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Pb-Stress Induced Oxidative Stress Caused Alterations in Antioxidant Efficacy in Two Groundnut (*Arachis hypogaea* L.) Cultivars

Ambekar Nareshkumar, G. V. Nagamallaiah, M. Pandurangaiah, K. Kiranmai, V. Amaranathareddy, U. Lokesh, B. Venkatesh, Chinta Sudhakar*

Plant Molecular Biology Unit, Department of Botany, Sri Krishnadevaraya University, Anantapur, India
Email: *chintasudhakar@yahoo.com

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Abstract

Lead (Pb) is an important environmental pollutant extremely toxic to plants and other living organisms including humans. To assess Pb phytotoxicity, a pot culture experiment was carried out using two groundnut cultivars (*Arachis hypogaea* L. cultivar K6 and cultivar K9) on plant growth, ROS levels, lipid peroxidation, and antioxidant metabolism using biochemical, histochemical methods. Plants were grown in pots for 14 days, in the botanic garden, and subjected to Pb-stress (0, 100, 200, 400 and 800 ppm) by adding Pb $(NO_3)_2$ solution and further allowed to grow for 10 days. The results showed that cultivar K6 registered lower Pb accumulation than cultivar K9, however, localization of Pb was greater in roots than leaves in both groundnut cultivars. The Pb-stress results in an increase in free radicals ($O_2^{\bullet-}$ and H_2O_2) generation in both groundnut cultivars, but more significantly in cultivar K9 than K6. Pb-stress also caused significant changes in the rate of peroxidation as shown in the levels of malondialdehyde (MDA) content in roots and leaves of both groundnut cultivars. Free proline, ascorbic acid (AsA) and non-protein thiol (NP-SH) contents were increased in cultivar K6 due to Pb-stress, but less in cultivar K9. Pb treated plants showed increased levels of antioxidant enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) glutathione reductase (GR) and glutathione S-transferase (GST). Isozyme band intensities of SOD, GPX and APX were more consistent with the respective changes in antioxidative enzyme activities. These results indicate that cultivar K6 possesses greater tolerance potential for Pb toxicity than cultivar K9.

Keywords

Groundnut (*Arachis hypogaea* L.), Pb-Stress, Oxidative Stress, Antioxidant Enzymes, Non-Protein Thiols, Reactive Oxygen Species

*Corresponding author.

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1. Introduction

In recent years heavy metal pollution has become one of the serious environmental problems worldwide. Unlike organic pollutants that can be easily degradable to harmless small molecules, toxic elements, such as lead, mercury, cadmium, copper and zinc, are immutable by biochemical reactions [1], hence, it is difficult to remediate these metals form the soil and water. The hot spots of soil contamination are located in the regions of large industrial activities, where surrounding agricultural lands are affected by the deposition of heavy metals and also agricultural practice, e.g., application of sewage sludges, phosphate fertilizers, liming, irrigation water and pesticides has lead to increased heavy metal concentration in soils [2]. The long time persistent nature of some heavy metals, such as Pb and Cd are leading to hazardous accumulation [3], and a huge variation in metal tolerance was observed between different crop plants [4] [5].

Among heavy metal ions, Pb is one of the most hazardous pollutants of the environment and Pb pollution in air, water and agricultural soil is an ecological concern due to its extreme impact on human health and environment. Naturally Pb occurs in soils, but in relatively low concentrations, in uncontaminated soils in the range of 2 to 200 ppm dry weight, whereas 0.1 - 10 ppm dry weight in agricultural crops [3]. The effect of Pb mainly depends on concentration, soil type, soil properties and plant species [6]. Heavy metal induced phytotoxicity or plant tolerant responses to heavy metal stress is a complex phenomenon, involving developmental changes as well as physiological and biochemical mechanisms [5] [7] [8].

Lead (Pb) is reported to produce reactive oxygen species (ROS) and enhance antioxidant enzyme activity in plants [9]. ROS production includes harmful effects in plant cells, such as inhibition of photosynthetic activity, inhibition of ATP production, lipid peroxidation and DNA damage [1]. In addition, enhanced production of ROS which causes damage to cell membranes, nucleic acids and chloroplast pigments [10], is a major consequence. Different ROS including superoxide anion ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet-}$), that have been proven to be important agents in the origin of tissue injury and membrane damage, are produced after the exposure of plants to heavy metals [11] [12]. Generation of excess ROS in heavy metal stressed plants may be a consequence of the distribution of the balance between their production and decomposition by antioxidant enzyme activities composed of antioxidants, such as superoxide dismutase (SOD), catalase (CAT) ascorbate peroxidise (APX) and guaiacol peroxidise (GPX) and glutathione reductase (GR) and the non-enzymatic antioxidants such as reduced glutathione (GSH), ascorbic acid (AsA) [1] [13]-[17]. Plants can tolerate Pb by chelation of the metal ions and compartmentalization into the vacuole [18]. It has also been shown that non-protein thiols (NP-SH) and glutathione (GSH) are known to play a pivotal role in metal detoxification mechanisms in terrestrial plants [19] [20]. In addition, phytochelatin complex metal ions through the thiolic group (-SH) of Cys and the PC-Cd complexes are found to be accumulated in the vacuole through the activity of ABC transporters, thereby limiting the free Cd inside the cytosol [21].

