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Antioxidative enzyme responses under single and combined effect of water and heavy metal stress in two *Pigeon pea* cultivars

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Abstract

Heavy metal toxicity and water stress are the two frequently co-occurring abiotic stresses restraining the growth of crop plants as the metal contaminated sites are over alarmingly increasing due to industrial pollution. The present study is to evaluate the effect of heavy metals (Cadmium, Chromium), PEG induced water stress and their combination on antioxidative enzyme activities in two Pigeon pea cultivars LRG-41 and Yashoda-45. The activities of antioxidative enzymes Superoxide dismutase, Catalase, Ascorbate peroxidase, Polyphenol Oxidase and Glutathione Reductase registered higher values in both roots and shoots with all the single stress treatments when compared to their controls. The levels of antioxidative enzyme activities and lipid peroxidation were high in cv. Yashoda-45 than cv. LRG-41. Overall, cv Yashoda-45 has shown better tolerance to both heavy metal and water stress. These results may show the novel insight in to the responses associated with combined stress in the cultivars of economically important crop, Pigeon pea.

Keywords: Antioxidative enzymes, Cadmium, Chromium, Water stress, *Pigeon pea*.

Introduction

Plants being stationary are exposed to multiple stresses simultaneously in nature. The stresses may bring about various responses in plants that could be additive, synergistic or antagonistic. In arid and semi-arid regions, water is usually one of the most important factors limiting crop production.[1]

Anthropogenic activities such as indiscriminate use of pesticides containing heavy metals in agriculture, discharge of untreated wastes and effluents, mining caused contamination of agricultural lands.[2] With the development of modern industry and agriculture, cadmium and chromium has become widespread pollutants in agricultural soils. Heavy metal toxicity and water stress are the two frequently co-occurring abiotic stresses restraining the growth of crop plants as the metal contaminated sites are over alarmingly increasing due to industrial pollution. However, most of the studies have been devoted to assess the physiological responses of plants in a single stress environment like drought, heavy metals and salinity. Studies on plant responses under combination of stresses are restricted to a few reports.[3]

During cell metabolism, reactive oxygen species (ROS) are generated as natural byproducts. The exposure of plants to environmental stresses enhanced the generation of ROS. To avoid the subsequent damage, cells are normally protected against by the

cellular antioxidant defense systems. The activities of antioxidant enzymes in plants under stress are usually regarded as an indicator of tolerance of genotypes against stress conditions.

In view of the above, an assessment of multiple stress effects is very important in obtaining a realistic view of the impact of current changes in the environment on crop plants. The present study was aimed to evaluate the interactive effects of two potential stress factors i.e. water stress and heavy metals (Cd / Cr) applied individually and in combination on the activities of antioxidative enzymes of two pigeon pea cultivars.

Materials and Methods

Seeds of Pigeon pea (*Cajanus cajan* cv.LRG-41 and Yashoda-45) were surface sterilized in 0.1% Hypo solution for 5 min followed by a thorough rinsing in distilled water 5-6 times. Seeds were equispacially arranged on filter papers placed in sterile petridishes for germination and maintained three replicates per treatment. The root and shoot were harvested 10 days after sowing and the antioxidative enzyme activities were recorded. Treatments included i) Control (C) ii)100 ppm Cadmium (Cd) iii) 100 ppm Chromium (Cr) iv) Water stress caused by PEG – 6000 according to Michel and Kaufmann (1973): Ws1 (-0.6 MPa) , Ws2 (-0.9 MPa). v) Water stress + Cadmium combination: Ws1+ Cd, Ws2+ Cd vi) Water stress+ Chromium combination: Ws1+ Cr, Ws2+ Cr. The concentrations, though rather high, were based on pilot experiments conducted prior to study, using a wide range of Cd and Cr levels (0.1 to 400 ppm).

