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STUDY OF EARLY RESPONSE IN CASTOR (*RICINUS COMMUNIS* L.) SEEDLINGS UNDER CADMIUM AND ZINC STRESS

A. Mondal[1] and R. Kundu[2*]

[1]Assistant Professor in the Department of Botany, Rishi Bankim Chandra College, Naihati-743165, North 24 Paraganas, West Bengal.

[2]Professor in the Department of Botany, Ballygunge Science College, University of Calcutta, 35 Ballygunge Circular Road, Kolkata -700019, West Bengal.

***Corresponding Author Email: kundu_rita@yahoo.co.in**

ABSTRACT

Cadmium (Cd) pollution is a source of worldwide concern because of its high toxicity to plants, animals and humans. Zinc (Zn) is an essential micronutrient required for several physiological processes. Rapid industrialization has resulted in the accumulation of essential and non-essential heavy metals such as Cd, Cr, Al, Zn, As etc. in the environment. Although the non-essential metals are required for normal growth in plants, supra-optimal levels of these heavy metals (for eg: - Zn, Cu, Al etc.) are detrimental for plants. The aim of this study is to investigate the early response of the antioxidant system of young castor (Ricinus communis L.) seedlings when exposed to Cd and Zn stress. These seedlings were grown in hydroponics for one month and later supplemented with $CdCl_2$ (10 mg kg $^{-1}$ Cd) and $ZnSo_4$ (300 mg kg $^{-1}$ Zn) solution, individually. The non-enzymatic antioxidants (such as carotenoids, proline, non-protein thiols, endogenous H2O2, malondialdehyde, phenolics and flavonoids) increased significantly in the treated seedlings than the control ones after 72 and 120 hours. The enhanced production of superoxide dismutase (SOD, EC1.12.1.1), ascorbate peroxidase (APX, EC1.11.1.11), guaiacol peroxidase (GPOD, EC 1.11.1.7) and glutathione reductase (GR, EC1.6.4.2) indicated that these enzymes played a key role in defense mechanism, against the toxicity of these elements due to their excess concentrations.

KEY WORDS

Antioxidants, heavy metal stress, non-protein thiols, Ricnius communis L.

1.INTRODUCTION

The term 'heavy metals' applies to the group of metals with atomic density greater than 4 g/cm^3 (Hawkes, 1997). Heavy metals like Cd, Cr, Ni, Sn, Hg etc. are non-essential elements whereas, metals like Cu, Zn, Mn, Co etc. are considered essential for plants in trace amounts (Niess *et al.,* 2009). Non-essential metals cause toxicity, sometimes, even in low concentrations. Among them, cadmium (Cd) is a very toxic metal, as it is highly mobile and has a long half-life (Salt *et al., 199*5, Patra *et al.,* 2004). When the concentrations of essential metals such as zinc (Zn) exceed beyond permissible limits, they can result in oxidative stress (Hassan *et al*., 2011, Todeschini *et al*., 2011; Cambrolle´ *et al*., 2012). To overcome this stress, plants adopt several enzymatic and non-enzymatic mechanisms to eliminate or reduce the harmful effects. These help the plants to successfully thrive in heavy metal contaminated areas.

Cadmium is ranked 7th in the list of Agency for Toxic Substances and Disease Registry (ATSDR, 2008). It has been classified as human carcinogenic group I. Cadmium is readily released into the environment by smelting, mining and cadmium-phosphate rich fertilizers (Huff *et al*., 2007). The phytotoxic limit for cadmium was reported in the range of 3-7 mg kg^{-1}, depending on the plant species (Environmental Protection Agency, 2009). Excess cadmium uptake may cause leaf chlorosis,

damage to plasma membrane permeability and alteration in the activity of many key enzymes (Das *et al.,* 1998, Chaoui *et al.,* 1997, Baryla *et al.,* 2001). Although cadmium has no known biological function, plants can readily take up Cd^{+2} ions through their roots and travel to the aerial shoots (Patra *et al*., 2004)

Zinc is a micronutrient essential for normal growth and metabolism for plants (Cakmak *et al.,* 2000). It serves as a catalyst for many essential enzymes such as superoxide dismutase (Cu/Zn SOD). Besides, it also has an important role in gene expression and DNA transcription (Coleman *et al.,* 1992). However, zinc in high concentrations, can be detrimental to plants (Rout *et al.,* 2003). The elevated zinc concentrations may cause altered mitotic activity, damage to membrane permeability and growth retardation (Jain *et al.,* 2010). Zinc levels above 100-200 mg kg^{-1} is generally considered toxic for plants (Kramer *et al.,* 2010). The potential sources of anthropogenic zinc emissions are emissions from power plants, commercial fertilizers, coal and bottom fly ash (Zinc in the Environment, 1997). *Ricinus communis* L. is an oil yielding, perennial shrub found in the wild. It has been reported to be invasive in many countries. It is an erect tropical plant and can grow up to 30 feet tall. In the recent times, this plant is being used to clean up toxic contaminated soils. These plants can successfully thrive in metal contaminated areas. They are capable of degrading at least 15 organic pollutants (Rissato *et al.,* 2015). It has been reported earlier that castor bean plants can accumulate up to 300 mg kg^{-1} lead (Pal *et al.,* 2013).