The effect of Pb-stress has been studied in various plant species including *Eichhornia crassipes* [1], *Oryza sativa* [14], *Sesbania drummondii* [22], *Triticum aestivum* [23], *Sesuvium portulacastrum* and *Brassica juncea* [24], *Salsola passerine* and *Chenpodium album* [25], and some Chinese groundnut cultivars [26]. Though, the groundnut (*Arachis hypogaea* L.) species has the potential for hyperaccumulation of various heavy metals, the effect of Pb exposure induced physiological and biochemical changes in this plant has not been studied yet. Earlier studies demonstrated that some groundnut cultivars were tolerant to heavy metals like Cd and Pb [26] [27]. Although, legumes are reported to be tolerant to some heavy metals [6] [28], there has been a considerable need in finding suitable groundnut cultivars that are able to grow on heavy metal contaminated soils for land reclamation and to study metal tolerance mechanisms exists in this crop. Owing to the economic importance of groundnut crop, and increasing international concern on the soil contaminations due to Cd, and Pb [29], it is very important to investigate the impacts of heavy metals on growth, physiological and biochemical aspects in groundnut under induced Pb-stress. This crop may further be useful in soil reclamation through the process of phytoremediation. However, selection of cultivars would be of great importance to reclaim the soil with lesser impact on plant metabolism. So far this plant species has been used in studies of the effects of heavy metals like Cd [26], and Pb [30], on metal accumulation and yield study parameters. In addition, there are no publications on the relationship between Pb toxicity and morphological, physiological and antioxidative responses of groundnuts. In this study, we initially aimed to investigate Pb induced tolerance mechanisms in two widely cultivated, local and high yielding groundnut cultivars (cultivar K6 and K9) subjected to Pb-stress. Therefore, we studied in detail about the role of antioxidant systems in detoxification of Pb ions in groundnut under pot culture experiment.

2. Materials and Methods

2.1. Plant Material and Pb Treatment

Groundnut (cultivar K6 and K9) seeds were procured from Regional Agricultural Research Station (RARS), Kadiri, India, was sown in earthen pots containing air dried red soil and farmyard manure in 3:1 proportion. The pots were kept under natural photoperiod (12 - 14 h and temperature 28˚C ± 4˚C) in the botanical garden and were irrigated once a day with water and maintained for 14 days. Fourteen-day-old plants were subjected to Pb-stress once by adding 0 (control), 100, 200, 400 and 800 ppm of Pb solution using lead nitrate ($Pb(NO_3)_2$). For each treatment, twenty five pots, each with three plants were maintained. Both control and treated pots were irrigated daily with tap water. Care was taken while adding water slightly less than field capacity (approximately 300 ml) to avoid leaching out of solution from treated pots. After 10 days of stress imposition, the plants were uprooted carefully; the leaves and roots were separated, flash frozen in liquid nitrogen and stored at −80˚C until further use.

2.2. Growth Parameters

The plants were carefully uprooted from pots and washed thoroughly with running tap water. Plant growth was determined by measuring the length of the root and shoot system. The dry weight (DW) was measured after the shoots and roots were dried at 80˚C to constant weight.

2.3. *In Situ* Histochemical Localization of $O_2^{\bullet-}$ and H_2O_2

In situ accumulation of $O_2^{\bullet-}$ and H_2O_2 was detected by histochemical staining with nitrobluetetrazolium (NBT) and diaminobenzidine (DAB) according to Romero-Puertas *et al.* [31], with minor modifications. For $O_2^{\bullet-}$ detection the leaves of control and Pb-stressed plants were excised and immersed in a 0.1% solution of NBT in 10 mM phosphate buffer (pH 7.8) at room temperature. The immersed leaves were illuminated for 1 - 2 h until the appearance of dark spots, characteristic of blue formazan precipitates. For localization of H_2O_2, excised leaves were immersed in a 0.1% DAB solution in 10 mM phosphate buffer (pH 3.8) and incubated at room temperature for 8 h until brown spots, derived from the reaction of DAB with H_2O_2. The leaves were then bleached in warm ethanol to visualize the blue and brown spots.

2.4. Superoxide Anion ($O_2^{\bullet-}$) Estimation

Superoxide anion content was determined using the method described by Doke [32]. The leaves were cut into pieces and placed in the test tubes and filled with 7 ml of mixture containing 50 mM of NaN_3. Next, the test tubes were incubated in dark for 5 min, and then 2 ml of the solution were taken from the tubes and heated up for 15 min at 85˚C. The samples were cooled down on ice for 5 min and the absorbance was measured at 580 nm against the control.

2.5. Hydrogen Peroxide (H_2O_2) Estimation

H_2O_2 levels were determined according to Singh *et al.* [33]. Fresh leaves (0.1 g) were homogenised in ice bath with 5 ml 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 × g for 15 min and 0.5 ml of the supernatant was added to 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. H_2O_2 was used as a standard and the concentration of H_2O_2 in the samples was calculated and expressed in $\mu mol \cdot g^{-1}$ dry weight.

2.6. Lipid Peroxidation

The level of lipid peroxidation products was determined by estimating thiobarbituric acid reactive substances (TBARS) as described by Hodges *et al.* [34]. Five hundred milligrams of leaf tissue was ground to a fine paste with 15 ml of ethanol: water (95:5 v/v and 0.1% butylated hydroxyl toluene). The contents were centrifuged at 3000 × g for 10 min and the supernatant was collected. One millilitre of supernatant was treated with 1 ml of 20% TCA along with 0.65% thiobarbituric acid (TBA) in a clean glass tube and mixed thoroughly. Another 1 ml of supernatant was treated only with 1 ml of 20% TCA and mixed thoroughly. The mixtures were heated at 95˚C

for 30 min in a water bath, cooled immediately on ice and centrifuged at 3000 × g for 10 min. Absorbance at 532 nm was recorded for MDA. In addition, the absorbance at 440 nm (carbohydrates) and 600 nm (phenylpropanoid pigments) were also recorded to avoid overestimation of MDA.

