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Cadmium induced changes on Growth, Lipid Peroxidation and Antioxidative Enzymes in the bioenergy crop, *Ricinus communis L.*

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Abstract: *Ricinus communis L. plants were grown in soil having different concentrations of Cd (5, 20, 60 and120 mgkg-1) in a pot culture experiment. Plants were harvested after 60 days. The effects of Cd on biomass, leaf area, photosynthetic pigments, protein, antioxidative enzymes and proline were analysed. High Cd level in soil cause significant reduction in chl a, chl b, total chl and carotenoid. MDA content and activities of antioxidative enzymes viz., SOD, CAT and POD increased at high Cd concentration. SEM-EDX study showed Cd accumulation and deposition in the roots and leaf tissues. Highest accumulation of Cd was observed in the roots. Overall Cd had negative effects on the morpho-physiological characteristics of R. communis.*

Keywords: *Chlorophyll, Biomass, Oxidative stress, Malondialdehyde, Protein, ROS, SEM-EDX*

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I. Introduction

Heavy metal contamination in the environment is a global problem and can have a severe toxic impact on the living systems. Heavy metals contamination in soil leads to loss in agricultural yield and enhances toxic health effects as they tend to enter into the food chain [1].

Cadmium (Cd) is most toxic metal among the other non-essential heavy metals. Cd is not biodegradable ad there is a high possibility of this metal to enter into the food chain affecting the higher trophic organisms. It is readily taken up by plant root and translocate to different plant parts[2].High levels of Cd in soil elicits various stress symptoms in plants, from growth inhibition to metabolic disturbances like photosynthesis[3],transpiration[4] ,carbohydrate metabolism[5]and other metabolic activities [6,].

Plants have well developed antioxidative system, including antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT) and peroxidise (POD) in scavenging ROS [7].Antioxidants in plants play unique roles in preventing cellular damage to stress conditions. Cadmium toxicity in plants induces oxidative stress in cells leading to enhanced production of ROS including superoxide radicals, hydroxyl radicals and hydrogen peroxide (H2O2) [8].These reactive oxygen species is harmful for plant cell and leads to inhibition of photosynthetic activity, inhibition of ATP production, lipid peroxidation and DNA damage [9].

Ricinus communis L. (Family: Euphorbiaceae) is an economically important crop with wide application in different fields. This plant has a strong ability to extract heavy metals like Cd and insecticide like DDT from contaminated soil [10].The plant is also tolerant to other stresses viz., salinity, drought and persistant organic pollutants [11].According to Olivare et al. [12], *R.communis* can phytostabilize heavy metal contaminated mine tailings.

This research has been executed to determine the effect of Cd on growth, physiology and bioaccumulation of Cd by *R. communis.* The study will highlight the potential of *R. communis* to deal with stress conditions at the cellular level and its capability to cope with oxidative stress caused by Cd ions at different soil concentrations. Very few studies have been carried out on the antioxidant enzymes in *R. communis.* The results thus obtained in the present study could be useful for better understanding the phytotoxic effect of Cd on *R. communis* antioxidative defence system and detoxification mechanism adopted by the plant.

II. Materials and Methods

The seeds of castor were obtained from Experimental farm of CSIR-North East Institute of Science and Technology (NEIST), Jorhat, Assam, India. Healthy seeds with uniform size, colour and weight were chosen for the experimental purpose. The soil used in the experiment was sandy loam in nature and pH of the soils was 7.2. The cadmium nitrate Cd $(NO_3)_2$ was used as cadmium source. Nitrate salts were preferred over other forms of salt because of their high solubility in the medium. Representative garden soils were collected by digging to a depth of about 0 - 30 cm depth from the surface of a soil profile. The soils were sun dried and the foreign particles were removed and mixed with sand (1:1). Seeds were sown in 30-cm-diameter earthen pots filled with 6kg of sand soil mixture. Soil was mixed with appropriate amount of Cd $(NO_3)_2$ to have Cd=0, Cd=5, Cd=20 Cd=60 and Cd=120 mg Cd kg1 soil. The calculated amount of heavy metal salt first mix thoroughly with 6 kg of sand soil mixture over a polythene sheet and then transferred to the earthen pot.