In the present work, we have aimed to study the early response of *Ricinus communis* seedlings when treated with cadmium and zinc and the detoxification mechanism adopted by the plants. For this purpose, we have chosen a concentration higher than the phytotoxic limit of Cd i.e, 10 mg kg^{-1} and for Zn, 300 mg kg^{-1}. The detoxifying defense machinery includes non-enzymatic (carotenoid, proline, flavonoids and phenols) and enzymatic antioxidants [superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPOD) and glutathione reductase (GR)]. Apart from this, oxidative damage at a cellular level is measured by endogenous hydrogen peroxide, non-protein thiol (NPSH) content and malondialdehyde (MDA) content.

2. Materials and methods

2.1 Plant material, growth conditions and treatments

Castor bean seedlings were grown in the laboratory condition for 30 days and maintained in hydroponics for seven days. The seedlings were treated with 10 mg kg^{-1} cadmium and 300 mg kg^{-1} zinc for 5 days separately. The seedlings grown in Hoagland's nutrient solution were maintained as untreated (control) set. The experimental data was recorded after 72 and 120 hours.

2.2 Estimation of total chlorophyll and carotenoid content

The total chlorophyll and carotenoid content was measured spectrophotometrically according to Lichtenthaler *et al*, 1987.

2.3 Estimation of proline content

0.5g shoot and 0.5g root tissue were homogenized in 5ml 0.1M sulfosalicylic acid and centrifuged at 5000 rpm in a cooling centrifuge (Remi C-24 Plus) for 30 minutes. To the supernatant (2ml), 5ml of glacial acetic acid and 5ml of ninhydrin solution were added. The resulting mix was heated in a water bath at 100º C for 1 hour. The mixture was extracted with toluene in a separating funnel and absorbance was taken at 520 nm. Free proline amount was determined (µg gm^{-1} tissue) from a previously prepared standard curve (Bates *et al.,* 1973).

2.4 Estimation of non-protein thiol (NPSH) content

The measurement of non-protein thiol compounds was carried out as described by Devi *et al*.,1998. 0.5 g shoot and 1.0 g root tissue were extracted with 5 ml of 5% meta-phosphoric acid, and centrifuged at 12,000 rpm for 15 minutes at 4°C. 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 of 6 mM 5,5'- dithiobis (2-nitro benzoic acid). This mixture was incubated at room temperature for 30 minutes and the colour produced was measured at 412 nm with a spectrophotometer.

2.5 Estimation of endogenous hydrogen peroxide (H_2O_2) content

Shoot and root tissue (0.5g each) were homogenized with 3 ml of 1% (w/v) TCA in an ice bath. The homogenate was centrifuged at 10,000 rpm for 10 minutes, and the supernatant (0.5 ml) was added with 0.75 ml of 50 mM potassium phosphate buffer (pH 7.0) and 1.5 ml of 1 M potassium iodide (KI), and the absorbance was measured at 390 nm. H_2O_2 content was expressed as nmol $H_2O_2\ g^{-1}$ of fresh weight tissue (Jessup *et al.,* 1994).

2.6 Estimation of Malondialdehyde (MDA) content

MDA is a direct measure of lipid peroxidation, which is produced by the thiobarbituric acid reaction as described by Heath *et al*., 1968. The crude extract was mixed with the same volume of a 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) tricholoroacetic acid. The mixture was heated at 95˚C for 30 min and then quickly cooled in an ice-bath. After cooling, the resulting mixture was centrifuged at 10,000 rpm for 10 minutes at 4˚C and the absorbance of the supernatant was measured at 532 and 600 nm. After subtracting the non-specific absorbance (600 nm) from the specific absorbance (532 nm), the MDA concentration was determined by its molar extinction coefficient (155 mM^{-1} cm^{-1}) and the results expressed as mol MDA g^{-1} FW.

2.7 Total phenolic content (TPC)

Crude extract was prepared by crushing 0.5g shoot and 0.5g root tissue in 80% hot ethanol. The extract was centrifuged at 10,000 rpm for 15 minutes at 4˚C. The supernatant was evaporated to dryness in a water bath and finally dissolved in minimum volume of water. An aliquot of 0.5ml was mixed with 5 times diluted Folin Ciocalteau reagent followed by addition of 20% of 1 ml of sodium carbonate. The resulting mixture was incubated for 1 minute in a water bath at 100˚C and the readings were taken at 650 nm. The results were expressed as gallic acid equivalents per gram (GAE g^{-1}) of the plant material (Mohsen *et al*., 2009).

2.8 Total flavonoid content (TFC)

The total flavonoid content (TFC) of shoot and root tissue was determined using the aluminium chloride assay. 0.5g shoot and 0.5 g root tissue was crushed in ethanol and the extract was kept overnight. The extract was then mixed with 1% aluminium chloride and kept in room temperature for 30 minutes. A light yellowish coloured solution was obtained. The readings were measured at 415 nm. The TFC was expressed in mg of quercetin equivalents (CE) per gram of extract (Lamaison *et al*., 1990).