$[(Abs_{532+TBA}) - (Abs_{600+TBA}) - (Abs_{532-TBA}) - (Abs_{600-TBA})] = A.$

$[(Abs_{440+TBA} - Abs_{600+TBA})0.0571] = B.$

$[\text{MDA equivalents } (\mu mol \cdot ml^{-1}) = (A - B/157000)10^3] = C.$

$\text{MDA equivalents } (\mu mol \cdot g^{-1}) = (C \times 15 \times 1/0.5).$

2.7. Free Proline Content

The extraction and estimation of free proline was done according to Bates *et al.* [35]. Fresh plant material (0.5 g) was homogenized in a pre-chilled mortar with a pestle using 3% aqueous sulfosalicylic acid. The homogenate was filtered through four layered muslin cloth and the filtrate was collected. The extraction was repeated twice, all the filtrates were pooled and made up to known volume. Two millilitres of filtrate was taken into a test tube and 2 ml of acid ninhydrin, 2 ml of glacial acetic acid were added. The tubes were incubated at 100˚C for 1 h in a boiling water bath. The tubes, after incubation, were transferred to an ice bath to terminate the reaction. Four millilitres of toluene was added to the contents of the tubes and mixed thoroughly using a cyclomixer (CM101, REMI India) for 15 sec. Chromophore containing toluene was aspirated from the aqueous phase. Then the absorbance was measured in a UV-spectrophotometer at 520 nm against toluene. Proline was measured from the standard curve prepared with authentic proline and its amount was calculated on dry weight basis.

2.8. Estimation of Ascorbic Acid (AsA) and Non-Protein Thiol Compounds (NP-SHs)

The measurement of total AsA and NP-SH groups were carried out as described in Cakmak and Marschner [36]. Five hundred milligrams of fresh leaf samples were extracted with 5 ml of 5% meta-phosphoric acid, and centrifuged at 15,000 × g for 15 min. For the assay of AsA, the reaction mixture contained 0.2 ml aliquot, 0.5 ml 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, 0.1 ml 10 mM DTT and 0.1 ml 0.5% (w/v) *N*-ethylmaleimide (NEM) to remove excess DTT. To this, 0.4 ml 10% trichloroacetic acid (TCA), 0.4 ml 44% ortho-phosphoric acid, 0.4 ml 2.1‘bipyridine in 70% ethanol and 0.2 ml 3% $FeCl_3$ were added to develop colour. The mixtures were then incubated in a water bath at 40˚C for 40 min and the colour produced was read at 525 nm. AsA was used as a standard and the concentration of AsA in the samples was calculated and expressed in $\mu mol \cdot g^{-1}$ dry weight.

For the assay of −SH groups, the reaction mixture contained 0.5 ml aliquot of the supernatant, 2.5 ml of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and 0.5 ml 6 mM 5-5’-dithiobis-(2-nitro-benzoic acid). Following incubation at room temperature, the colour produced was measured at 412 nm with a spectrophotometer. Reduced glutathione (GSH) was used as a standard. The concentration of NP-SH compounds in the samples were calculated and expressed in $\mu mol \cdot g^{-1}$ dry weight.

2.9. Antioxidant Enzyme Assays and In-Gel Enzyme Activity Staining

Fresh leaf tissue was homogenized in 50 mM Tris-HCl (pH 7.5) buffer containing 40 mM phenyl methyl sulfonyl fluoride (PMSF), and 2% (w/v) polyvinyl poly pyrrolidone (PVPP) with the addition of 2 mM AsA for the APX assay. The extract was centrifuged at 15,000 × g for 20 min at 4˚C and the resultant supernatant was used for all the enzyme assays. The amount of protein was calculated according to Lowry *et al.* [37].

All enzymic activities were measured spectrophotometrically at 25˚C. Superoxide dismutase (SOD) activity was assayed using the method described by Urbanek *et al.* [38]. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction rate. Guaiacol peroxidase (GPX) activity was determined in terms of oxidation of guaiacol by H_2O_2 [39], as measured by the increase in absorbance at 420 nm ($E = 26.6\ mM^{-1} \cdot cm^{-1}$). Ascorbate peroxidase (APX) activity was determined as described by the method of Nakano and Asada [13]. The oxidation of AsA in the reaction mixture was measured using the rate of decrease in absorbance at 290 nm and was calculated using an extinction coefficient of $2.8\ mM^{-1} \cdot cm^{-1}$.

Glutathione reductase (GR) activity was assayed as per the method of Foster and Hess [40], the activity was measured at 340 nm and calculated using the extinction coefficient for NADPH of $6.22\ mM^{-1} \cdot cm^{-1}$ and expressed as µmol NADPH oxidized per gram fresh weight. Glutathione S-transferase was assessed by the method

of Habig *et al.* [41], the enzyme was assayed by its ability to conjugate GSH and CDNB, the extent of conjugation caused a proportionate change in the absorbance at 340 nm. GST activity was calculated using the extinction co-efficient of the product formed (9.6 $mM^{-1} \cdot cm^{-1}$) and was expressed as nmol. GSH oxidized/h^{-1}/g^{-1} DW.

Poly acrylamide gel electrophoresis (PAGE) was performed on 1.5 mm thick gels on the mini slab gel electrophoresis apparatus (Hoefer, USA) according to Laemmli [42], except the addition of SDS, and the gels were captured in a gel documentation system (UvItec, UK). SOD isoforms were visualized following the method described by Beauchamp and Fridovich [43]. Activity of GPX isoforms was visualized on 7.5% gel according to staining procedure of Birecka [44]. In-gel APX activity staining was performed according to Lee and Lee [45]. GR isoforms were visualized following the method described by Ye *et al.* [46].

2.10. Estimation of Pb Content

Oven dried powder sample (0.5 g) of roots and leaves was taken in a 50 ml boiling test tube and 5 ml of concentrated nitric acid (70%) was added and incubated at RT overnight. The next day, 5 ml of di-acid mixture (HNO_3:H_2O_2:10:4) was added to it and placed on a mantle (REMI, India) till all the white fumes evaporated and the thick white residue was left out in flask. These diluted samples were used for Pb estimation using ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrophotometer, Optima 8000, Perkin Elmer India). The concentration of Pb was expressed as $\mu g \cdot per \cdot g^{-1}$ dry weight.

2.11. Statistical Analysis

All data were analyzed using the SPSS (Statistical Package for the Social Sciences) version 16.0. Data presented here are mean values and standard deviation (±SD). One-way ANOVA was carried out using Post hoc multiple comparison from the Duncan's test at a significance level of $p < 0.05$.

3. Results

The two groundnut cultivars differed from each other in terms of morphological, physiological and antioxidative responses to Pb-stress.