The healthy seeds of Castor *(R. communis*) were surface sterilized with 0.1 % mercuric chloride for 5 min and subsequently washed with sufficient distilled water to avoid fungal contamination. 10 seeds were sown into each pot. After two week of seed sowing, seedlings were thinned to a maximum of five per pot. Each treatment including the control was replicated 3 times. The pots with seeds of *R. communis* were kept directly in naturally illuminated greenhouse of Medicinal and Aromatics Plant Division, CSIR-NEIST, Jorhat (November 2015–February 2016) with the temperature ranged between 21^0C - 27^0C and with relative humidity 70-86 percent. Plants were watered to maintain soil moisture at 60-70% water holding capacity. The drainage collected at the bottom of the pot was also added in the pots to avoid loss of metals from leachate. All the measurements were performed at 60 days after sowing (DAS).

2.1Plant sample preparation and chemical analysis

Plants were harvested after 60 days of sowing. Following the harvest, the plants were washed with distilled water and immerged in 20 mM Na EDTA for 15 to 20 minutes to remove Cd adhered to root surface. Plants were washed thrice with distilled water and finally with deionised water. Roots stem and leaves of the plants were separated for further analysis.

2.2 Leaf area and Tolerance Index (TI)

Leaf area (LA) was determined by multiplying leaf length with leaf width and correction coefficient (r) which is 0.72 as proposed by Hoyt and Bradfield [13]. Leaf Area was calculated as LA = L x W x r. The tolerance index (TI) is the ratio of plant biomass after Cd treatments to that of the control plant biomass expressed in percentage.

2.3 Photosynthetic pigment and MDA content

The effects of Cd stress on photosynthetic pigments of *R.communis* were determined according to the method described by Arnon [14].200mg of fresh leaves were blended and extracted with 10ml of 80% acetone and left for 15min. The liquid protein was decanted into another test tube and centrifuged at 2,500rpm for 3min. The supernatant was then collected and the absorbance was taken at 645nm and 663nm for chlorophyll a, b and 480nm, 510nm for carotenoid using a UV-visible spectrophotometer (Thermofisher, Evolution 201).

2.4 Determination of lipid peroxidation rate

Oxidative damage to leaf lipids due to Cd stress was estimated by the content of total 2-thiobarbituric acid reactive substances (TBARS) expressed as equivalents of malondialdehyde (MDA). TBARS content was estimated by the method of Cakmak and Horst [8] with some modifications. Fresh leaf samples (0.2g) were ground in 5ml of 0.1% (w/v) trichloroacetic acid (TCA) at 4°C. Following the centrifugation at 12,000 × g for 5 min, an aliquot of 1ml from the supernatant was added to 4 ml of 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) TCA. Samples were heated at 90°C for 30 min. Thereafter, the reaction was stopped in ice bath. Centrifugation was performed at 10,000×g for 5 min, and absorbance of the supernatant was recorded at 532 nm on a UV-visible spectrophotometer and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The following formula was applied to calculate malondialdehyde content using its absorption coefficient (ε) and expressed as nmol malondialdehyde g^{-1} fresh mass –

MDA (nmol g-1 FM) = [(A532-A600) ×V×1000/ε] ×W

where ε is the specific extinction coefficient (=155 mm cm^{-1}), V is the volume of crushing medium, W is the fresh weight of leaf, A600 is the absorbance at 600 nm wavelength and A532 is the absorbance at 532 nm wavelength.

2.5 Scanning electron microscopy and energy dispersive X-ray spectroscopy

In order to determine the cellular localization of Cd in Cd treated plant, the leaves and roots were sectioned to 2-3 mm and immediately fixed in formalin–acetic acid–alcohol (FAA) for 24 hrs. The samples

were then dehydrated in a series of alcohol in ascending order. After dehydration, the samples were mounted on aluminium stubs with double-sided carbon tape vacuumed ($1x10^{-3}$mBar) and plated with gold. The SEM observations were performed using a Carl Zeiss Microscopy (SIGMA) Scanning electron microscope in a low vacuum mode using backscattered electron imaging.

2.6 Determination of antioxidative enzyme activities and soluble protein

2.6.1Antioxidative enzyme

Leaf tissues (0.2g) were homogenized separately in a pre-chilled mortar and pestle under ice-cold conditions with 2.0 ml of extraction buffer [50 mM phosphate buffer (pH 7.5), 0.5 mMascorbate and 1 mM EDTA]. The homogenate was centrifuged at 10,000 rpm for 15 minutes.