2.9 Assay of antioxidative enzymes

2.9.1 Superoxide dismutase (SOD)

0.5 g shoot and 0.5 g root tissue were homogenized in extraction buffer (pH 6.8) containing 0.1 M Tris, 0.25 M sucrose, 1% PVP, 0.1% ascorbic acid, 0.1% cysteine HCl,1mM EDTA, 0.4 mM $MgCl_2$ and 0.4 mM DTT. This homogenate was centrifuged at 12,000 rpm at 4˚C for 3 minutes and the supernatant was used for SOD assay. SOD activity was measured in a 3 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM nitroblue tetrazolium, 2 µM riboflavin, 0.1 mM EDTA and a suitable aliquot of enzyme extract. The test tubes were shaken and placed under illumination and the readings were taken at 560 nm after one hour. The activity of SOD was expressed as unit per milligram protein. One unit of activity is the amount of protein required to inhibit 50% initial reduction of nitroblue tetrazolium under light (Beauchamp *et al*., 1971).

2.9.2 Ascorbate peroxidase (APX)

0.5g shoot and 0.5 g root tissue were homogenized in 50 mM sodium phosphate buffer (pH 7) containing 2% PVP and 0.1 mM EDTA. The homogenate was centrifuged at 14,000 rpm for 30 minutes at 4˚C. Activity of APX was assayed by taking 1 ml of 50 mM sodium phosphate buffer (pH 7), 0.8 ml of 0.5 mM ascorbic acid, 0.2 ml of enzyme extract and 1 ml of H_2O_2 solution in a total volume of 3 ml. Absorbance was recorded at 290 nm at an interval of 10 seconds up to 3 minutes. APX activity was expressed in moles of monodehydroascorbate formed min^{-1} gm^{-1} FW using molar extinction coefficient, ε = 2.8 mM^{-1} cm^{-1} (Nakano *et al*., 1981).

2.9.3 Guaiacol peroxidase (GPOD)

1 g shoot and root tissue were homogenized in 3ml of 0.05M sodium phosphate buffer (pH 7.8) including 1mM EDTA and 2% (w/v) PVP. The homogenate was centrifuged at 14,000 rpm for 20 min at 4˚C. The reaction mixture in a total volume of 2ml contained 25mM (pH 7.0) sodium phosphate buffer, 0.1mM EDTA, 0.05% guaiacol, 1.0mM H_2O_2 and 100 ul enzyme extract. The increase of absorbance due to oxidation of guaiacol was measured at 470 nm using molar extinction coefficient, ε = 26.6 mM^{-1} cm^{-1}).

2.9.4 Glutathione reductase (GR)

0.2 g shoot and 0.5 g root tissue were homogenized in 50 mM Tris HCl buffer (pH 7.6) and centrifuged at 14,000 rpm for 30 minutes at 4˚C. The reaction mixture in total volume of 1 ml contained 50 mM Tris HCl buffer (pH 7.6), 0.15 mM NADPH, 1mM oxidized glutathione (GSSG), 3mM $MgCl_2$ and 200 µl of enzyme extract. The readings were taken at 340 nm using a spectrophotometer. The specific activity of enzyme was expressed as µmol NADPH oxidized $min^{-1}mg^{-1}$ protein using molar extinction coefficient, ε= 6.2 mM^{-1} cm^{-1} (Schadle *et al*., 1977).

2.10 Protein content

Protein concentration of shoot and root extracts was determined according to Lowry *et al.* 1951 using bovine serum albumin as standard protein

2.11 Statistical analysis

The experiments were performed in triplicates. Statistical significance of the difference between treatment means were evaluated by one-way ANOVA at a 0.05 probability level.

3. RESULTS

3.1 Effects on chlorophyll and carotenoid content

The total chlorophyll (chlorophyll a+b) content of the treated seedlings were not significantly affected after 72 and 120 hours. Accumulation of carotenoids had significantly increased by 86.4% in Cd treated seedlings and by 132.1% in Zn treated seedlings, only after 120 hours. At 72 hours, the carotenoid content of the treated seedlings has not changed significantly than the control seedlings (Fig 1).