3.1. Effect of Pb on Plant Growth

Effect of different concentration of Pb on the growth of both groundnut cultivars in terms of shoot and root length was measured (**Figure 1(a)**). In general, both cultivars showed reduced growth under Pb-stress compared to unstressed conditions. Growth of cultivar K6 was less affected due to Pb treatments compared with cultivar K9. After exposure to 800 ppm Pb, the reduction in the root growth was 24% and 46% in cultivar K6 and K9 respectively, compared to their respective controls. Whereas the shoot growth of both cultivars remained less affected than root growth and the percent reduction was 14% in cultivar K6 compared to 38% in cultivar K9 (**Figure 1(a)**).

3.2. Effect of Pb on Plant Drymass

Increased Pb metal concentration significantly reduced the biomass of two groundnut cultivars. Pb induced root and shoot biomass reduction in cultivar K6 was lower than that of cultivar K9. At 800 ppm Pb treatments the reduction in root dry mass was 13% in cultivar K6 compared to 30% in cultivar K9 and when compared to their controls (**Figure 1(b)**). Similarly, the reduction in shoot dry mass was 18% in cultivar K6 and 47% in cultivar K9.

3.3. Histochemical Detection of Leaves for $O_2^{\bullet -}$ and H_2O_2

Histochemical staining was employed to bring out *in situ* accumulation of $O_2^{\bullet -}$ and H_2O_2, two important reactive oxygen species. Under control conditions there was no detectable difference between cultivar K6 and K9 in the accumulation of $O_2^{\bullet -}$ and H_2O_2 radicals. **Figure 2** illustrates that there was an increase of $O_2^{\bullet -}$ and H_2O_2 levels in both cultivars exposed to higher Pb concentrations compared with their controls. However, remarkable differences were observed between the cultivars with the increasing Pb treatment, in which cultivar K9 showed more local blue spots (**Figure 2(c)**, indicator of $O_2^{\bullet -}$) and brown spots (**Figure 2(d)**, indicator of H_2O_2) than

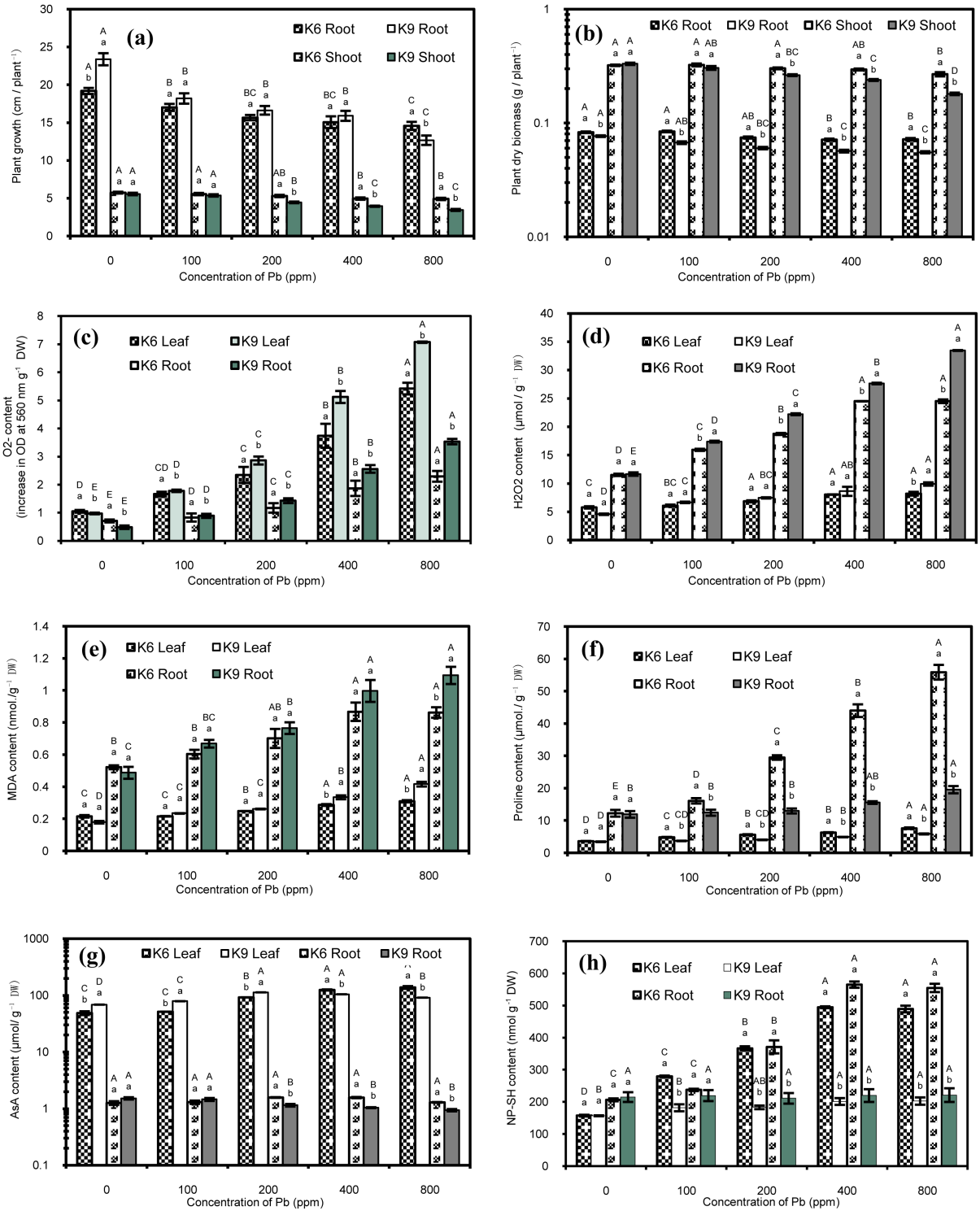

Figure 1. Effect of increasing concentrations of $Pb(NO_3)_2$ on (a) plant growth (cm·plant^{-1}), (b) plant biomass (g·plant^{-1}); (c) superoxide anion production (increase in OD at 590 nm h g^{-1} DW); (d) hydrogen peroxide production (µmol·g^{-1} DW); (e) malondialdehyde (MDA) content (µmol·g^{-1} DW); (f) free praline content (µmol·g^{-1} DW); (g) ascorbic acid content (µmol·g^{-1} DW) and (h) non-protein thiol content (µmol·g^{-1} DW) of two groundnut cultivars. Error bars indicate SD (n = 5). Different uppercase letters indicate significant differences between cultivars in response to treatments (Student's t test; JMP 8.0 software), and lowercase letters indicate significant differences within the cultivar in response to treatments (Tukey-Kramer HSD test; JMP 8.0 software).

