Cadmium induced antioxidant activity and alteration in DNA pattern in biodiesel plant: *Jatropha curcasL.*

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Abstract— In the present study, effect of cadmium on glutathione reductase, catalase, peroxidase activity, MDA content and genotoxicity effect using Random amplified polymorphic DNA (RAPD) techniques in *Jatropha curcas* were investigated. Seedling were grown in vitro in MS medium solidified with agar containing various concentration of Cadmium as $CdCl_2$(0, 50, 100, 150, 200 and 250 µMol L^{-1}) for two week. Atomic absorption spectrometry data suggest the uptake and accumulation of Cd by root and shoots. Glutathione reductase activity was inhibited while CAT and POX activity were enhanced at higher concentration of Cd (50-250 µMol L^{-1}). MDA content in all concentration was increased. RAPD technique was utilized to evaluate the genotoxicity effect of Cd on Jatropha. DNA polymorphisms were observed at 100 and 150 µMol L^{-1} concentration of Cd where 6 and 7 new band were appeared respectively while 9 band were absent at 200 µMol L^{-1} of Cd compared to control. The results, suggest the importance of enzymatic and MDA content in response to cadmium toxicity and RAPD analysis revealed that the toxic effect of Cd at molecular level.

Keywords—Cadmium, *Jatropha curcas*, Antioxidant, MDA, DNA polymorphism, Ecotoxicity, Biodiesel

1 INTRODUCTION

With the advent of industrialization and increment of mining activities, heavy metal pollution became an extensive problem. Heavy metal pollutant derived from various anthropogenic sources adversely affects the crop productivity and human health. Among all the anthropogenic sources power station, heating system, metal industries, disposal of batteries play a significant role to discharge heavy metal in to the environment [1-2]. Among all the other heavy metals cadmium (Cd) is considered as most carcinogenic and mutagenic at higher concentration in many species. Cd is a divalent heavy metal cation also causes the phytotoxicity in plants [3]. Cdaccumulation adversely affects the plant growth and causes morphological, physiological, biochemical changes in plants [4-5]. Low concentrations of Cd affect negatively on mineral uptake and homeostasis of plant development as well as shoot and root growth [6]. Heavy metal uptake causes the molecular damage to plants directly or indirectly through the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), super oxide ions (O_2^{-}), singlet oxygen ($_1O^2$) etc[7-8]. These reactive free radical species impart oxidative stress which changes the plant metabolism and lower the crop productivity. To scavenge ROS and to avoid the oxidative stress plants possess the antioxidative enzymes such as catalase (CAT), glutathione reductase (GR), and peroxidase (POX). The antioxidative enzyme GR reduces the glutathione disulfide (GSSH) to the sulfhydril form GSH. CAT is synthesized in tissue specific and age dependent manner and scavenges H_2O_2, generated throughout the photorespiration and beta oxidation of fatty acids [9]. Peroxidase is usually located in cytosol, vacuole cell wall as well as in extracellular matrix. It utilizes guaiacol as electron donor, H_2O_2 in the oxidation of various organic and inorganic substrates. The hydroxyl radical ($OH^{\cdot}$) is the most reactive species that can initiate the lipid peroxidation and damage the nucleic acids and proteins.Lipid peroxidation can be explained as the oxidative deterioration of lipid containing any number of C=C double bond. A variety of antioxidants with itsmultidirectional function inhibits lipid peroxidationand its deleterious effect caused by the product of lipid peroxidation. Toxic heavy metal also induces the damage of cellular components such as proteins and DNA [10-11]. Several studies have been carried out to evaluate the genotoxicity effect of heavy metal viz. chromosome aberration, comet assay or micronucleus[12-13]. The development of molecular marker technology such as RAPD has provided new tools for the detection of genetic alteration in response to heavy metal tolerance directly at the level of DNA structure and sequence [5, 14-15]. RAPD is PCR based technique which is efficient for DNA analysis. To evaluate genetic variation RAPD technique is capable of detection of point mutation as well as temporary alteration of DNA [16-17].