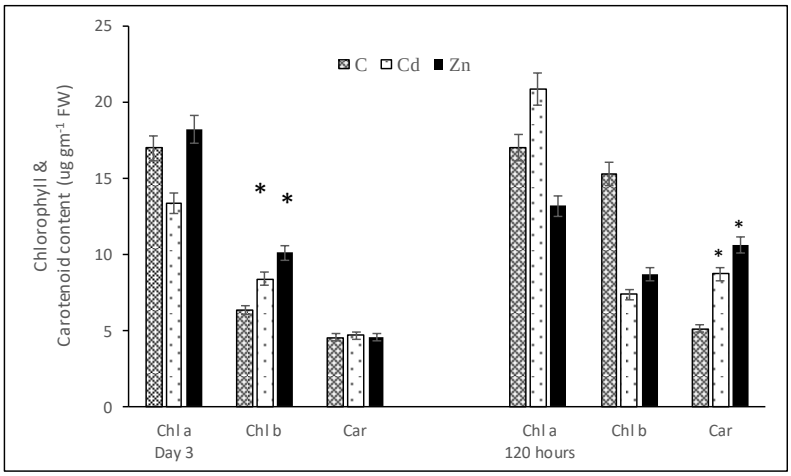
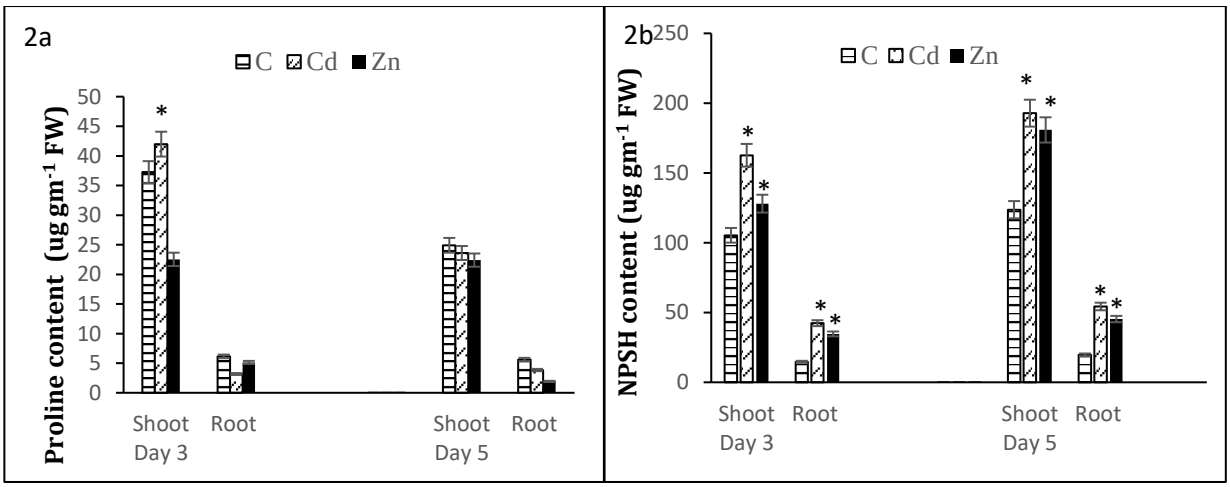


Fig 1: The effect of Cd and Zn on the total chlorophyll and carotenoid content in the shoots of castor seedlings exposed for 72 and 120 hours, individually. Values are mean of three replicates ±S.E. Asterisks denote significance at P ≤ 0.05.



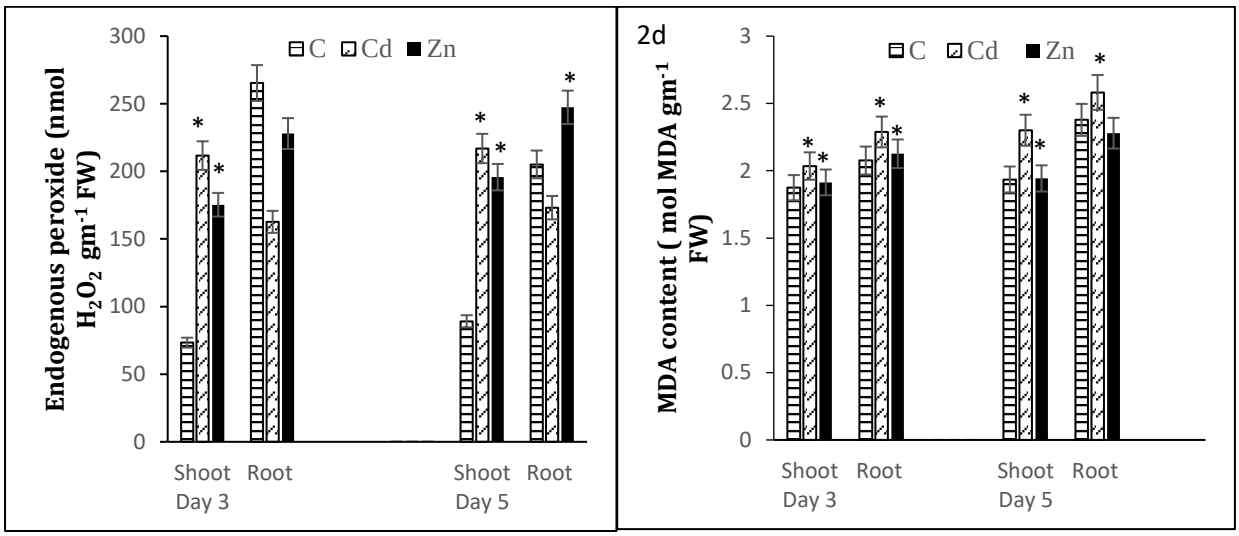


Fig 2: The effect of Cd and Zn on the (a) proline (b) NPSH (c) endogenous H_2O_2 and (d) MDA content in the shoots and roots of castor seedlings exposed to 72 and 120 hours individually. Values are mean of three replicates±S.E. Asterisks denote significance at P ≤ 0.05.