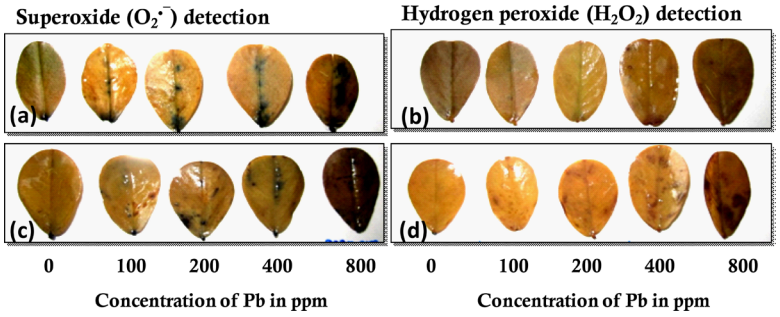

Figure 2. Effect of increasing concentration of $Pb(NO_3)_2$ on free radical generation in cultivar K6 (a) (b) and cultivar K9 (c) (d) leaves.

cultivar K6 (**Figure 2(a), (b)**).

3.4. Effect of Pb on $O_2^{\bullet-}$ and H_2O_2 Production

Pb treatment caused a significant increase in $O_2^{\bullet-}$ production in both groundnut cultivars compared to their controls. A linear increase in $O_2^{\bullet-}$ accumulation with increasing concentration of Pb was observed in both cultivars. However, a more pronounced increase was observed in the leaves of cultivar K9 compared to cultivar K6. At 800 ppm Pb, the per cent increase in the $O_2^{\bullet-}$ production was 624% in cultivar K9 and 415% in cultivar K6 respectively, compared to their respective controls (**Figure 1(c)**). Whereas, the production of H_2O_2 was 42% in cultivar K6 compared to 117% in cultivar K9 (**Figure 1(d)**).

3.5. Lipid Peroxidation (MDA Content)

Malondialdehyde content (**Figure 1(e)**) in roots and leaves of both groundnut cultivars was elevated due to Pb toxicity and the magnitude of elevation was concentration dependent in both the cultivars. However, the per cent increase in MDA content was relatively less in cultivar K6 than in K9. The root MDA content was increased more than leaf MDA content in both the cultivars under Pb-stress conditions.

3.6. Free Proline Content

There was a linear increase in free proline accumulation with increasing Pb concentration was observed in groundnut cultivars. However, a more pronounced increase was noticed in the cultivar K6 compared to cultivar K9. After expose to 800 ppm Pb, the percent increase was 104% in cultivar K6 and 70% in cultivar K9 with respective to their controls (**Figure 1(f)**).

3.7. Effect of Pb on Ascorbic Acid (AsA) and Malondialdehyde (MDA) Content

Significant changes in the AsA content between two groundnut cultivars due to Pb treatments were observed. Cultivar K9 showed higher AsA content at 200 ppm Pb when compared to 400 and 800 ppm Pb treatments (**Figure 1(g)**). Whereas, cultivar K6 showed gradual increase in the AsA content with increasing Pb concentration. Nevertheless, the per cent increase in AsA content was more in cultivar K6 than K9 due to Pb treatments.

3.8. Effect of Pb on Non-Protein Thiols Content (NP-SH)

The concentration of NP-SH in leaves and root of cultivar K6 steadily and consistently increased with increasing Pb-stress when compared to controls. In contrast, levels of NP-SH contents were remained unchanged due to Pb treatment in leaves and roots of cultivar K9 (**Figure 1(h)**).

3.9. Effect of Pb on Antioxidant Enzyme Activities

Pb-stress caused a significant increase in the total SOD enzyme activity in cultivar K6. The total SOD enzyme activity was increased due to Pb-stress up to 400 ppm, but decreased at 800 ppm Pb-stress (**Figure 3(a)**). Similarly, SOD activity was increased in cultivar K9 up to 400 ppm Pb but decreased at 800 ppm Pb concentration.