The supernatant was used for the measurement of SOD, CAT and POD antioxidative enzyme activities. The protein content was measured according to the method of Bradford [15], using bovine serum albumin (BSA) as standard. The SOD activity was quantified by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium [16].The reaction mixture (3 ml) was prepared with 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 2.25 mM NBT, 60 µM riboflavin and enzyme extract. After mixing, the mixture in the cuvette were illuminated (40 watts light) for 15 minutes. Enzyme extract kept in the dark served as blank, while buffer with no enzyme extract kept in the light served as control. The absorbance was measured at 560 nm against a blank using a UV–visible spectrophotometer. NBT reduction in the light was measured in the presence and absence of enzyme extract. SOD activity was calculated as absorbance of control minus absorbance of sample, giving the total inhibition. One unit of activity was the amount of enzyme required for 50% reduction in colour and was expressed in units of the enzyme (mg/protein/h).

Catalase activity (CAT) was determined by measuring the decomposition of hydrogen peroxide. About 100 µl of enzyme extract was added into the reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 20 mM H_2O_2. The decrease of the absorbance at 240 nm was recorded. Activity was calculated using an extinction coefficient of 39.04 mM^{-1} cm^{-1}. One unit of CAT activity was defined as the amount required for decomposing 1 µmol of hydrogen peroxide/min.

The method proposed by Reddy et al. [17], was adopted for assaying the activity of peroxidase (POD). Leaf tissues (0.2g) were homogenized in 0.1M phosphate buffer (pH 6.5) centrifuged at 12,000 rpm and the supernatant was used for the assay. The reaction mixture contains 3.0ml of pyrogallol solution, 0.1ml of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 430 nm. The reaction mixture was further mixed with 0.5ml of H_2O_2 in the test cuvette. The change in absorbance was recorded every 30 seconds up to 2 minutes in a UV-spectrophotometer. One unit of peroxidase is defined as the change in absorbance/minute at 430 nm.

2.7 Proline determination

Accumulation of proline was determined according to Bates et al. [18].For this 0.5g of fresh plant leaves were homogenized in 10ml of aqueous sulfosalicyclic acid and centrifuged at 5000 rpm. 2ml of the supernatant, 2.0ml each of glacial acetic acid and ninhydrin was mixed and heated in a boiling water bath for 1 h at 1000C. The ninhydrin reaction mixture was partitioned against toluene and the absorbance of the toluene phase was read at 520 nm. Proline concentrations were determined after the realization of a standard curve and are expressed in µmol g^{-1} fresh weight of plat leaf.

2.8 Cd determination in plant parts

The plant parts used for estimation of Cd concentration were dried in a hot air oven at 65°C for 48 hours to remove all the moisture. The roots, stems, old leaves, and young leaves were separately ground into fine powder and then digested in digestion flask at 60°C -70°C and after the disappearance of brown fumes heated to 90°C -100°C to near dryness. The sample volume was adjusted to a 25 ml volume with deionised water. The Cd concentration in each sample was measured by an Atomic Absorption Spectrophotometer (Perkin-Elmer A, Analyst, 700).

2.9 Statistical analysis

Statistical analysis of the data generated was performed by using the IBM SPSS Statistics 23. Data were presented as mean value ±SE and were compared using the LSD test at the 5% probability level. A p-value less than 0.05 were considered to indicate a significant difference.

III. Results

3.1Effects of Cd on plant leaf area, biomass and tolerance index (TI)

After 60 days of sowing, the potted plants grown in the soils containing different levels of Cd concentrations were harvested. The leaf area and the biomass content of each plant were determined and then the tolerance index was calculated. The results are summarised in Table 1. Seedling growth was significantly reduced by Cd treatments. A significant reduction in leaf area was observed in the treated plants compared to control. Exposure of castor plant to high concentration of Cd level in the soil leads to reduce biomass production compared with the control. With increasing Cd concentrations, the tolerance index (TI) of castor plant decreased from 82.5 to 20.2.

3.2 Effect of Cd stress on photosynthetic parameters

The impact of different concentration of Cd treatment (5 to 120 $mgkg^{-1}$) on the photosynthetic pigment of castor is shown in the figure 1. There was significant reduction in the chlorophyll content on application of Cd. The reduction of chlorophyll a, b, total chl and carotenoid contents was significant at different concentrations of Cd i.e. 5, 20, 60 and 120 $mgkg^{-1}$ compared to the control. The impact of Cd deposition is more visible in Chl b reduction.