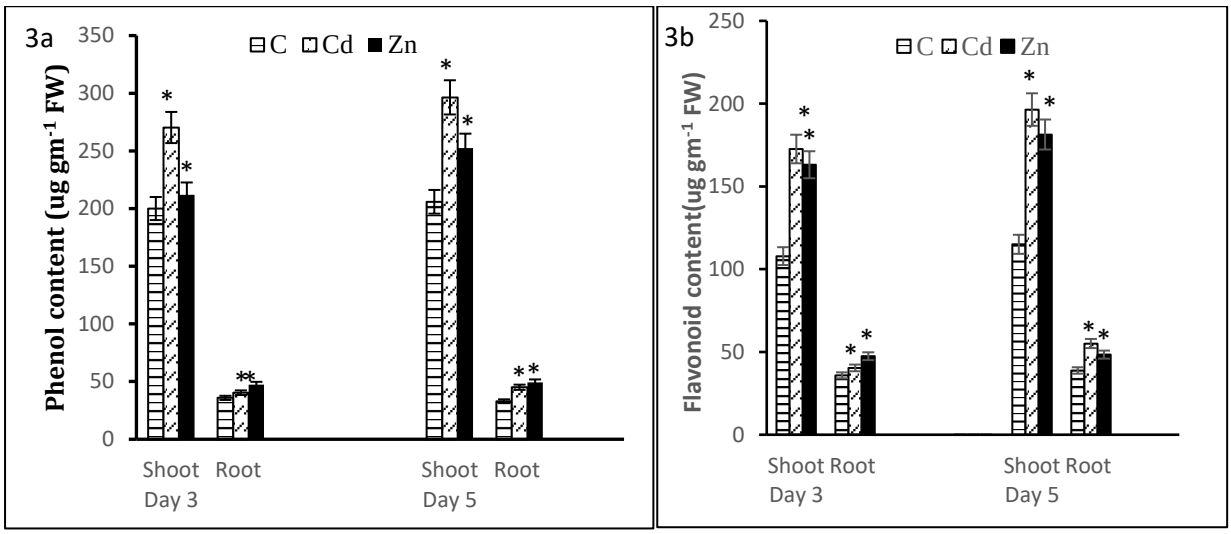
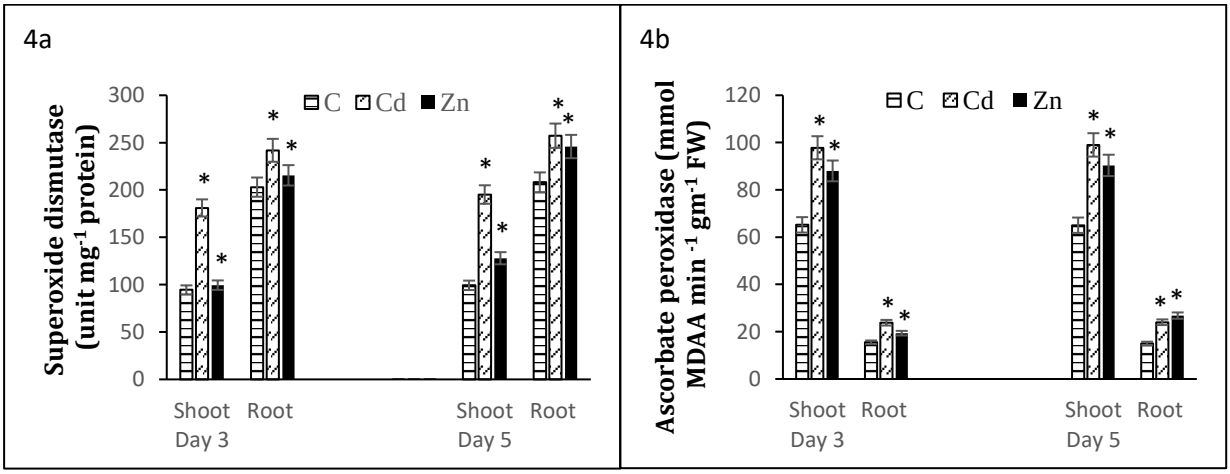


Fig 3: The effect of Cd and Zn on the (a) total phenol content and (b) flavonoid content in the shoots of castor seedlings exposed for 72 and 120 hours, individually. Values are mean of three replicates ±S.E. Asterisks denote significance at P ≤ 0.05.



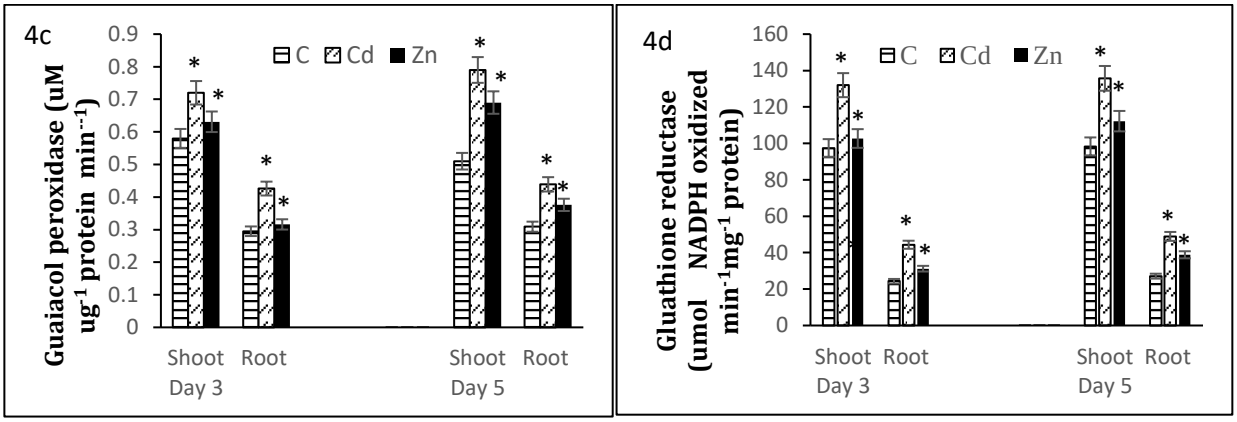


Fig 4: The effect of Cd and Zn on the (a) superoxide dismutase and (b) ascorbate peroxidase (c) guaiacol peroxidase and (d)glutathione reductase in the shoots and roots of castor seedlings exposed for 72 and 120 hours, individually. Values are mean of three replicates ± S.E. Asterisks denote significance at P ≤ 0.05.

