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ORIGINAL PAPER

Oxidative stress induced by cobalt in *Jatropha curcas* seedlings: response of growth and chemical changes*

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Abstract

Experiments using *in vitro* embryo germination and culture have been designed to investigate the effects of cobalt levels of 0, 100, 200, 400 and 800 μM on some growth parameters and chemical changes in *Jatropha curcas* seedlings. The results showed that excess cobalt levels in MS media produced visual symptoms of toxicity in *J. curcas* seedlings after 7 d with increasing levels of cobalt ion. The fresh weights of cotyledons and radicles were significantly affected except for hypocotyls at the cobalt concentration of 100 μM. The activity of superoxide dismutase (SOD) significantly increased in the radicles when compared to the control, but decreases in the activity of the hypocotyls were found at the tested cobalt levels. Increase of guaiacol peroxidase (GPX) activity in the cotyledons and hypocotyls was observed at the tested cobalt levels, but a decrease of the activity in the radicles except for at cobalt concentration of 100 μM. Increase in the activity of catalase (CAT) of radicles with increasing cobalt concentrations, and a significant decrease in activity in the hypocotyls was also observed. Increase in phenylalanine ammonia-lyase (PAL) activity of cotyledons and radicles with increasing cobalt concentrations in the range 100-800 μM, but a decline in the activity of hypocotyls was also observed when hypocotyls at the cobalt concentrations of 400 and 800 μM. The present results indicated that SOD, GPX, CAT, and PAL may play an important role in the defensive mechanisms of *Jatropha curcas* seedlings exposed to excessive cobalt. These findings will help to provide useful bioassays to help in assessment of cobalt contamination in agricultural environments.

Keywords: *Jatropha curcas*, cobalt, anti-oxidative enzyme, toxicity effects, growth retardation

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INTRODUCTION

Cobalt (Co) is not classified as an essential element for plants; however, it is usually described as beneficial. The biological importance of cobalt was first recognized by the discovery that small amounts of the elements would cause certain deficiency symptoms in plants (Lison et al. 2018). It was shown that excessive Co was able to display negative effects on plant growth and metabolism in different degrees, depending on the concentration and status of Co in the rhizosphere and soil. Co is known to cause irreversible damage to a number of vital metabolic constituents, plant cell walls, and cell membranes. Co can be incorporated into the active site of urease and render the enzyme inactive (Pilon-Smits et al. 2009, Lange et al. 2017). Excessive Co induces yield reduction and inhibition in assimilate production in leaves, and even inhibits the export of photoassimilates to roots and other sinks (Tewari et al. 2002, Li et al. 2009). On the other hand, some beneficial effects are also reported for plants. For example, Co is unequivocally essential for leguminous crops as it is required for nitrogen fixation by bacteria in root nodules (Ali et al. 2010). Other reports showed that growth and pigment content were slightly increased at low concentrations and inhibited by high Co^{2+} concentrations in *Monoraphidium minutum* and *Nitzhia perminuta* (El-Sheekh et al. 2003). Like studies on other heavy metals, most of the recent literature focuses on the mechanisms through which plants can cope with Co stress. Excess Co also causes oxidative stresses, and may result in phytotoxity to plants (Pratima et al. 2012, Karuppanapandian, Kim 2013, Rajeev 2014). These reports suggested that Co, as a trace element, is necessary for normal metabolic functions in plants, but at higher concentrations these metals are toxic and may severely interfere with physiological and biochemical functions.

In abiotic stress, the metal response will result in the production of reactive oxygen species (ROS). The generation of ROS, such as superoxide, H_2O_2, and hydroxyl molecules, causes rapid cell damage by triggering a free-radical chain reaction (Wang et al. 2018). ROS scavenging depends on the detoxification mechanism provided by an integrated system of non-enzymatic reduced molecules, including ascorbate, and enzymatic antioxidants, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), etc. These antioxidant enzymes or molecules may provide a strategy to enhance metal tolerance in plants. The elevated concentration of Co has been found to cause toxicity in plants. It causes a marked inhibition of growth together with declines in CAT activity, and deteriorates the quality of produce (Seneviratne et al. 2019). Excess cobalt may alter the photosynthetic activity of the plants at multiple levels such as pigments, stomatal functioning, electron transport chain, enzymes, and thylakoid membrane (Fryzova et al. 2018). Lower concentrations of cobalt enhanced the antioxidative enzyme activities whereas higher concentrations generated negative effects in *Indian mustard*

(Karuppanapandian, Kim 2013) and *Vigna radiata* plants (Jaleel et al. 2009). These studies will help to understand the behaviour of those enzymes in the presence of highly toxic Co ion.