3.3 Effect of Cd on protein and MDA content

The effect of Cd on total soluble protein of castor plant is shown in Figure (2a). Protein content decreases significantly ($P<0.05$) in the leaves of *R.communis* as the concentration of Cd in the soil increased.

Lipid peroxidation was estimated in terms of malondialdehyde (MDA) generated and electrolyte leakage was considered as indicative of oxidative damage. Results showed that lipid per oxidation were significantly influenced by cadmium stress ($P\leq0.05$) (Fig 2b). Leaf MDA was higher under stress conditions, compared with control. Cd levels at 5, 20, 60 and 120 $mgkg^{-1}$ caused 162%, 166%, 190% and 235% increase, respectively in leaf MDA content in the castor leaves compared to control.

3.4 Effect of Cd on proline

Proline plays an important role in stress tolerance mechanism in plants. In the current experiment, proline concentrations (Fig 3a) increase in leaves of *R. communis* exposed to increasing Cd concentrations. There is a significant increase in proline in the treated plants compared to control ($p<0.05$).

3.5 Effect of Cd exposure on antioxidative enzyme activity

Significant changes ($p<0.05$) in the antioxidative enzymes activity (SOD, POD and CAT) were observed in castor leaves treated with Cd treatment and it was found that with increasing concentration of Cd treatment, the activity of the enzymes also increased (Fig 3b-3d). The SOD, POD and CAT activity showed 325.34%, 204.03% and 163.6% increases at 120 $mgkg^{-1}$ Cd treatment of leaf compared to the control.

3.6 Scanning electron microscopic observations

Scanning Electron Microscopy equipped with Energy Dispersive X-ray Spectrometer (EDX) analysis was performed to determine the location and transport of Cd ions in castor root and leaf tissues grown at 120 mg/kg Cd concentration (Fig 4 a-d). It was observed that most of the Cd metal ion had accumulated in the roots. Elemental analysis on the surface was also performed by an energy dispersive X-ray analysis (EDX) system attached to SEM.

3.7 Cd concentration and accumulation

In the present study Cd accumulation increases in *R.communis* with increase in soil Cd concentration (Fig: 5) .Roots accumulated highest Cd (271 mg/kg^{-1}) than compared to its aerial parts. Accumulation of Cd in different parts of R.communis was in the order roots>stem>leaves. Translocation factor was found to be less than 1. An increase of 634.3%, 498.3 and 303.9 of Cd in roots, stems and leaves at the higher Cd stress ($120mgkg^{-1}$) was observed in the present experiment.

IV. Discussion

Cadmium is a potent environmental pollutant which inhibits biosynthesis of chlorophyll and carotenoid in plants. The study shows significant reduction in the leaves of castor under Cd stress condition. Chlorophyll b, which constitutes a smaller fraction also exhibited similar trend. Cadmium was reported to affect chlorophyll biosynthesis and inhibit protochlorophyll reductase and aminolevulinic acid (ALA) synthesis [19]. Cadmium induced changes on chlorophyll and carotenoid contents may be due to the inhibitory effect of Cd on enzymes involved in pigment biosynthesis and results in leaf senescence [20, 21]. Our results of decrease in chlorophyll content are in support with the findings of Siedlecka and Krupa [22] who also found a decrease in chlorophyll content with heavy metal stress in *Zea mays* and *Acer rubrum.*

Malondialdehyde (MDA) is an organic compound occurring naturally and is a oxidative stress marker. The present study shows significant increase in leaf MDA production when treated with Cd. This indicated the oxidative damage of membrane due to production of free radicals .The present findings show that an electrolyte leakage significantly increased in leaf. These findings are in agreement with similar findings by several research workers [23,24].A significant increase in the MDA with respect to control in *Hydrillaverticillata*at the highest copper treatment was reported by Srivastavaet al.[23].Heavy metals adversely affect protein synthesis in plants. The findings of our study are in support with the results of Verma et al. [25], who showed that soluble protein content was decreased in seedlings with increasing concentration of Cadmium Chloride over the control seedlings. Palma et al. [26] found a reduced protein content in *Lemnapolyrrhiza* which was caused by enhanced protein degradation process as a result of increased protease activity that was found to increase under stress conditions. Proline is an amino acid and appears to be the most widely distributed metabolite accumulated under stress conditions [27].Proline plays an important function as an osmoticum, a nitrogen storage compound, a hydroxyl radical scavenger, and a compatible solute that protects enzymes. Free proline has been reported in a wide range of plants in response to heavy metal toxicity [28].