3.2 Effects on some oxidative stress markers

In our works, we have investigated the following oxidative stress markers *(i)* proline *(ii)* non-protein thiols *(iii)* endogenous hydrogen peroxide and *(iv)* MDA content.

Proline content showed a significant increase of 12.7% after 72 hours, only in the shoots of Cd treated seedlings (by 12.7%), as compared to control (Fig 2a). After 72 hours of Cd and Zn treatment, non-protein thiol content increased significantly, in comparison to control. After 72 hours, cadmium induced a 54.4% increase (in shoots) and a 188.6% increase (in roots). Zinc treatment produced a 21.5% and 136.3% rise in the shoots and roots respectively. Similarly, NPSH levels had significantly increased after 120 hours in the treated seedlings, in comparison to control. An increase of 68.9% and 176.3% was observed in the shoots and roots of Cd treated seedlings. An increase of 45.9% and 130.5% was observed in the shoots and roots of Zn treated seedlings, respectively. It is to be noted that NPSH content was much higher in the shoots of metal stressed seedlings compared to the roots (Fig 2b).

An increase by 188.6% and 139.1% of endogenous H_2O_2 levels was estimated in the shoots of Cd and Zn treated seedlings after 72 hours, in relation to control (Fig 2c). It was more pronounced in the shoots than in the roots. A significant rise in H_2O_2 production was noticed after 120 hours, when compared to control. Cd treated seedlings showed a significant increase of 132.09%, only in the shoots. Zn treated seedlings showed a 191.51% increase in the shoots and 25.57 % increase in the roots.

MDA, a product of lipid peroxidation, was measured to study the extent of membrane damage. Seedlings exposed to Cd for 72 hours resulted in 8.59% increase in the shoots and a 10.21% increase in the roots, in comparison to control. Similarly, the Zn treated seedlings also showed a significant increase in MDA content (2.1% in shoots and 2.4% in roots). It is to be noted that the MDA content was highest in shoots on cadmium treatment. An increase in exposure period of 120 hours resulted in significant increase in lipid peroxidation in both Cd and Zn treated seedlings. The roots of Cd treated seedlings showed a 8.57% rise, in comparison to control. The roots of Cd treated showed a 18.91% rise while that of Zn treated seedlings showed a small significant rise of 0.41%, when compared to control seedlings (Fig 2d).

3.3 Effects on phenolic and flavonoid content

After 72 hours, the total phenolic content increased by 35.1% (in shoots) and 12.47% (in roots) in Cd treated seedlings and by 12.5% (in shoots) and 32.16% (in roots) in Zn treated seedlings compared to control (Fig 3a). After 120 hours, Cd treatment led to about 43.9% in shoots and 36.75% in roots while, Zn treatment resulted in a 22.5% rise in shoots and 49.92% rise in roots, compared to after 72 hours. Shoots maintained a higher phenolic content than roots in the treated seedlings at both 72 and 120 hours. For Cd treated seedlings, it was 270.29 ug gm^{-1} FW (in shoots) while for roots, it was 40.356 ug gm^{-1} FW.

As compared to control, Cd and Zn treatment had significantly increased flavonoid activity after 72 hours (Fig 3b). The total flavonoid content increased by 59.9% (shoots) and 12.4% (roots) in Cd treated seedlings and by 51.2% (shoots) and 35.16% (roots) in Zn treated seedlings compared to control. Similar to the total

phenolic content, the shoots of treated seedlings also produced a higher flavonoid content than roots (172.61 ug gm^{-1} FW in shoots and 40.356 ug gm^{-1} FW in roots for Cd treated seedlings).

Comparatively after 120 hours, the flavonoid content recorded was significantly higher in both Cd and Zn treated seedlings. Cd treatment shows a 95.03% rise (in shoots) and a 41.9% rise (in roots), as compared to control. Likewise, Zn treatment shows a marked rise of 57.6% rise (in shoots) and 24.7% rise (in roots).

3.4 Effects on some antioxidant enzymes

3.4.1 Effect of Cd and Zn on SOD activity

In comparison to control, a significant elevation of SOD activity was observed in the treated seedlings after 72 hours, when compared with control (Fig 4a). The seedlings treated with Cd showed a 91.4% increase in shoots and a 19.15% increase in roots. The Zn treated seedlings showed a 5.25% increase in shoots and a 6.14% increase in roots.

In contrast, SOD activity was more enhanced after 120 hours. Cd treated seedlings showed a 96.5% (in shoots) and 23.62% (in roots) rise while, Zn treated seedlings showed a 28.81% (in shoots) and 18.13% (in roots) increase in enzyme activity, in comparison with control.

3.4.2 Effect of Cd and Zn on APX activity

Similar to superoxide dismutase, the activity of ascorbate peroxidase was markedly elevated under Cd and Zn treatment, when compared to control seedlings (Fig 4b). After 72 hours, the shoots of Cd treated seedlings resulted in a 50% increase whereas, the roots resulted in a 53.35% increase. Under Zn treatment, after 72 hours, a 34.98% increase in enzyme activity was observed in the shoots and a 24.8% increase was observed in the roots.