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Jatrophacurcas is a potential candidate for biodiesel production and it belongs to the family of *Euphorbieaceae*. Its seeds contain high amount of storage oil (up to 45%) including both saturated and unsaturated fatty acid. Because of its high oil yield and much similarity with petro diesel it could be the best replacement of conventional diesel [18]. *Jatropha* thrives well on degraded soil making it an attractive crop for production of biodiesel as it can be planted on land unsuitable for food crop such as abounded mining/ industrial sites [19. Therefore, industrial and mining sites can be

utilized for the large scale cultivation of *Jatropha*. Present study was undertaken to evaluate the oxidative stress and DNA polymorphism in *Jatropha* seedlings under the elevated concentration of Cd**.**

2 MATERIALS AND METHODS

2.1Plant materials and growth condition

J. curcas **s**eeds were decoated and soaked in distilled water, overnight at room temperature. Decoated seeds were surface sterilized with 0.1% sodium hypochlorite of supplemented with 4-5 drop of Tween-20 for 10-15 min, followed by washing with distilled water for 15-20 min. Subsequent sterilization of seed were carried out with 0.2% mercuric chloride for 2 min and rinsed with sterile distilled water for 4-5 times. Seed were then blot dried with autoclaved filter paper. Endosperm was carefully dissected out to expose embryos with papery cotyledonary leaves and germinated on MS medium [20].

2.2Cadmium solution and media preparation

Cd stock solution was prepared from the $CdCl_2$ (Merck) with the stock concentration of 20 mM, and sterilized through 0.22µm syringe filter (Milipore).MS medium (supplemented with vitamins, sucrose solidified with 0.7% agar) was used for the germination of Jatropha.

The cotyledonary leaves were germinated in MS medium with the elevated concentration of Cd (0, 50. 100, 150, 200, 250 µMol L^{-1}) for two weeks. The experiment was laid out in three replicates, germination percentage, shoot length and biomass yield.

2.3 Cadmium analysis using flame ionization atomic absorption spectroscopy

Plant sample were dried at 80^{o}C for 48 hrs to measure the dry weight. Dried plant samples (root and shoots separately) were incubated in hydrochloric acid and nitric acid (3:1) to extract the Cd cation. Cd content in plant tissues was determined by atomic absorption spectrometer (AA 240, Varian). A series of standard solutions was prepared (0, 0.5, 1.0, 1.5, 2, 2.5, and 3 µg/ml) of Cd^{2+}. The absorbance of the standard solutions were measured at 228.8 nm and used to prepare a calibration curve. The operating parameters for Cadmium were set according to the manufactures instructions. Cadmium concentration expressed in µgg^{-1}.

2.4 Enzymatic assay

2.4.1 Glutathione reductase (GR) was determined by the method of Smith et al (1988)[21]. The reaction mixture contained 1.0 ml of 0.2 M potassium phosphate buffer (pH 7.5) containing 1mM EDTA, 0.5 ml of 3 mM DTNB (5, 5-dithiobis-2 nitrobenzoic acid) in 0.01 M potassium phosphate buffer (pH 7.5), 0.1 ml of 2 mM NADPH, 0.1 ml of enzyme extract and distilled water to make up final volume of 2.9 ml. Reaction was initiated by adding 0.1 ml of 2 mM GSSG. Absorbance was measured at 412 nm ((Perkin Elmer- lambda 25 UV-Vis spectrometer).

2.4.2 Catalase assay (CAT) was determined by using the standard method of Chance and Maehly (1955) [22]. The reaction mixture contained 1 ml of 0.1 mM phosphate buffer (pH 7.4), 0.25 ml of 30 mM hydrogen peroxide (H_2O_2), 0.25 ml enzyme extract and the absorbance was recorded at 240 nm (Perkin Elmer- lambda 25 UV-Vis spectrometer).

2.4.3 Peroxidase (POX) Activity was measured by estimation of the oxidation of guaiacol [23]. The reaction mixture (0.996 ml) was made of 25% (v/v) guaiacol in 10 mM sodium phosphate buffer (pH 6.0) containing 10 mM Hydrogen peroxide as substrate. The crude enzyme (0.034 ml) extract was taken and absorbance was recorded at 470 nm (Perkin Elmer- lambda 25 UV-Vis spectrometer).

2.4.4Lipid peroxidation assay: The level of lipid peroxidation was determined by using 2-thiobarbituric acid (TBA), which measure the accumulation ofreactive metabolites mainly malondialdehyde [24]. The 0.2 g of the tissue was homogenised with 5 ml of 0.25% TBA made in 10% trichloro acetic acid (TCA).The homogenised sample was boiled for 30 min at 95^{o}C and centrifuged at 10,000 rpm for 10 min. The absorbance of supernatant was recorded at 532 nm and corrected by subtracting absorbance at 600 nm (Perkin Elmer-lambda 25 UV-Vis spectrometer).