Superoxide dismutase (SOD; E.C.1.15.1.1.) assay

The activity of superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium, adopting the method of Beauchamp and Fridovich.[4]

Catalase (CAT; E.C. 1.11.1.6.) assay

Ascorbate Catalase activity was determined according to the modified method of Aebi.[5]

Peroxidase (APX; E.C.1.11.1.11.) assay

The H_2O_2 dependent oxidation of ascorbate was followed by a decrease in absorbance at 290 nm using the molar extinction coefficient 2.8 mM cm^{-1} according to modified method of Zhu *et al*.[6] One gram each of roots and shoots, were homogenized in 50 mM phosphate buffer containing

1 mM EDTA and 2% PVP (w/v), pH 7.8 at a proportion of 1:3 (w/v). The homogenate was centrifuged at 13,000 X g for 20 min at 4ºC and the supernatant used for the estimation of enzyme activity. The reaction mixture in a total volume of 2 ml consisted of 25 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1 mM H2O2 and100 μl extract.

Polyphenol oxidase (PPO; E.C.1.14.18.1.) assay

The polyphenol oxidase activity was assayed by using the method of Sadasivam and Manickam.[7]

Glutathione Reductase (GR; E.C.1.6.4.2.) assay

The assay of Glutathione reductase in the crude tissue homogenate was carried out according to Smith *et al*.[8]

One gram each of roots and shoots were homogenized separately in a mortar using 5 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 0.5 mM EDTA. The brie was filtered with cheese cloth and the filtrate was centrifuged for 10 min at 20, 000 X g to sediment particulate matter. The supernatant is referred to as crude extract. The protein content of the enzyme extract was estimated following the method of Lowry *et al*., 1951. The reaction mixture contained 1.0 ml of 0.2 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.5 ml of 3 mM 5, 5 - dithiobis (2-nitrobenzoic acid) in 0.01 M phosphate buffer (pH 7.5), 0.1 ml of 2 mM NADPH, 0.05 ml of enzyme extract and 0.1 ml of 20 mM oxidised glutathione (GSSG). The components were added to a cuvette in the order listed and the reaction was initiated by the addition of GSSG. GR activity was determined by measuring the increment in the absorbance at 412 nm when 5, 5'- dithiobis (2-nitrobenzoic acid) (DTNB) was reduced by GSH, generated from glutathione disulfide (GSSG). The activity was calculated as the amount of reduced DTNB, in μmol / min mg-1 protein, ε420 = 13.6 mM^{-1} cm^{-1}.

Lipid peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde content following the method of Heath and Packer[9] with slight modification. One gram of material was macerated in 5 ml of 0.1% trichloroacetic acid. The homogenate was centrifuged at 10,000 x g for 5 min. For every 1 ml aliquot of the supernatant, 4 ml of 20% TCA containing 0.5% thiobarbituric acid was added. The mixture was heated at 95ºC for 30 min and then cooled quickly on ice- bath. The resulting mixture was centrifuged

at 10,000 x g for 15 min and the absorbance of the supernatant was measured at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The concentration of malondialdehyde was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Results

Superoxide dismutase activity of the roots and shoots of Pigeon pea cultivars exposed to water stress, Cd, Cr and Ws + Cr exhibited a rise when compared to control. But, Ws + Cd combination significantly decreased the SOD activity in both roots and shoots of Pigeon pea cultivars except in LRG-41 with Ws3 + Cd where, a slight increase in SOD activity was found (Figure 1).

Catalase activity of roots and shoots of Pigeon pea cultivars is shown in Figure 2. The catalase activity of the roots and shoots of Pigeon pea cultivars treated with PEG, Cd and Cr registered higher values when compared to their respective controls except in Ws + Cd and Ws + Cr combinations.

Figure 3 demonstrates that the activity of Ascorbate peroxidase showed a slight increase in response to individual treatments and also in combination (Figure 3).

Both the stresses individually and in combination showed a marked increase in the activities of Polyphenol oxidase (Figure 4) and Glutathione reductase (Figure 5) in the cultivars of the Pigeon pea studied.

