible symptoms. Young leaves of plants treated with low Zn turned chlorotic and eventually white (bleached) and necrotic along the margins. About 35 DAS, the leaves of high Zn plants also showed necrosis at the margins. Difference in Zn supply was reflected in Zn accumulation in leaves. Plants treated with 0.1, 10 and 20 \(\mu\)M of Zn contained 18.2 ± 5.2; 58.8 ± 2.9 and 141.4 ± 8.5 \(\mu\)g of Zn g\(^{-1}\) dry wt respectively.

Low and high Zn, both caused decrease in chlorophyll (chl) concentration. As seen in Table 1, Zn effect on chl b was more than on chl a. Low Zn plants showed increase in the concentration of MDA, AS and DHAS in leaves. Increase in AS content was relatively more marked than DHAS, lowering DHAS:AS ratio in leaves of low Zn plants.

| Table 1—Effect of Zn supply on concentration of chlorophyll, protein, malondialdehyde, ascorbate and dehydroascorbate in maize leaves |
|---------------------------------|---|---|---|
| Zn supply (\(\mu\)M) | 0.1 | 10 | 20 |
| Total chlorophyll (mg g\(^{-1}\) fresh wt) | 3.3±0.13 | 1.13±0.04 | 0.85±0.50 |
| Chl a:b | 1.40±0.21 | 1.30±0.15 | 1.65±0.45 |
| Protein (mg g\(^{-1}\) fresh wt) | 0.41±0.21 | 0.92±0.21 | 0.71±0.12 |
| Malondialdehyde (nmol g\(^{-1}\) fresh wt) | 17.12±0.85 | 13.56±0.12 | 28.90±1.43 |
| Ascorbate (nmol mg\(^{-1}\) fresh wt) | 24.2±0.43 | 17.2±0.28 | 21.5±0.26 |
| Dehydroascorbate (nmol mg\(^{-1}\) fresh wt) | 5.0±0.81 | 3.8±1.01 | 4.2±0.80 |
| Dehydroascorbate/ascorbate | 0.25±0.04 | 0.28±0.09 | 0.18±0.04 |

Table 2—Effect of Zn supply on the activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase in maize leaves

<table>
<thead>
<tr>
<th>Zn supply ((\mu)M)</th>
<th>0.1</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (units mg(^{-1}) protein)</td>
<td>19.0±0.45</td>
<td>24.0±0.49</td>
<td>18.4±0.28</td>
</tr>
<tr>
<td>Cu/Zn superoxide dismutase (units mg(^{-1}) protein)</td>
<td>9.1±0.10</td>
<td>17.2±0.60</td>
<td>14.7±0.46</td>
</tr>
<tr>
<td>Ascorbate peroxidase ((\mu)mol ascorbate oxidized mg(^{-1}) protein)</td>
<td>2.33±0.35</td>
<td>3.45±0.52</td>
<td>2.81±0.12</td>
</tr>
<tr>
<td>Glutathione reductase ((\mu)mol NADPH oxidized mg(^{-1}) protein)</td>
<td>4.31±0.58</td>
<td>3.44±0.44</td>
<td>1.79±0.19</td>
</tr>
</tbody>
</table>

Low Zn supply caused decrease in the activities of APX and SOD (Table 2). Low Zn effect on Cu/Zn SOD activities was more than on total SOD activity. Contrary to the effect on APX and SOD, GR activity in leaves of low Zn plants was higher than in control (Table 2). High supply of Zn led to decrease in the activities of each of the three antioxidative enzymes.

Plants treated with low and high Zn showed increased accumulation of MDA, which is a major TBA reactive metabolite, resulting from lipid peroxidation caused by enhanced production of reactive oxygen species. Lipid peroxidation causes damage to membrane integrity, functioning of proteins and other membrane associated structures\(^\text{15}\). Increased accumulation of MDA in Zn stressed plants is indicative of a role of Zn in maintaining the integrity of cellular membranes by preventing MDA production, possibly by stabilizing the –SH groups\(^\text{16}\).
Increase in concentration of AS observed in leaves of Zn stressed plants was explained by concomitant decrease in the activity of APX that catalyses peroxidative degradation of photosynthetically generated H$_2$O$_2$ as part of ascorbate-glutathione cycle. Low activity of APX, associated with decrease in DHAS:AS ratio contributed to enhanced H$_2$O$_2$ production, which is known to cause oxidative damage. Our results with maize are in consonance with decrease in APX and SOD activity in Zn deficient tobacco plants.

Observed increase in GR activity in plants treated with low Zn and decrease in GR activity in leaves of high Zn maize plants was contradictory to Cakmak and Marschner, who have observed low levels of GR under Zn deficiency in beans, but agrees with elevated levels of the enzyme in response to oxidative stress reported in peas and wheat. High activities of SOD and GR have also been reported to contribute to tolerance of maize inbreds to environmental stress such as drought, herbicide and SO$_2$.

Low activity of total and Cu/Zn SOD in plants treated with low Zn is well documented and is attributed to Zn as the metal constituent of the enzyme. It has been suggested that Zn prevents oxidative stress by restricting generation of O$_2^-$ ions, via an NADPH dependent oxidase and efficient scavenging of O$_2^-$ ions through Cu/Zn SOD. The visible symptoms exhibited by low Zn maize plants such as bleaching and necrosis of young leaves might be a result of accelerated oxidative damage to cellular components caused by enhanced generation of ROS resulting from alterations in the activities of antioxidative enzymes under Zn deficiency. This is also substantiated by high levels of MDA, produced under Zn deficiency.

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References