2.5 Genomic DNA extraction and RAPD

From each treatment, a total of 100 mg of juvenile leaves from five individual plants were bulked for DNA extraction.The total genomic DNA was extracted from homogenized leaf samples of five different treatments along with control plant of *Jatropha* following the CTAB method with minor modifications. The quality and concentration of the DNA samples were analysed by spectrophotometric analysis and agarose gel electrophoresis on ethidium bromide stained 1% agarose gel to confirm DNA integrity and absence of RNA contamination. The extracted DNA was diluted to final concentrations of 100 ng μl^{-1} which were used in RAPD analysis.

Five RAPD primers were selected for the main experiments (**Tab. 1**). The RAPD assay was carried out in 25 µl reaction volume containing 100 ng genomic DNA, 100 µM of dNTP mix, 1 µM of random primer and 1.0 U of Taq DNA polymerase (Bangalore Genei, India). Amplification was performed in thermal cycler (Applied Biosystems 2720). The standardized amplification for RAPD was: initial denaturation at 94ºC for 3 min, followed by 35 cycles of denaturation at 94ºC for 1 min; primer annealing at 34º C for 1 min; primer extension at 72ºC for 2 min and final primer extension at 72ºC for 10 min. The PCR products were size-separated on 1.5% agarose gel in 1X TBE buffer by electrophoresis at 100 V for 30 min and documented in gel documentation system (Bio-Rad Laboratories).

Table 1. Primer sequences used for the RAPD analysis.

Primer no.	Primer ID	Primer sequence
1	OPS- 09	TCC TGG TCC C
2	OPG- 02	GGC ACT GAG G
3	OPK- 07	AGC GAG CAA G
4	OPK- 16	GAG CGT CGA A
5	OPL- 04	GAC TGC ACA C

2.6 Statistical analysis

Each experiment was repeated thrice and data presents are mean ± standard error (SE). The results were subjected to ANOVA. Tukey test was performed for comparison between set of experiments. The data analysis was carried out using statistical software SPSS 20.

3 RESULTS

3.1 Plant growth and Cd toxicity

Results indicated the plant germination rate (**Tab. 1**) and plant growth was inhibited with elevated concentration of Cd (**fig. 1**). Shoot length and Root length decreased significantly by 55.56 and 52.38 % respectively at 200 µMol L^{-1} (**fig. 2a and 2b**). At higher concentrations 250 µMol L^{-1} we have observed the plant has not able develop the shoot as well as roots. Furthermore, with increasing dose of Cd, the total fresh and dry biomass showed a linear negative response. Total fresh weight decreased by 46.37, 57.38 & 70.48 % and total dry weight was decreased by 54.91, 62.97 & 71.56 % respectively at 150, 200 and 250 µMol Cd L^{-1} compared to control (**fig. 2c and 2d**).

3.2 Cadmium accumulation and translocation

Cd content were analyzed by the AAS in root and shoots of *Jatropha* seedling exposed to various concentration of Cd. Root and shoot Cd content significantly increased in response to increasing concentration of Cd in growth medium (**tab.2**).

Table. 2 Percentage germination of *Jatropha curcas*cotyledonary leaves on growth medium, containing increasing concentration of Cadmium.

Cd (µM)	% germination
Control	93.3
50	86.6
100	80.0
150	66.6
200	46.6
250	0

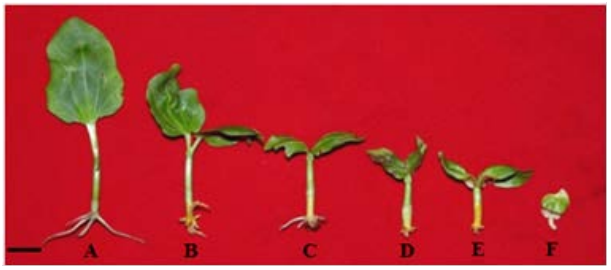

Fig. 1 Effect of increasing concentration of cadmium on the growth of *Jatropha curcas* (A, B, C, D, E and F represents 0, 50, 100, 150, 200and 250 µMol L^{-1} Cd respectively). (Bar = 1 cm).

Table. 3 Cadmium concentration in root and shoot tissues (µg g^{-1} dry weight) of *Jatropha* seedlings.