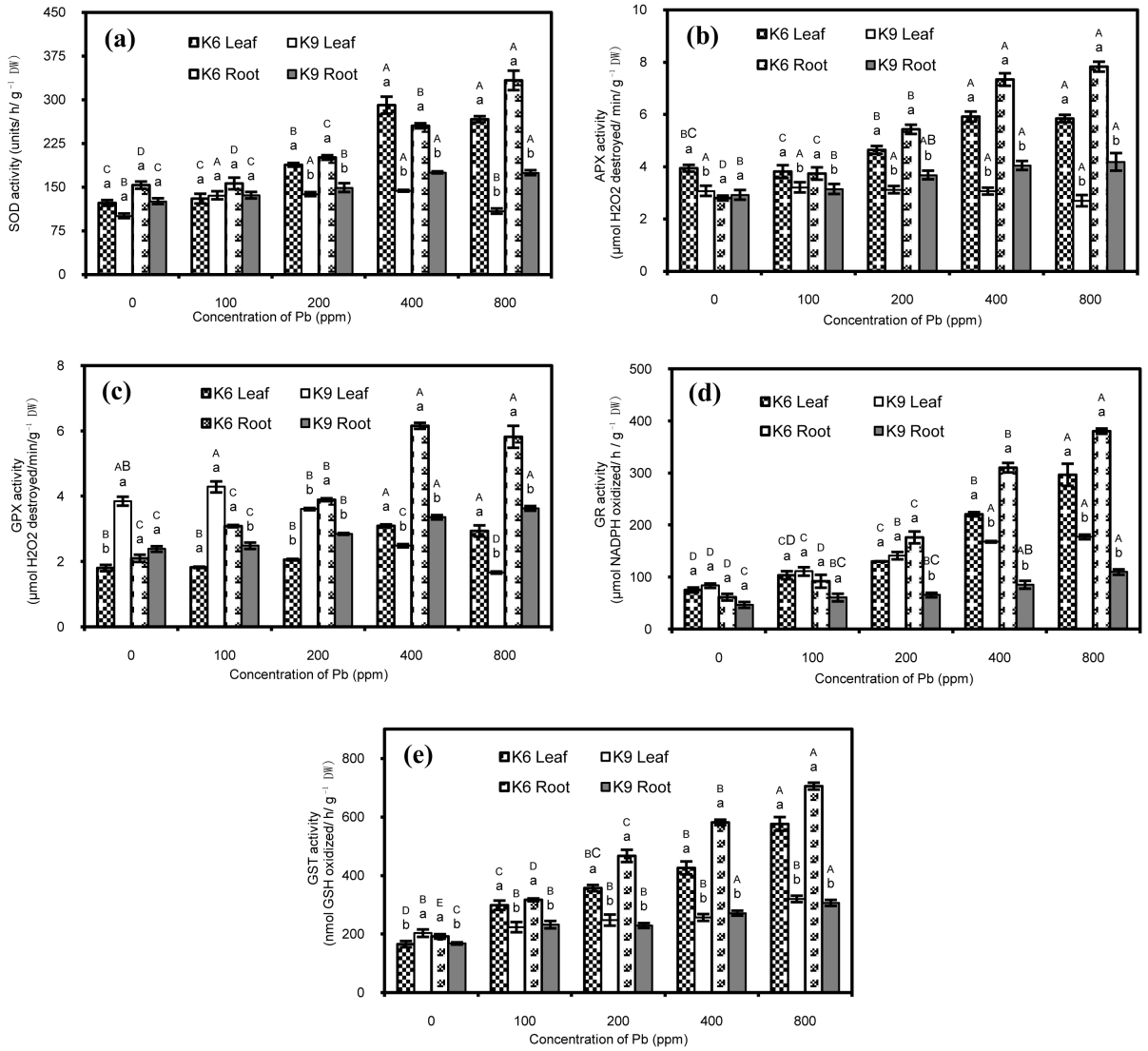

Figure 3. Effect of increasing concentrations of $Pb(NO_3)_2$ on (a) superoxide dismutase (SOD) activity (units $h \cdot g^{-1}$ DW); (b) ascorbate peroxidase (APX) activity (µmol H_2O_2 reduced $min \cdot g^{-1}$ DW); (c) guaiacol peroxidase (GPX) activity (µmol H_2O_2 reduced $h \cdot g^{-1}$ DW); (d) glutathione reductase (GR) activity (µmol NADPH oxidized $min \cdot g^{-1}$ DW) and (e) glutathione S-transferase (GST) activity (µmol GSH $h \cdot g^{-1}$ DW) of two groundnut cultivars. Error bars indicate SD (n = 5). Different uppercase letters indicate significant differences between cultivars in response to treatments (Student's t test; JMP 8.0 software), and lowercase letters indicate significant differences within the cultivar in response to treatments (Tukey-Kramer HSD test; JMP 8.0 software).

However, in both cultivars the activity was remained higher in Pb-stressed plants compared to those controls. The percent increase in SOD enzyme activity was more in cultivar K6 than in cultivar K9.

Marked differences in APX activity were found between two cultivars after 10 days of Pb treatments (**Figure 3(b)**). Leaf APX activity in cultivar K9 was unaffected under Pb-stress but root APX activity was increased at higher Pb concentration (400 - 800 ppm) when compared to respective controls. In cultivar K6 the leaf and root APX activities were increased with increasing Pb-stress when compared to controls.

Pb-stress caused differential response in GPX activity in two groundnut cultivars (**Figure 3(c)**). GPX activity was significantly increased in leaves and roots cultivar K6 and peaked maximum activity at 400 ppm of Pb-stress. The GPX activity was significantly decreased in leaves and increased in roots of cultivar K9 at higher Pb concentration.

The activities of glutathione reductase (GR, **Figure 3(d)**) and glutathione S-transferase (GST, **Figure 3(e)**)

increased in Pb-stressed plants of cultivar K6 and cultivar K9. However the per cent increase was relatively more in cultivar K6 than in cultivar K9 under Pb-tress conditions. And, the degree of elevation in the activities of GR and GST was more in roots compared to leaves of Pb-stressed plants of both the cultivars.

Pb-induced oxidative stress was further corroborated by the antioxidant isozyme profiling by native-PAGE (**Figure 4**). At least six SOD (**Figure 4(a)**), four APX (**Figure 4(b)**) and five GPX (**Figure 4(c)**) isozymes were visualized on in-gel enzyme activity staining. The staining intensities of these isozymes were differed between unstressed and Pb-stressed conditions in both cultivars. An enhanced isozymic activity staining was observed for all three enzymes in Pb-stressed plants compared to unstressed plants. The increased SOD, APX and GPX activities were relatively greater in cultivar K6 compared to cultivar K9.

3.10. Pb Accumulation

The Pb concentration (**Table 1**) of roots and leaves in two groundnut cultivars were increased under Pb-stress when compared to controls. The magnitude of increase in the Pb content of both the cultivars was found to be dependent on soil Pb concentration. Furthermore, the Pb content was relatively more in roots than in leaves. At 800 ppm of Pb, there was a 4.5-fold increase in leaves and 30-fold increase in Pb content in roots of cultivar K6, whereas, 18-fold and 55-fold increase in leaves and roots of cultivar K9, respectively when compared to corresponding controls.