Jatropha curcas L., commonly known as the physic nut, belongs to the family *Euphorbiaceae*, and is today recognized as a fuel substitute. Its cultivation can also help in the reclamation of wastelands, degraded lands and mine contaminated lands (Navarro-Pineda et al. 2016). Panzhihua, an important industrial and mining base with abundant mineral resources, is located in the southwest of China. In particular, the area around mine facilities in Panzhihua has higher levels of environmental Co, due to the centuries-long extraction and processing of Co-mineral ore (Dai et al. 2021). In Panzhihua, *J. curcas* is often grown on the riverside and in mining areas, where soils are usually contaminated by heavy metals. Our previous research suggested that *J. curcas* seedlings were adaptable to higher concentrations of copper under sand culture condition as well as to heavy metal toxicity in an *in vitro* embryo culture (Gao et al. 2008, 2010). Although no scientific paper reporting the results of those field experiments has been published, it is known that the test results have been promising (Yamada et al. 2018, Álvarez-Mateos et al. 2019, Martín et al. 2020). Comparatively little information is available on the response of many plant species commonly used in ecological restoration, and *J. curcas* might be a good candidate for eco-toxicological research. Although there have been many studies on the effects of Co stress on the growth and development of plants, the effects of Co on the growth and antioxidant responses of *J. curcas* plants have not been studied. Thus, the present study developed an *in vitro* embryo process to understand the growth and antioxidant defensive responses in *J. curcas* seedlings subjected to different levels of Co, with emphasis on the changes in SOD, POD, CAT, and PAL activities.

MATERIALS AND METHODS

Plant material and chemicals

Mature and healthy *J. curcas* seeds were collected in October 2020, from more than 10 individual wild trees in the town Datian, situated in District Renhe, Panzhihua, China, at latitude 26°21′182″ N, longitude 101°46′221″ W and altitude 1348 m. These mature seeds were stored in plastic boxes (Labelled, No. 20201015) at 4°C until being used for tests. The chemicals: guaiacol, L-phenylalanine, methionine and nitro blue tetrazolium (NBT), were purchased from Sigma (St. Louis, MO, USA). Others reagents were grade or higher.

Embryo germination and seedling growth

J. curcas seeds were surface sterilized with 70% ethanol for 30 s, and then kept in 0.1% mercuric chloride for 8 min. 150 seeds were rinsed several times with distilled sterile water, and soaked in water at room temperature for 24-36 h. Embryos were dissected from the seeds. These embryos were placed on Murashige and Skoog (MS) medium in wide-neck bottles (100 ml) for germination and growth in an *in-vitro* culture for 7 days. The culture medium was separated into five lots. One lot was allowed to grow MS medium with 30 g l^{-1} sucrose and 0.6% agar powder to serve as the control. The remaining four lots were cultured on basic MS medium supplemented with cobalt added as $CoCl_2$ at concentrations of 100, 200, 400, and 800 μM, respectively. The pH value of these media was adjusted to 5.8±0.1 prior to autoclaving at 121±2°C for 15 min. The cultures were incubated at 30±2°C, under a 12-h photoperiod with cool, white fluorescent light. Rotten and contaminated embryos were removed promptly. When cotyledons of these seedlings had developed, cotyledons, hypocotyls, and radicles were washed with double distilled water, blotted, and immediately frozen in liquid nitrogen or stored at -80°C for analysis. The experiments were arranged in a completely randomized design with three replicates per treatment, and each replicate contained 15 embryos (27.8 mg per embryo).

Protein extraction and estimation

Fresh samples were homogenized and extracted with 50 mM sodium phosphate buffer (pH 7.0, m/v, 1/10) including 0.1 mM EDTA and 150 mM NaCl. The crude extract was centrifuged at 12 000 rpm for 5 min at 4°C and the supernatant was analyzed for further use. Protein content was determined according to the Lowry's method with bovine serum albumin as the standard.