Cd (µM)	Root	Shoot
Control	-	-
50	1673.3± 4.4	428.3± 6.4
100	3475.3± 2.6	496.3± 8.6
150	4460.3± 2.9	686.7± 7.2
200	6686.6± 5.9	1094.7± 4.2
250	-	1487.3± 7.6

3.3 Changes in antioxidant enzyme activity

The activities of antioxidant enzymes in *Jatropha* seedlings under stress condition were significant.Glutathione reductase (GR)activity increased at 50 µMol Cd L^{-1} (121.53%) and then significantly decreased by 87.54% at 250 µMol L^{-1} Cd supply compared to control (**fig. 3a**). It showed the reduced activity at higher concentrations. Catalase (CAT)activity was significantly increased in all Cd stress treatment at higher concentration (250 µMol Cd L^{-1}) it increased by 110 % than control plant. A fluctuation in CAT activity has been observed in concentration in 50 and 150 µMol L^{-1} Cd. It maybe due to stress management of plant cells against the higher Cd concentrations. It has been also observed that there is a gradual increase in CAT activity with an increase in Cdconcentrations (**fig. 2b**). Peroxidase (POX) mainly removes hydrogen peroxide due to induced stress condition. *Jatropha* seedling germinated, under higher concentrations of Cd, significantly increases in POX activity compared to control plants by 257.9% at 250 µMol Cd L^{-1} (**fig. 2c**).
Lipid peroxidation was measured by MDA accumulation levels; result showed that MDA level is proportionally increased in *Jatropha* by 71.08% compared to control plant at higher concentration of Cd. Increased MDA level shows the damage of cellular membrane caused by Cd. Significant differences were observed between control and the Cd treated plants (**fig. 2d**).

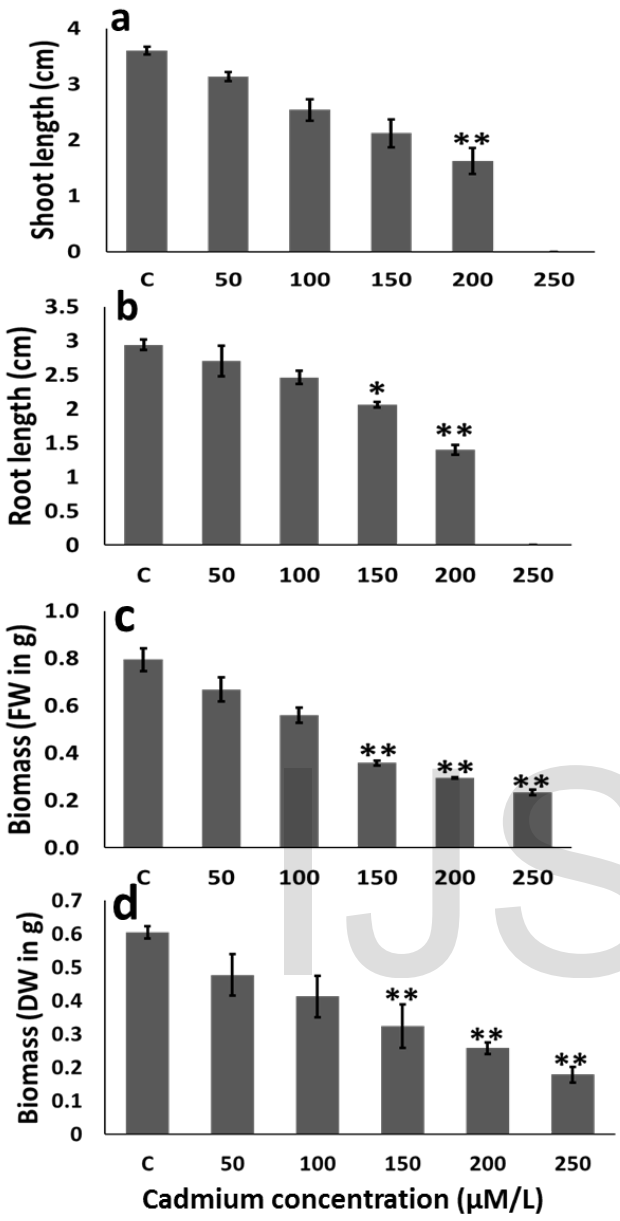

Fig. 2 Effect of increasing concentration of Cd in the growth medium on shoot length (**a**), root length (**b**), fresh mass (**c**) and dry mass (**d**) of Jatropha curcas seedlings. Values are mean ± SE (n=3). Difference between treated (Cd) and control plants were significant at $P< 0.05$ (**) by Tukey test.