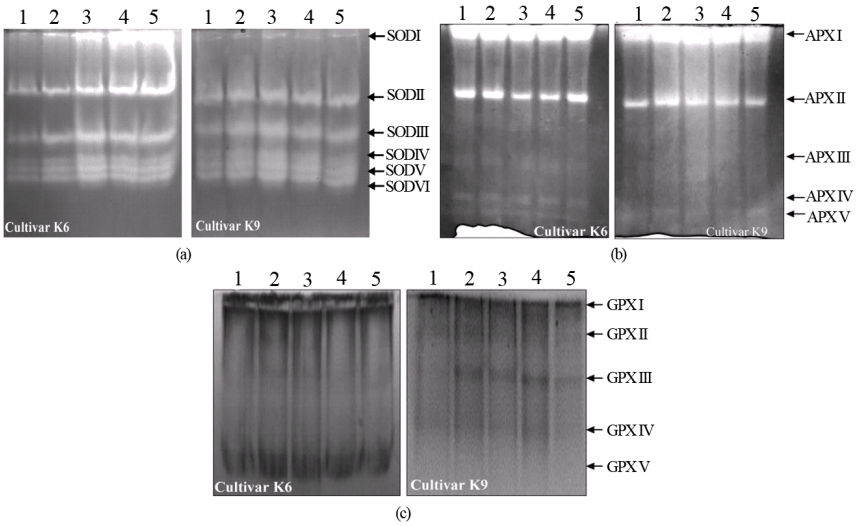

Figure 4. Native gel stained for the activity of (a) SOD; (b) APX and (c) GPX of groundnut (cultivar K6 and K9) leaves. Equal amount of protein (200 µg) was loaded on the gel. Lane 1—control; lane 2—100 ppm; lane 3—200 ppm; lane 4—400 ppm; lane 5—800 ppm $Pb(NO_3)_2$.

Table 1. Pb content ($mg \cdot g^{-1}$ DW in roots and $ug \cdot g^{-1}$ DW in leavews) of control and Pb-stressed groundnut cultivars (±SD).

Organ	Cultivar K6					Cultivar K9				
	0	100	200	400	800	0	100	200	400	800
Leaf	0.199c ±0.009 (100)	0.561b ±0.16 (261.5)	2.74a ±0.046 (284.6)	3706a ±0.302 (407.7)	6.106a ±0.166 (469.2)	0.693d ±0.046 (100)	3.46c ±0.122 (346.1)	8.05b ±0.122 (776.9)	17.33b ±0.092 (1149)	38.1a ±0.323 (1800)
Root	0.13c ±0.046 (100)	0.34c ±0.046 (281.4)	0.37b ±0.046 (1376.8)	0.53b ±0.046 (1862.3)	0.61a ±0.046 (3068.3)	0.13e ±0.046 (100)	0.45d ±0.092 (499.27)	1.01c ±0.092 (1161.6)	1.49b ±0.046 (2500.7)	2.34a ±0.092 (5497.8)

The mean values (n = 5) in a row followed by different letter for each cultivar are significantly different ($P \leq 0.05$) according to Duncan's multiple range (DMR) test. Figures in parenthesis represent per cent of control.

4. Discussion

Morphological changes such as root and shoot growth in response to Pb-stress has been studied by several investigators. It has been reported that root and shoot growth was reduced in plants [23] [27] [47], by Pb-stress. The growth of legume plants grown on Pb ore tailings was reported to be drastically affected [28]. Pb also inhibited root and shoot growth in tobacco [48], and wheat [7]. Similarly, in the present study, the root and shoot growth of cultivar K6 and cultivar K9 were inhibited by Pb-stress and the reduction was found to be concentration dependent. However, the per cent decrease in root and shoot growth was less in cultivar K6 than in cultivar K9, which indicates the better adaptation of former one to Pb-stress. The reduction in root length and shoot length under Pb-stress may be due to the inhibition in cell elongation process [49], or due to reduced mitotic activity as observed in lupin roots [50].

Generally, excess concentration of heavy metals in soil results in lowering plant biomass production. Biomass production has been considered as an index of tolerance level of plants growing on metal enriched soils. Cox and Hutchinson [51], have reported that the dry mass production in non-tolerant plant was significantly negatively correlated with log metal concentration in soil, but the same relationship with tolerant plant was positive. Similarly, in the present study, the dry mass accumulation was much less affected in tolerant cultivar K6 than susceptible cultivar K9 with increasing stress intensity. In concomitant to this, Ekmekci *et al.* [23], reported that the shoot and root ratio (dry mass) was affected by increasing Pb concentrations in maize. Inhibition of fresh and dry mass accumulation under Pb-stress conditions was also reported in cotton seedlings [52], and in sunflower [53]. An increase in the metal supply resulted in inhibition of leaf area in the cotton [52], garden cress [3], and tomato [47] [54]. In contrast, in the present study Pb-stress caused no significant inhibition in the leaf area in tolerant cultivar K6 which reveals it tolerant nature to Pb-stress.

Proline continues to be the most studied molecule under abiotic stresses in plants. A pronounced increase in proline content has been noticed in *Solanum nigrum* [55], and wheat [5], when the plants were exposed to heavy metals. Similarly, in the present study, the proline content was increased in leaves and roots of both cultivars with increasing concentration of Pb. Further, accumulation of proline is more pronounced in root than in leaves of both cultivars. However, the proline accumulation was greater in cultivar K6 than in cultivar K9. It has been reported previously that proline accumulation in stress tolerant plants is higher than in stress sensitive plants [56]. AsA is the major primary antioxidant in all sub-cellular compartments, which reacts directly with ROS, and also acts as a secondary antioxidant by reducing the oxidized form of α-tocopherol and preventing membrane damage [57]. In this study, the AsA content was significantly higher (**Figure 2(d)**) in cultivar K6 than in cultivar K9 in response to Pb-stress. These results suggest that the higher level of AsA in cultivar K6 could be the reason for its relative tolerance to Pb-stress than cultivar K9. Similar results have been reported by Li *et al.* [58] in two cultivars of *Brassica* species grown under copper stress.