In the present study, MDA content increased (Figure 6) in all the treatments when compared to control in Pigeon pea cultivars.
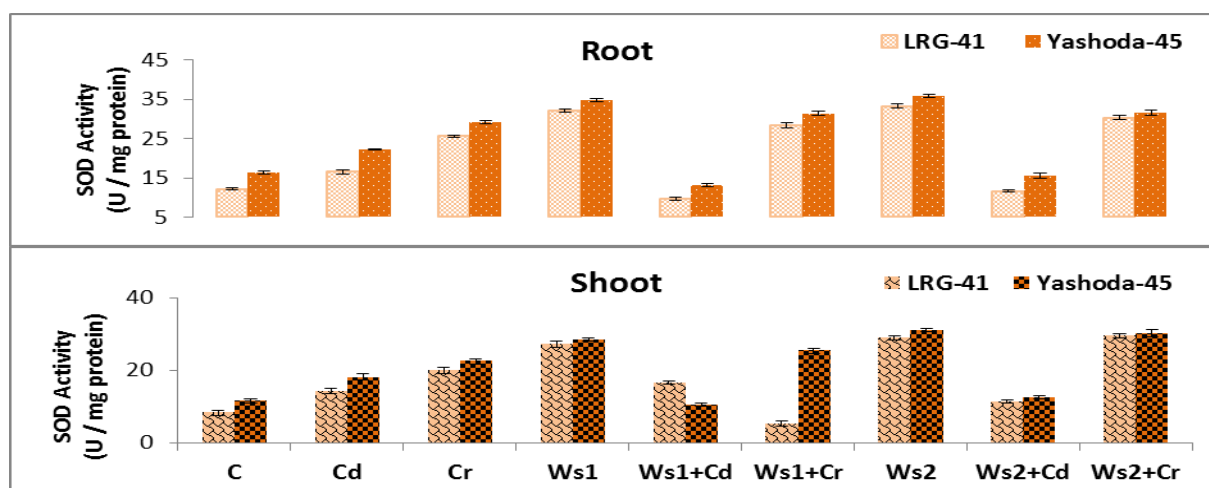

Figure 1: Effect of Water stress, Cd and Cr on SOD activity in roots and shoots of two Pigeon pea cultivars
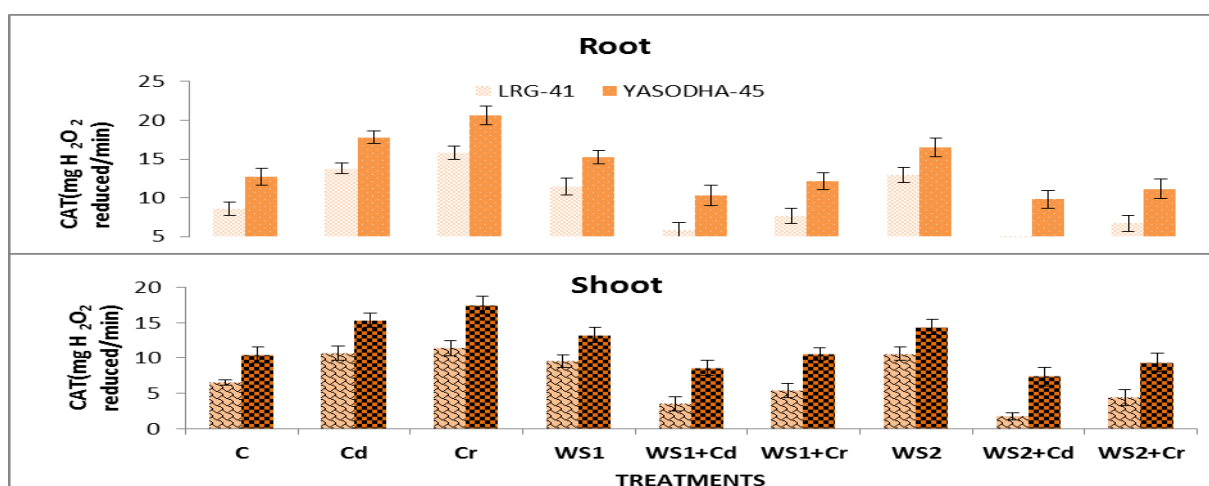