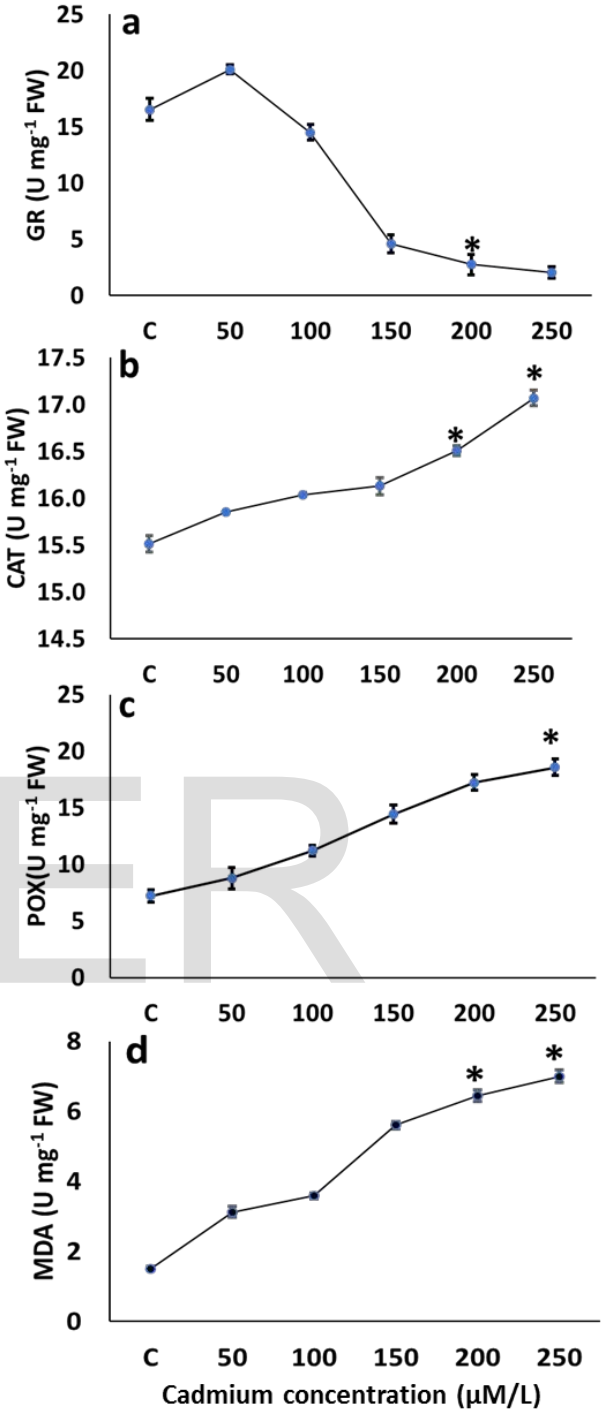

Fig. 3 Effect of increasing concentration of Cd on antioxidative enzymes in crude leaf extracts, glutathione reductase(GR) (**a**), Catalase (CAT) activity (**b**), Peroxidase (POX) activity (**c**) and non-enzymatic lipid peroxidation (MDA) content (**d**) of *Jatropha curcas* seedlings. Values are mean ± SE (n=3). Difference between treated (Cd) and control plants were significant at $P<0.05$ (*)by Tukey test.

3.4 RAPD analysis

The RAPD analysis was performed on DNA extracted from the leaves of 3-5seedling of each replicate treated with cadmium in 50,

100, 150, 200 and 250 µMol Cd L^{-1}. Eleven 10-mer oligonucleotide primers were utilized for screening of the *Jatropha* genome for changes. Only five primers generated specific and stable results. The total number of bands was 29 (control) and 134 (all treatments) ranged from 20 kb to 831bp. RAPD profile showed substantial difference between untreated control and treated seedlings with difference in the band intensities, appearance or disappearance of bands with different primers (**fig. 4**).

RAPD profiles in the leaves of *Jatropha* seedlings exposed to cadmium showed a deviation in disappearance and/or appearance of bands (**tab. 3**). The number of disappeared band was low (-2) and high (-8 and -5) with 50, 200 and 250 µMol L^{-1} of Cd respectively. Appearance of new band was also observed in 100 and 150 µMol L^{-1} Cd (+2 each) whereas absences of DNA bands were observed at higher Cd concentration. With the increasing concentration of cadmium the number of disappeared band was increases.