Assay of antioxidant enzymes

An SOD assay was performed according to the McCord and Fridovich's method with some slight modifications (Mccord, Fridovich 1969). In brief, a 3 ml reaction mixture contained 50 mM sodium phosphate buffer, pH 7.8, 13 mM methionine, 75 μM NBT, 2 μM riboflavin, and 50 μl protein extract. Absorbance was recorded at 560 nm using a UV/vis spectrophotometer. The protein volume corresponding to 50% inhibition of the reaction (one unit) was calculated. Guiacol peroxidase (GPX) activity was determined by measuring the increase in absorbance at 470 nm due to the formation of tetraguaiacol (GAO et al. 2010). The reaction mixture (3 ml) consisted of 2.8 ml 3% guaiacol in 50 mM Tris-HCl (pH 7.0) and 100 μl 2% H_2O_2. The reaction was started by adding 100 μl protein extract, and the absorbance at 470 nm was measured. One unit of enzyme activity was defined as the amount of enzyme which produces a 1.0 absorbance change at 470 nm per min in assay conditions. CAT activity was measured with the Montavon method

(Montavon et al. 2007). The activity was assayed in a 3 ml reaction solution containing 2.8 ml phosphate buffer (50 mM, pH 7.0), 100 µl 2% H_2O_2 and 100 µl of protein extraction. One unit of CAT was defined as the amount causing the decomposition of 1 µM of H_2O_2 per min. The SOD, GPX, and CAT activities were expressed as enzyme units per gram fresh weight (U g^{-1} FW).

Enzyme extraction and PAL activity assay

Fresh samples were ground in ice-cold 50 mM Tris-HCl buffer of pH 8.8 containing 1% polyvinylpolypyrrolidone and 0.1 mM EDTA. The homogenate was centrifuged at 12 000 rpm for 5 min at 4°C, and the supernatant was assayed for enzyme activity. PAL activity was determined by monitoring the reaction product *trans*-cinnamate at 290 nm. One unit of enzyme activity was defined as the amount of enzyme that increased the absorbance by 0.01 min^{-1} under assay conditions (GAO et al. 2010).

Polyacrylamide gel electrophoresis (PAGE)

Samples of crude protein extracts were electrophoresed in 8% (w/v) polyacrylamide slab gel under non-denaturing conditions. Staining protocols of SOD and GPX isoenzymes are generally followed by Beauchamp and Fridovich (1971), and Ros Barcelo (1987), respectively.

Statistical analysis

All treatments were arranged in a completely randomized design with three replicates. Data were analyzed using one-way analysis of variance (one-way ANOVA) and expressed as means ± S.E. The Waller-Duncan multiple interval tests at $P \leq 0.05$ were performed for evaluating the significance differences among different groups using SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS AND DISCUSSION

Effects of different cobalt levels on the seedling growth

Cobalt is an essential micronutrient for normal plant growth, development and metabolism, and its negative effects on seedling development and growth have been well documented at higher concentrations (Pilon-Smits et al. 2009, Lange et al. 2017). Excessive cobalt not only reduces soil fertility, but also disrupts the physiological and biochemical functions of plants, thereby having a significant reducing impact on plant yields (El-Sheekh et al. 2003, Chatterjee et al. 2006, Pratima et al. 2012). Thus, the present study was undertaken to contribute to the understanding of the relationship between Co toxicity, growth parameters and antioxidative defense system.

As shown in Figure 1, *J. curcas* seedlings showed no apparent morphological changes observed at 100 μM Co. However, significant differences were found in the lengths of hypocotyls and radicles depending on Co levels. Increasing salt concentrations severely affected hypocotyls and radicle elongation. Growth of the cobalt concentration in a medium to 200, 400, and 800 μM Co levels caused significant growth inhibition and toxicity effects on the growth and development of seedlings in comparison with the control. Seedlings cultivated under 200~800 μM Co developed leaf chlorosis, which