Figure 2: Graphical representation of CAT activity in roots and shoots of two Pigeon pea cultivars under Water stress, Cd and Cr
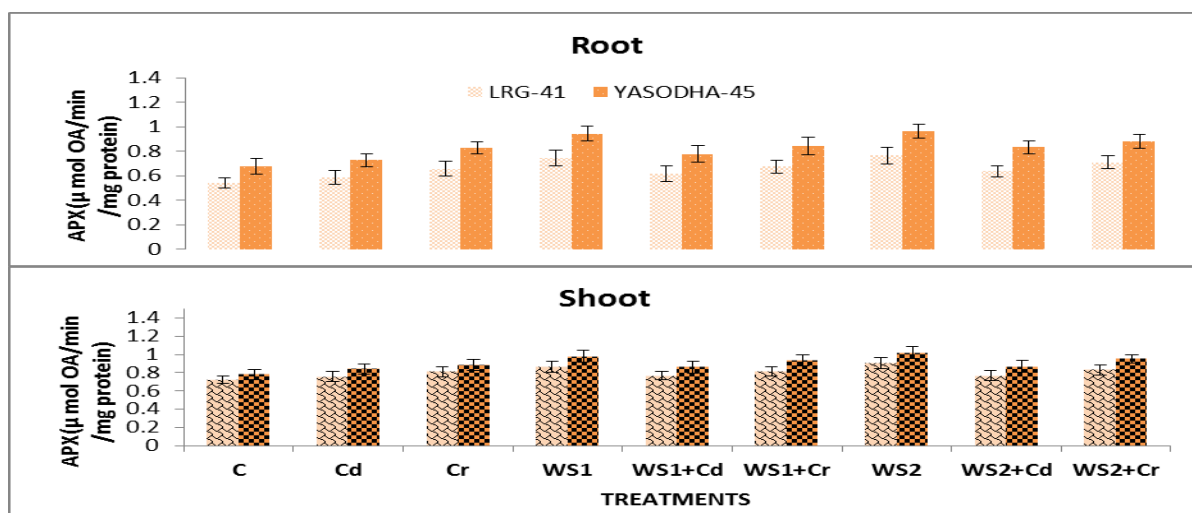
Figure 3: Effect of Water stress, Cadmium and Chromium on APX in roots and shoots of two Pigeon pea cultivars