A significant increase in APX activity was observed in the treated seedlings after 120 hours, in comparison to control. Cd treatment resulted in a 52.2% increase in shoots and 59.8% increase in roots. Zn caused a significant increase of 38.89% in shoots and 78.4% increase in roots.

3.4.3 Effect of Cd and Zn on GPOD activity

The same pattern of increase was again observed in GPOD activity for the treated seedlings, in relation to control, after 72 hours. An increase of 24.13% and 44.4% was observed in the shoots and roots, respectively for Cd treated seedlings. An increase of 8.79% and 7.11% was observed in the shoots and roots, respectively for Zn treated seedlings.

After 120 hours, an increase of GPOD activity was recorded in Cd and Zn treated seedlings, as compared to after 72 hours. There was an increase of 54.9% and 42.07% observed in the shoots and roots, respectively for Cd treated seedlings. An increase of 35.29% and 21.68% was observed in the shoots and roots, respectively for Zn treated seedlings (Fig 4c).

3.4.4 Effect of Cd and Zn on GR activity

A similar trend was observed in GR activity of the treated seedlings at 72 hours, as compared to control. An increase of 35.41% rise (in shoots) and a 82.1% rise (in roots) was noted in Cd treated seedlings. Exposure to zinc for 72 hours resulted in a 5.3% rise in shoots and a 28.1% rise in roots.

After 120 hours, there was a highly enhanced GR activity in the treated seedlings, when compared to control. Cd treatment caused an increase of 37.98% in shoots and 80.57% in roots. An increase in Zn exposure from 72 to 120 hours produced an increase of 14.08% in shoots and 43.29% in roots, when compared with control seedlings (Fig 4d).

4. DISCUSSION

Abiotic stress, such as exposure to heavy metals, can cause damage to seedlings directly or indirectly through the burst of reactive oxygen species (ROS) (Dazy *et al.,* 2009). As a non-essential and potently toxic metal, cadmium is known to induce oxidative stress (Hatata *et al.,* 2008). High concentrations of Zn was toxic and inhibited plant growth in seedlings grown in soil near smelting areas rich in Zn (Bia *et al.,* 2006). The role of enzymatic and non-enzymatic defense mechanisms in young castor seedlings grown hydroponically were studied after a short term exposure to Cd and Zn for 72 and 120 hours.

4.1 Chlorophyll and carotenoid content

The total chlorophyll content did not increase significantly in the treated seedlings after 72 and 120 hours. An increase in carotenoid content have occurred to possibly overcome the metal induced oxidative stress. Carotenoids scavenges free radicals, mainly singlet oxygen and can also quench the excited state of chlorophyll (Taiz *et al.,* 2002). Excess zinc supply (10 mM Zn) increased carotenoid production in *Vigna mungo* after 10 days of treatment (Gupta *et al.,* 2009).

4.2 Oxidative stress markers

Proline has been reported to be associated with environmental stress such as drought and salinity (Costa

et al., 1994). Cadmium is known to affect membrane integrity that can cause water stress, resulting in the accumulation of free proline (Cordeiro *et al.,* 2009). Heavy metals like cadmium can induce accumulation of proline, as observed by us in the present study (Xu *et al.,* 2009, Sharma *et al.,* 2010, Anjum *et al.,* 2011, Muneer *et al.,* 2011, Zhao, 2011). With increasing cadmium concentration, proline accumulation had increased in wheat seedlings after 24 hours of Cd exposure (Lesko *et al.,* 2002). In the present study, castor seedlings have showed significantly higher proline production only after 120 hours of Cd exposure. However, NPSH content was significantly higher in the treated seedlings, both after 72 and 120 hours. Such similar reports of high non-protein thiol accumulation with the increase in Cd concentration and duration of Cd supply in *Pisum sativum* seedlings have been observed earlier (Pandey *et al.,* 2012). Glutathione is one of the most important non-protein thiol groups found in plants (Grill *et al.,* 1979). Glutathione is involved in the detoxification of harmful reactive oxygen species (ROS). It is also essential for the synthesis of Cd-binding peptides such as phytochelatins, which sequesters toxic Cd ions by forming stable Cd-complexes in the vacuole (Cobbett, 2000, Hall, 2002).

Plant cell membranes are the primary target for metal induced injuries. Lipid peroxidation *in vivo* results in a massive production of free radicals (Gill *et al*., 2010). The effect of Cd and Zn on cell membrane integrity was determined through measurement of MDA. In our results, increased levels of H_2O_2 in Cd and Zn treated seedlings both after 72 and 120 hours could account for the formation of hydroxyl ($OH^{\cdot}$) radicals. This may have led to increased lipid peroxidation and aggregation of thiobarbituric acid reactive substances (TBARS) in castor seedlings exposed to Cd and Zn for 72 and 120 hours. Increased MDA formation in seedlings exposed to Cd has been verified in sunflowers (Gallego *et al*., 1996), in mustard (Bauddh *et al.,* 2011), in *Thlaspi caerulescens*, *Oryza sativa* L. (Fei-bo *et al.,* 2006) and also *Ceratophyllum demersum* L. after a 7-day treatment (Mishra *et al.,* 2007).