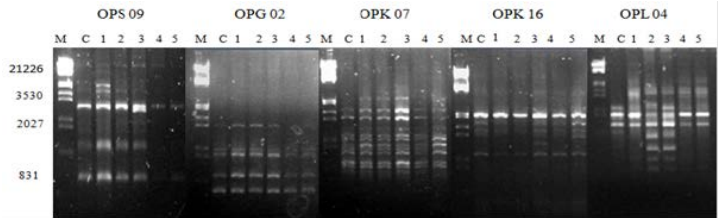

Fig. 4 RAPD profiles of genomic DNA from leaves of *Jatropha curcas*seedlings exposed to varying Cd concentration. Lane 1= 50, 2= 100, 3= 150, 4= 200 and 5= 250 µM Cd. Lane C, control and Lane M, molecular marker.

Table. 3 The number of band in control and disappearance (-) and appearance (+) of DNA bands for all primers in the leaves of Cd-treated *J. curcas* seedlings.

Primer	Cadmium concentration (µM)					
	Control	50	100	150	200	250
OPS- 09	7	0	2 (-)	3 (-)	4 (-)	5 (-)
OPG- 02	5	0	0	1 (-)	1 (-)	0
OPK- 07	8	1 (+)	3 (+)	2 (+)	1 (+)	0
OPK- 16	5	2 (-)	3 (-)	0	3 (-)	0
OPL- 04	4	1 (-)	4 (+)	4 (+)	1 (-)	0
Total	29	1 (+); 3 (-)	7 (+); 5 (-)	6 (+); 4 (-)	1 (+); 9 (-)	5 (-)

5DISCUSSION

Cadmium (Cd) treatmentshowed retarded growth, chlorosis, inhibition of antioxidant enzymes and the production of lipid peroxidation components in *Jatropha* seedlings and it also responsible for DNA alteration. Morphometric observations such as plant height, root length, biomass yield are noticeable response to heavy metal toxicity. These outcomes directed that the phytotoxicities of Cd, were directly related with the capability of heavy metal tolerance of the plant and accompanied by an occurrence of a variety of morphological, biochemical or molecular events. Elevated concentration of Cd showed the reduced plant height, and low biomass yields. Present observation revealed that at elevated Cd concentrations, the plant height was significantly reduced, and the biomass was decreased. Root length was significantly affected by increased concentration of Cd as plants uptake water and nutrients through its root system therefore roots are the primary tissue for heavy metal stress. Root system absorbed Cd with water and accumulates in root. Similar results were also reported in *Typhaangustifolia*[25] and *Jatropha curcas*[26]. Accumulation of Cd in root give rises an abnormal rooting system to *Jatropha* seedlings during germination and affects the shoot: root ratio. However, *Jatropha* seedling gives four main roots during germination but it could be higher in such stress conditions [27].

Cadmium uptake in root has been considered a key process in whole plant Cd accumulation, result revealed that *Jatropha* seedlings grown in increasing concentration of Cd capable to uptake by root and translocate to the leaves. The uptake and accumulation of Cd in root has been demonstrated in various plants [28-30]. Plant has indigenous biochemical/ physiological properties that help to tolerate the heavy metal toxicity [31]. Increasing accumulation rate in response to Cd concentration medium retarted growth and chlorosis at higher concentration suggest the Cd toxicity in *Jatropha* seedlings.

Metal toxicity is often driven by ROS generation [32, 33]. The imbalance between ROS generation and elimination may responsible for oxidative stress. Plant possesses an antioxidative system that comprises enzymatic and non-enzymatic antioxidants to scavenge ROS [34]. The involvement of oxidative stress in expression of Cd- toxicity has been suggested in many plant species such as Indian mustard [35-36], Lettuce [37], Maize [38], *Medicagotruncatula* [39] and Arabidopsis [40].These defense systems are composed of enzymatic and non-enzymatic scavengers of activated oxygen such as glutathione reductase, catalases and peroxidases, [41-44]. Enhanced antioxidant activity could prevent oxidative damage and improve tolerance capacity of plants. Response of GR, CAT, and POX activates the essential component of the plant antioxidative defense systems [45].