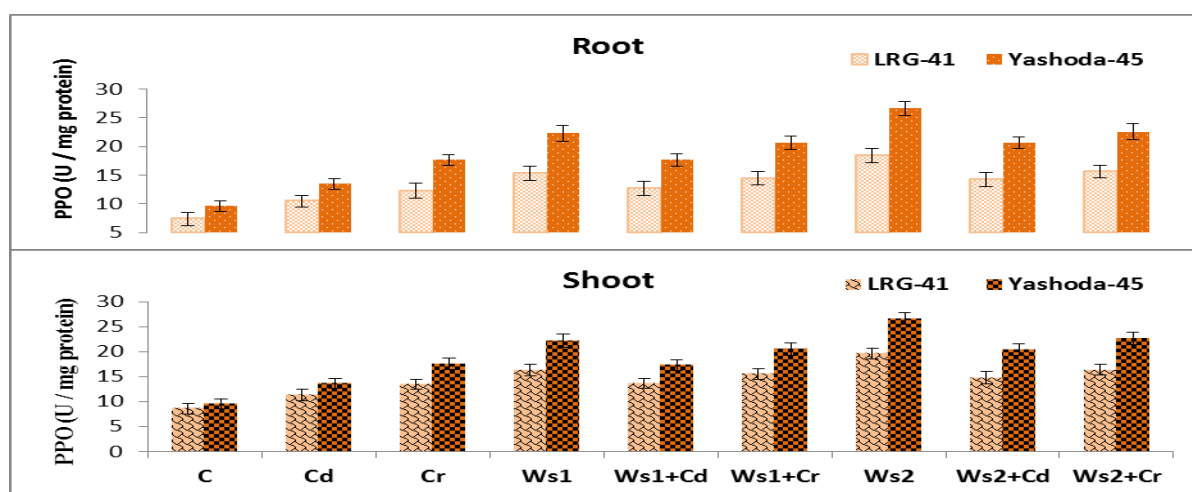
Figure 4: Effect of Water stress, Cd and Cr on PPO activity in roots and shoots of two Pigeon pea cultivars
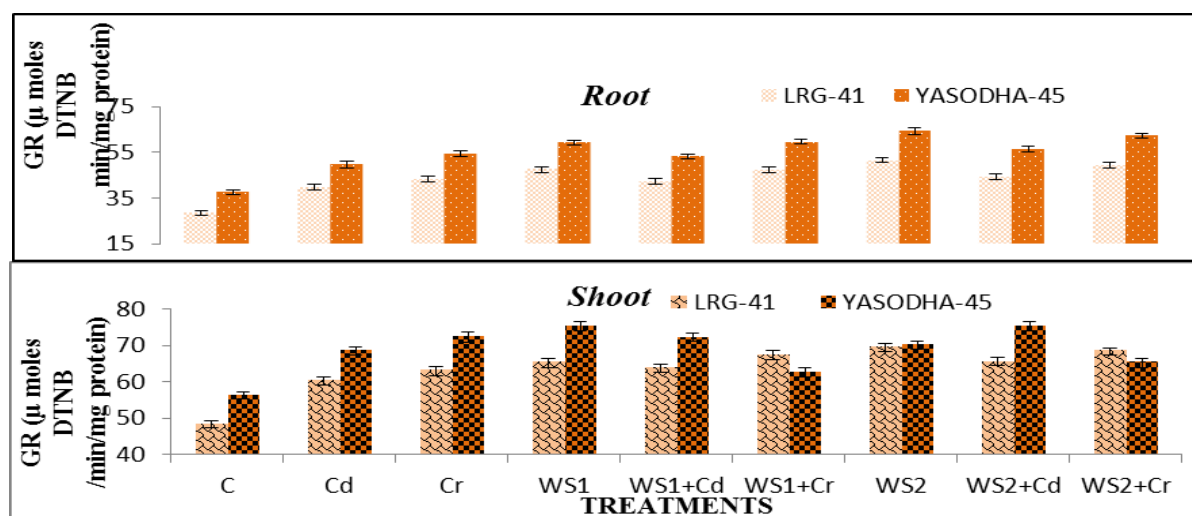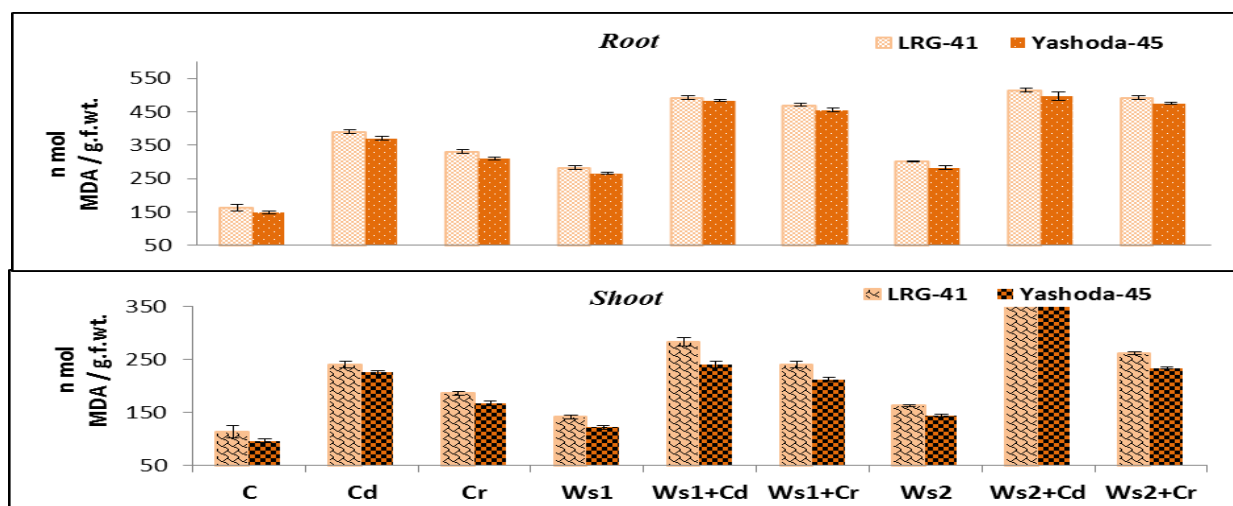

Figure 5: Graphical representation of GR activity in roots and shoots of two Pigeon pea cultivars under Water stress, Cd and Cr

Figure 6: Effect of Water stress, Cadmium and Chromium on lipid peroxidation in roots and shoots of two Pigeon pea cultivars

The F ratio of ANOVA shows that there is a change in antioxidant activity due to cultivar with higher values in Yasodha-45 than LRG-41(in both root and shoot). The effect of treatments in all the cases is significant and the interaction between cultivar and treatments is significant. Comparision of treatments with control was done by Dunnet's test.