H_2O_2 and $O_2^{\bullet-}$ are two kinds of reactive oxygen species causing oxidative stress in plants. Increased reactive oxygen species have been detected in the roots of maize and soybean [59], and in leaves of *Vallisneria natans* [60], *Alocasia macrorrhiza* [61] under heavy metal stress. In this study, we further confirmed that Pb induced generation of H_2O_2 and $O_2^{\bullet-}$ *in vivo* in leaves by histochemical staining. Through the Haber-Weiss reaction H_2O_2 and $O_2^{\bullet-}$ can be transformed to the highly reactive oxidant $OH^{\bullet-}$, which causes lipid peroxidation in plant cell [62]. MDA is a cytotoxic product of lipid peroxidation and an indicator of membrane damage from oxidative stress. Pb toxicity is reported to induce the increase of MDA in *Talinum triangulare* [63]. As excess of heavy metals stimulate the formation of free radicals [64]. A constitutive amount of $O_2^{\bullet-}$ is considered as a signal molecule in signal transduction and programmed cell death [65], however, if $O_2^{\bullet-}$ not scavenged efficiently, excessive accumulation of $O_2^{\bullet-}$ might impair biological molecules. SOD can catalyze the dismutation reaction of $O_2^{\bullet-}$ into molecular oxygen and H_2O_2, and thus represent primary antioxidant defence in the plant cells. The level of $O_2^{\bullet-}$ and H_2O_2 were increased significantly, in the leaves and roots of both groundnut cultivars in all Pb treatments were observed.

Plant possess a well organized ROS scavenging systems comprising enzymatic such as SOD, APX, GR, GPX and CAT, and non-enzymatic antioxidants such as AsA, GSH, NP-SH and PCs. A coordinated function of these systems plays an important role in scavenging ROS and maintaining redox status of the cell [66]. In the current study, the ROS levels in groundnut are controlled by a complex enzymatic and non-antioxidant system. The present results show an increase of AsA and NP-SH coupled with enhanced SOD, GPX and APX activities in groundnut cultivars growing in the presence of Pb-stress could be attributed to the increased $O_2^{\bullet-}$ and H_2O_2

radical concentration. Increased activity of APX may efficiently scavenge H_2O_2 to protect against oxidative damage, SOD, APX and GPX, in general, show simultaneous induction and decline, which may be due to their co-regulation as reported earlier [67].

Further, the variations in isozyme profiles and increase in the isozyme activities are considered to play important role in the cellular defence against stress induced oxidative stress. The intensity of the isozyme bands can reflect relative quantity and activity of the isozyme [68]. Pattern of SOD, GPX and APX isozyme expression obtained from leaf tissue of both the cultivars are shown in **Figure 4**. Although we did not identify the specific activity of each isozyme, the total antioxidant isozymes in different tissues might be connected with increased requirement to combat Pb toxicity. In the present study, the involvement of free radicals in membrane lipid peroxidation in both the cultivars subjected to Pb-stress could be a possible reason for the increase in MDA content. Generally, free radical generation and membrane damage would be low in tolerant plants and thereby the formation of lower levels of MDA content.

The reduced form of glutathione (GSH) is one of the most important components of metabolism of NP-SH and play important roles in heavy metal tolerance and sequestration [69] [70]. GSH is the direct precursor of phytochelatins (PCs) in a reaction catalyzed by phytochelatin synthase (PCS). It is known that the metal stress alleviates the depletion of GSH [60], and enhance PCs synthesis, resulting in an increased metal tolerance of plants [71]-[73]. Nouairi *et al.* [71], reported that the level of PC-SH in tolerant plants was significantly increased under Cd stress in *Brassica juncea*. Similarly, in the present investigation, thiol (-SH) compounds were significantly higher in cultivar K6 under Pb-stress compared to cultivar K9. This may be ascribed to increased levels of PCs content in tolerant cultivar K6.

Pb uptake studies in plants have demonstrated that roots have an ability to take up significant quantities of Pb simultaneously, greatly restricting its translocation to the above ground parts [49]. In this study, both groundnut cultivars have the ability to accumulate Pb primarily in their roots and transport it to their leaves in much lesser concentration. Similar results were reported by [6] and [23]. The results also indicated that the accumulated Pb in roots of cultivar K9 was higher than the roots of cultivar K6 in all Pb treatments. Based on comparative studies of metal content in plant parts Baker and Walker [74], suggested that uptake, translocation and accumulation mechanisms differed for various heavy metals and for the species. It is known that the root system partially defends the above ground parts from Pb [75], as shown in the present study. Generally, the plants with highest tolerance take-up the smallest ratio of the total soil-metal and had the lowest shoot metal contents [6] [28]. In parallel to this, although both the cultivars in this study accumulated low Pb levels in the leaves, cultivar K6 accumulated still a less proportion of Pb by restricting the uptake and further translocation from roots to leaves. Our study also indicated that the uptake and translocation properties of Pb indicated the retaining abilities of the roots to Pb.

5. Conclusion

In conclusion, the two groundnut cultivars showed differential tolerance to Pb-stress. The higher tolerance of cultivar K6 to Pb-stress was due to the lower levels of Pb accumulation in the leaf and root tissues and coordinative interaction between the non-enzymatic and enzymatic antioxidants systems, which serves efficiently to protect cultivar K6 from the oxidative stress and thus the relatively better growth than the susceptible cultivar K9. Overall, antioxidant machinery plays an important role in Pb-stress tolerance of plants. In the present study, we hypothesized that: enhanced levels of non-enzymatic antioxidants and MDA content in both groundnut cultivars differ in sensitivity to Pb exposure, to activate the multi tolerance mechanism of antioxidative enzymes under Pb-stress, to induce genotoxic changes caused by Pb-stress, potential detoxification strategies are evolved. The results of the present study also demonstrate the effect of Pb exposure in cultivar K6, the cultivar capability to activate multi defense mechanism against oxidative damage and genotoxicity caused by Pb ions may be a key factor in the detoxification mechanism of plant tolerance to unfavourable conditions.

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