In present study, CAT and POX activity was significantly increased at higher concentration of Cd however GR activity is decreased significantly (87.54%) compared to control. GR catalyse the NADPH dependent reduction of GSSG to GSH. This reaction is very crucial for the activation of ascorbate glutathione cycle and for GSH/GSSG ratio in cells [46-47]. We observed that GR activity was significantly decreased in *Jatropha* under stress condition. The reduced activity of GR with elevated concentration of Cd shows the effect of Cd toxicity in plant metabolism. Similar observation has been found in *Oryza sativa*and *Phragmitesaustralis*[48-49]. Therefore, GR is the essential for the maintenance of the GSH/GSSG ratio [50] and the reduction of the activity in presence of Cd might explain the decrease in the percentage of the reduced GSSH in the presence of Cd. The activity of CAT gradually increased with increasing supply of Cd. CAT is sensitive to O^{-}_{2} radicals and thus their increasing level under Cd stress, response of inactivation of enzymes. CAT is an important enzyme for elimination of toxic peroxides. In response of increased activity of CAT has been reported in several plant species exposed to toxic concentration of heavy metals Cd, Cu, Pb and [51] under different environmental conditions such as CAT enzyme, POX also catalyses the H_2O_2 breakdown and convert into H_2O and O_2. Cadmium induced the activity of peroxidase (POX) in soybean [52], bean leaves [53] and in roots and leaves of *Oryza sativa* [54], *Brassicajuncea*[55] and in the leaves of *Calamus tenuis*[48].

Lipid peroxidation is considered as an indicator of antioxidative damage, which is initiated due to over production of

ROS and involves oxidative degradation of polyunsaturated fatty acid acyl residues of membrane lipids [56]. In rice seedling grown in sand culture containing 500 µM Cd(NO3) showed 40-57% enhancement of lipid peroxides in the shoots compared to control [57]. The present result revealed that the increase in level of MDA in the leaves of *Jatropha*, showed that Cd induced oxidative stress. Similar response was observed in the sunflower and Indian mustard treated with Cd [36, 41]. It has been reported that toxic oxygen species initiate chain reaction with polyunsaturated fatty acid which leads the peroxidation of lipids (f). The Cd toxicity due to production of ROS, which convert fatty acids to toxic lipid peroxides, and responsible for membrane damage in Cd susceptible plants. The increased level of MDA at higher concentration of Cd is toxic to the *Jatropha* seedling but result also revealed the in increasing concentration (50-200 µMol L^{-1}) of Cd, *Jatropha* seedling were able to survive but at lower rate.

In the field of genotoxicity, RAPD describe the changes in genome such as differences in band intensities, appearance and disappearance of bands [16]. RAPD techniques has been utilized successfully to detect various type of DNA damage and mutation in plants induced by cadmium, lead, nickel, zinc, chromium and mercury [5, 59,60, 61]. In the present study, we have measured the potential of RAPD assay to evaluate the toxicity of cadmium with different concentration on the *Jatropha* seedlings. The variations into genomic DNA were detected by RAPD profile through the random primers. The number of disappeared band was higher than the extra bands compared with the control. The disappearance of bands exposed to higher concentration of Cd may related to DNA damage due to single and double strand break, oxidized bases and DNA protein cross link and also due to the point mutation [14]. Appearance of new bands of DNA in the present study showed that the effect can be involved in DNA repair and replication. The appearance of new band can be due to the mutations if they occur at same locus in sufficient number of cells and can be amplify by PCR. Disappearance of band could be attributed by DNA damage and appearance of new band attributed by mutations [16]. Present study revealed that the RAPD primers are able to amplify the PCR product of *Jatropha* genome, exposed to Cd which caused by the DNA damage as well as the mutations. This observation revealed that the toxicity of Cd was directly related to with heavy metal tolerance ability of plant.

6 Conclusion

The present study revealed that the different concentration of Cadmium directly affects the plant morphology & biomass production. Biochemical assay show the generation of ROS and causes the oxidative stress. Plant defense mechanisms try to reduce the oxidative stress and maintain the homeostasis environment. Uptake and accumulation of Cd shows the toxicity and imbalance the plant metabolism further RAPD analysis can be applied as suitable biomarker assay for the detection of genotoxicity effect of cadmium on *Jatrophacurcas*. Molecular characterization of these markers would be able to indicate that such primers could amplify cadmium induced changes in DNA. Current study helps us to understand the heavy metal tolerance of *Jatropha* plant. It would be appreciable if we can cultivate Jatropha for natural compounds, medicinal purpose as well as a potential feedstock for biodiesel.

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