Discussion

The ability of plants to overcome oxidative stress partly relies on the induction of SOD activity and subsequently on the upregulation of other downstream antioxidant enzymes.[10] According to the fact that SOD processing is known to be substrate inducible[11], an increase in the SOD activity in the individual stress treatments may be attributed to the increased production of active oxygen species as substrate that lead to increased expression of genes encoding SOD. The enhanced SOD activity observed is consistent with previous reports in which other plant species were treated with Cd.[12-14] The results are consistent with other studies reporting the increased SOD activity in response to drought stress in sunflower[15], poplar[16], cowpea[17], liquorice[18], wheat[19, 20] and pea[21]. According to the results, the maximum increase in the SOD activity was observed in Yashoda-45 cultivar, which might lead to their higher protection against water deficit. However, the cultivars of Pigeon pea showed a significant decrease in SOD activity in Ws + Cd and this may be related to the low potential of these cultivars to remove O_2^- under the combination of stresses.

H_2O_2, which results from the action of SOD, is toxic to cells. Therefore, it is important that H_2O_2 be scavenged rapidly by the antioxidative defense system to water and oxygen.[22] The over expression of SOD, accompanied by enhanced H_2O_2 scavenging mechanisms like CAT and APX enzyme activities, has been considered as an important antidrought mechanism to cope with oxidative stress during water deficit conditions.[23]

The increased catalase activity can be associated with H_2O_2 scavenging. This increase suggests a compensatory mechanism of defence against oxidative stress caused by toxic metal concentrations and can be explained by increase in its substrate to maintain the level of H_2O_2 as an adaptive mechanism of the plants.[24] Furthermore, the combined action of CAT and SOD is critical in mitigating the effects of oxidative stress, since their roles in the cell metabolism are complementary.[25] Both SOD and CAT activities increased in Cd treated Pigeon pea seedlings and it is widely agreed that plants resist oxidative stress by increasing components of their intrinsic defense system. It was suggested that the heavy metal induced decrease in catalase activity can be attributed to inhibition of the synthesis of catalase and other oxidase proteins. The decline in CAT activity is regarded as a general response to many stresses.[26-28] The reduction of CAT activity is supposedly due to the inhibition of enzyme synthesis or change in the assembly of enzyme subunits under stress conditions. It may also be associated with degradation caused by induced peroxisomal proteases or may be due to the photo-inactivation of the enzyme.

APX is thought to play the most essential role in scavenging ROS and protecting cells in higher plants, algae, Euglena and other organisms. APX is involved in scavenging of H_2O_2 in water-water and Ascorbate-glutathione cycles and utilizes ASH as the electron donor. APX has a higher affinity for H_2O_2 (mM range) than CAT

and POD (mM range) and it may have a more crucial role in the management of ROS during stress.

The oxidative damage to different cellular components by H_2O_2 could be minimised either by catalase and peroxidase activities or by a reaction sequence known as ascorbate–glutathione cycle involving the redox pairs of ascorbate–dehydroascorbate and glutathione–glutathione disulphide. Hydrogen peroxide and superoxide radical (O_2) by themselves are relatively less damaging, but they can form species damaging the essential cellular components such as hydroxyl radicals (OH.) that can initiate lipid peroxidation and also attack DNA, proteins and many small molecules.

The higher activity of APX in treated plants would partly explain the lower H_2O_2 concentration, protecting the plants against oxidative stress damage.

Enhanced expression of APX in plants has been demonstrated during different stress conditions. Increased leaf APX activity under Cd stress has been reported in *Ceratophyllum demersum*[29], *Brassica juncea*[30], *Triticum aestivum*[31] and *Vigna mungo*[32]. Hsu and Kao[33] reported that pretreatment of Oryza sativa seedlings with H_2O_2 under non-heat shock conditions resulted in an increase in APX activity and protected rice seedlings from subsequent Cd stress. Significant increase in APX activity was noted under water stress in three cultivars of *Phaseolus vulgaris*[34] and *Phaseolus asperata*[35]. The findings of Koussevitzky *et al.*[36] suggest that cytosolic APX1 plays a key role in the protection of plants to a combination of drought and heat stress. Simonovicova *et al.*[37] also reported increase in APX activity in *Hordeum vulgare* L. cv. Alfor root tips under Al stress at 72 h.