Plants develop different types of tolerance to heavy metal exposure, and the enzymes in these plants exhibit varying response to heavy metal stress to avoid cellular damage. The increased activity of antioxidative enzymes in metal stressed plants appears to serve as an important component of antioxidative defence mechanism of plats to combat metal induced oxidative injury [29].

Plants exposed to heavy metals leads to oxidative injury inducing enzymatic and non-enzymatic reaction responses and lipid peroxidation. Yurekli and Porgali [30] reported increased activity of SOD, POD and CAT in leaf tissue of bean. Superoxide is one of the main reactive oxygen species in the cell. SOD serves a key antioxidant role to plant defence against cellular damage. It is a metalloprotein and catalyses the dismutation of O^{2-} to H_2O_2 and molecular oxygen and maintains cell membrane stability. Plants contain all three types of SODs viz. MnSODs, FeSODs and Cu/Zn SODs and located in different cell compartment. Increase in SOD activity may result from enhanced production of O2 or from de novo synthesis of enzyme proteins [8, 31]. The findings of the present work is in agreement with several earlier works which reported elevated level SOD activity under stress condition [32,33].

Catalase (CAT) is a water soluble enzyme found in all aerobic cells (animals, plants, fungi and microbes) and located in peroxisomes, cytosol and mitochondria [34].It catalyzes the decomposition of hydrogen peroxide to water and oxygen [35]. It is a very important enzyme in protecting the cell from oxidative by reactive oxygen species (ROS). POD decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants [36].Increased POD activity was observed by various workers in different stress condition [37, 38]. In the present study, an increase activity of CAT and POD was observed at high Cd concentrations. An increased activity of CAT under cadmium stress has been reported in *Phaseolusaureus, Pisumsativum, Lemna minor*, barley and sunflower [39]. The activity of peroxidase and CAT increased progressively with the increase in $CdCl_2$ concentration in *Vigna radiate* [40].

Scanning Electron Microscope provides details surface information by tracing a sample in a raster pattern with an electron beam. SEM is an essential research tool in fields such as life science, biology, geology, medical, forensic science and metallurgy. Analysis by scanning electron microscopy coupled with the elemental X-ray analysis (SEM-EDX) is an important technique in determining various elements in geological and environmental samples. The results of the present study showed the presence of Cd ($120 mgkg^{-1}$) in the root tissue and its translocation to leaves of R.communis. SEM techniques were used by Raize et al. [41], to evaluate the surface of *Sargassum vulgaris* before and after Cd, Ni and Pb binding. Baruah et al. [42], also studied SEM EDX analysis in *Eichhorniacrassipes* which showed upward Pb transport by root vascular tissues to leaf.

Cd is a non-essential element for plant growth and it enters to the plant system with other essential nutrients through the medium. Most of the Cd was accumulated in the roots of *R.communis.* Zhu et al. [43] also observed higher accumulation of Cd into the roots of *Brassica juncea* as compared to above ground parts by over expressing gamma-glutamylecyteinesynthetase. The BAF in plants shows the contaminant concentration comparing with the environment concentration in soil [44].Cd uptakes by R.communis increased with increasing the Cd doses in soil. Cd uptake and accumulation in four maize cultivars was also found to vary at different Cd concentrations and increased significantly [45].Translocation factor (TF) is an important indicator of pollutant transfer to the shoots from the roots [46]. All translocation factor values were found less than one. Increased concentration of Cd resulted in increased translocation of Cd form root to shoot and form shoot to leaves. Highest translocation was from root to shoot as compared to that of shoot to leaves. The result of this study was found according to the finding of Turan [47] andFazal [48].

V. Conclusion

Cd has negative effects on the morphological and physiological changes in *R. communis*. Cd induces oxidative stress via increasing antioxidative enzymes activities and lipid peroxidation. This suggests that heavy metal stress could overcome metal toxicity from ROS detoxification. Cd accumulation reduces photosynthetic activity of plant. Moreover proline content increased in the treated plants of *R. communis*. Plant height, root length and biomass were reduced by Cd. The plant was able to accumulate and translocate Cd in the tissues which increase with the increase of external Cd levels in the soil.

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