4.3 Phenolic and flavonoid content

It is known that stress conditions elevated phenolic activity in plants (Reid *et al.,* 1992, Siddiqui *et al*., 1996). This is evident in our present study where the castor seedlings have produced a high phenolic and flavonoid content in response to Cd and Zn stress. Phenolics are secondary plant metabolites. They are capable of scavenging ROS. Flavonoids are the most widely distributed group of plant phenolic compounds. They are found in all plant parts, particularly in the shoots and fruits. Their best property is that they act as antioxidants. They are able to suppress ROS formation and scavenge it. They are also known to protect lipids against oxidative damage (Kumar *et al*., 2012). Cd treated seedlings have showed higher phenolic and flavonoid accumulation in the shoots compared to the roots. This may have occurred due to the high mobility of Cd^{+2} ions. Cd ions are reported to travel upward through the aerial shoot and into the leaves. In *Brassica juncea* L. seedlings exposed to 0.6 mM Cd, the total phenolic content increased in a dose dependent manner (Kapoor et *al.,* 2014). Zinc stress induced increased phenolic content in *Verbascum thapsus L.* seedlings after 96 hours of Zn exposure (Morina *et al.,* 2008). The leaf extracts of *Erica andelvalensis* showed increased flavonoid production with increasing Cd concentrations after 120 hours of Cd stress (Marquez-Garcia *et al.,* 2012).

4.4 Antioxidant enzymes

In order to scavenge ROS, plants have a proper antioxidative defense system comprising of several enzymatic and non-enzymatic antioxidants. The co-operative action of these antioxidants played a crucial role to maintain the redox status of plant cells. The non-enzymatic antioxidants mainly comprise of carotenoids, non protein thiols, phenolics and flavonoids. The enzymatic antioxidants comprise of superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase and glutathione reductase. Stress imposed by ROS causes an upregulation of certain antioxidative enzymes. In the first line of defense, SOD plays the main role for detoxification (Gratão *et al.,* 2005). In our study, metal treated seedlings showed a significant increase in SOD activity in roots compared to control, both after 72 and 120 hours. SOD converts superoxide to hydrogen peroxide and molecular oxygen. Increased SOD activity was observed in two species of *Alternanthera* sp., namely *A. sessilis* and *A. tenella* by different concentrations of cadmium (20,30 and 50 mg kg^{-1} Cd) (Chinmayee *et al*., 2013). It was also reported that addition of Zn from 0.5 to 5 uM showed increased SOD activity in hydroponically grown rice seedlings (Asadi *et al*., 2012). It has been reported that an excess of zinc

may act as a producer of reactive oxygen species in plant tissues, resulting in oxidative injury (Rao *et al.,* 2000).

Enhanced SOD activity results in increased H_2O_2 production. This H_2O_2 may be detoxified by other enzymes such as APX and GR. In the glutathione-ascorbate pathway, APX is responsible for reducing H_2O_2 into water using ascorbic acid as an electron donor. In our results we have noticed elevated levels of APX in the treated seedlings after 72 hours and 120 hours compared to control seedlings. Phytotoxic concentrations of cadmium induced an increase in the level of antioxidative enzymes in *Ceratophyllum demersum* L. (Mishra *et al.,* 2008).

The main function of peroxidases (both APX and GPOD) is to scavenge H_2O_2. This is e vident by the present experiment which shows enhanced GPOD activities when subjected to Cd and Zn stress. Zn had induced increased GPOD in *Phaseolus vulgaris* (Kasim., 2007) while Cd induced GPOD activity was reported in *Lemna minor* (Paczkowska *et al*., 2007). Thus, we can establish that peroxidase activity had increased in castor seedlings to diminish the deleterious effects of ROS.

In addition, our findings indicate significantly higher GR activity in the treated seedlings. Results obtained in the present study are in agreement with reports found in *Helianthus annus* under Cd stress (Hatata *et al.,* 2008). GR is very essential for maintaining the redox status of the cell. It catalyzes the reduction of oxidized glutathione (GSSG) in a NADPH-dependent reaction to regenerate GSH. Therefore, GR plays an important role in the protection of chloroplasts by maintaining a GSH/GSSG ratio.

5. CONCLUSION

The results obtained from our work after 72 and 120 hours of zinc and cadmium treatment suggests that these heavy metals had induced an upregulation of certain antioxidative enzymes like SOD, APX, GPOD and GR. The castor seedlings have an efficient system for scavenging free radicals. From our results, it is quite clear that *Ricinus communis* L. is able to combat these damaging radicals by providing a defensive antioxidant system. It is still not known whether zinc has an alleviating effect on cadmium toxicity. Therefore, much more information is needed at a subcellular level to gather more knowledge about cadmium and zinc toxicity.

Summarising our results, it can be concluded that phytotoxic concentrations of cadmium and zinc have produced certain biochemical changes in young castor seedlings. We had witnessed the role of enzymatic and non-enzymatic components to combat the stress imposed by heavy metals. A deeper knowledge in how seedlings can thrive and breed in such heavy metal contaminated areas is certainly required. Further research is needed to find out the relationship between heavy metal stress and the biochemical changes induced in castor seedlings and whether zinc has any alleviating role in reducing cadmium stress.

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***Corresponding Author:**
R. Kundu*
Email: kundu_rita@yahoo.co.in