PPO is a terminal oxidase which can directly pass electrons to O_2 when the intermediate products of plant respiration are oxidized. It could catalyze the oxidation of such a group of compounds as phenol to quinone. PPO has some relationship with the synthesis of cell wall compounds containing phenol groups such as lignin.

It has been reported that PPO showed increased activity in response to various types of stresses[38]. The enhanced activity of PPO due to the presence of toxic heavy metals, is responsible for binding and detoxification of heavy metals. Therefore, the mechanism of Cd detoxification is by the formation and trapping of Cd -Ca crystals during the process of phenol polymerization by phenol oxidases. Increase in phenol concentration due to heavy metal stress has also been noted in maize[39] and pine[40].

GR is a flavo-protein oxidoreductase, found in both prokaryotes and eukaryotes[41]. It is a potential enzyme of the ascorbate-glutathione cycle and plays an essential role in defence system against ROS by sustaining the reduced status of GSH. GR catalyzes the reduction of GSH, a molecule involved in many metabolic regulatory and antioxidative processes in plants where GR catalyses the NADPH dependent reaction of disulphide bond of GSSG (oxidized glutathione) and is thus important for maintaining the GSH (reduced glutathione) pool[42]. GR is involved in defence against oxidative stress, whereas, GSH plays an important role within the cell system, which includes participation in the ASH-GSH cycle, maintenance of the sulfhydryl (-SH) group and a substrate for Glutathione-S-transferses. These reactions also reduce or avoid the formation of reactive OH- radical. GR and GSH play a crucial role in determining the tolerance of a plant under various stresses[43].

Similar increase in GR activity was reported in the presence of Cd in *Arabidopsis thaliana*[44], *Vigna mungo*[45]. Eyidogan and Oz, [46] reported increased GR activity in the leaf tissue of *Cicer arietinum* L. cv. Gokce under salt stress, Whereas, Kukreja *et al.*[47] noted increased GR activity in *Cicer arietinum* roots following salt stress. Sharma and Dubey noted a significant increase in GR activity in drought stressed *O. sativa* seedlings[48].

The peroxidation of lipids is considered as the most damaging process known to occur in every living organism. During LPO, products are formed from polyunsaturated precursors that include small hydrocarbon fragments such as ketones, MDA (malondialdehyde) and compounds related to them[49]. LPO, in both cellular and organelle membranes, takes place when above-threshold ROS levels are reached, thereby not only directly affecting normal cellular functioning, but also aggravating the oxidative stress through production of lipid-derived radicals[50]. The overall effects of LPO are to decrease membrane fluidity; make it easier for phospholipids to exchange between the two halves of the bilayer; increase the leakiness of the membrane to substances that do not normally cross it other than through specific channels and damage membrane proteins, inactivating receptors, enzymes, and ion channels.

It has also been noted that plants exposed to various abiotic stresses exhibit an increase in LPO due to the generation of ROS. The present study showed that the levels of LPO increased in both CVS. of Pigeon pea but its level was higher in LRG-41 than Yashoda-45 and they correlated the

higher free radicals scavenging capacity and more efficient protection mechanism of Yashoda-45 against stress with lower level of LPO in comparison to LRG-41. Similar results has been reported that water stress increased the LPO, membrane injury index, H_2O_2 and OH- production in leaves of stressed *Phaseolus vulgaris*[34] and tomato[51] Simova-Stoilova *et al.*[52] reported that the weakening of membrane integrity and oxidative damage to lipids were more pronounced in the sensitive varieties under field drought conditions in wheat plants.

SOD and GR showed more substantial activity than CAT and APX

The shoots of both the cultivars registered lower levels of SOD, CAT and higher levels of APX, GR activities than roots resulting in relatively less damage in shoots suggesting that roots might act as a kind of filters of stress inducing factors for the protection of plants.

Conclusion

The present study showed that water stress and heavy metals both caused oxidative damage to plants through excessive ROS generation. Water stress caused more damage than the heavy metals and their effect in combination was less than additive in both the cultivars of Pigeon pea. Ws+Cd treatment triggered more stress than Ws+Cr. Further biochemical and molecular studies are required to elucidate the effect of combination of stresses in Pigeon pea cultivars.

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