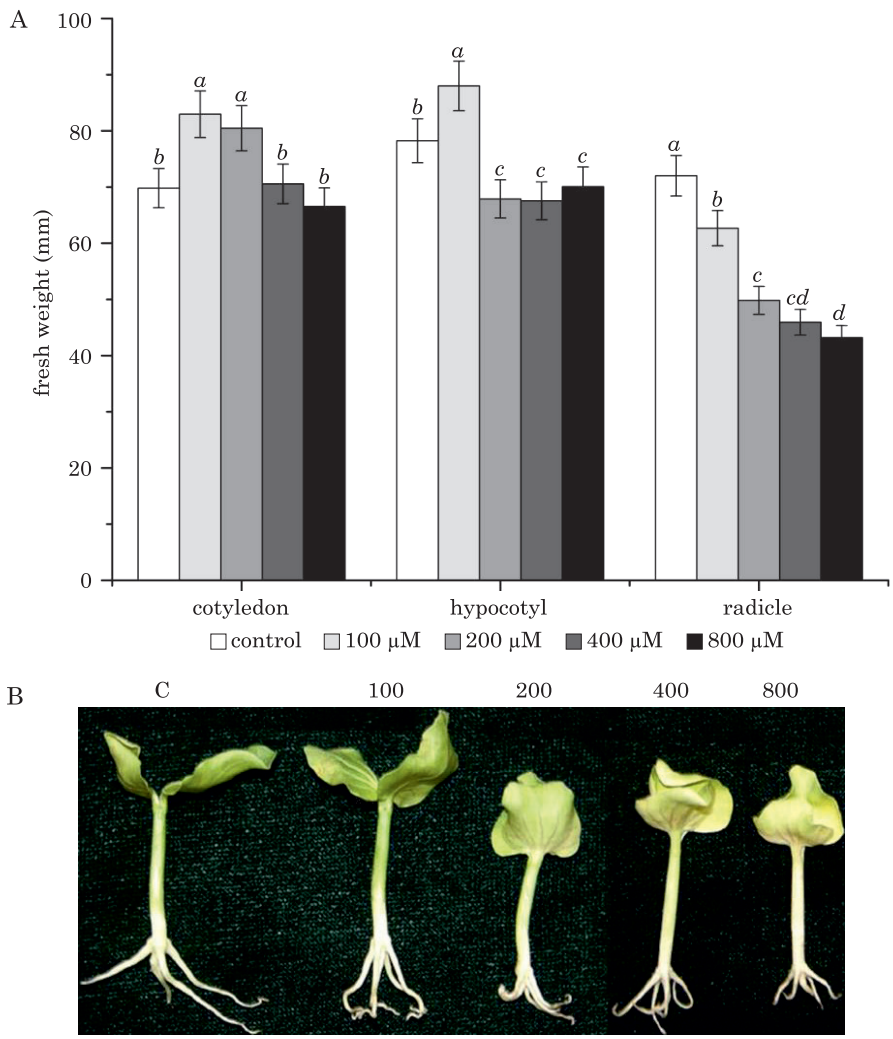

Fig. 1. Effects of cobalt on fresh weight (A) and growth (B) of the cotyledons, hypocotyls and radicles in *J. curcas* seedlings germinated and grown in MS medium containing 100, 200, 400 and 800 μM Co. Data points and error bars represent means ± S.D. of three replicates (n=3). Lowercase letters represent significant differences among different treatments, with a significance level of 0.05

can progress to yellow patches in the basal leaves. In the cotyledons, the fresh weight increased by 18.9% and 15.3% at Co levels of 100 and 200 μM compared to the control, respectively. However, the values showed no significant changes with the increasing Co levels. In the hypocotyls, the fresh weight increased by 12.5% at Co levels of 100 μM, and other tested Co levels decreased it by 10.4-13.7% with respect to the control. In the radicles, the fresh weight gradually decreased by 13-40% with the increasing Co levels up to 800 μM (Figure 1A). The present results indicated that there was a decrease in all growth parameters such as seedling height, leaf area, and fresh weights of aerial parts and roots, except for cotyledons and hypocotyls at 100 and 200 μM (Figure 1B). However, if present at high levels, cobalt may inhibit seedling growth by interfering with normal cellular metabolic events and inducing visible injuries and physiological disorders, as reported by our and other studies (El-Sheekh et al. 2003, Ali et al. 2010). The first visible damage due to excessive cobalt was on root growth due to a reduction in cell division (Mico et al. 2008). Significant morphological aberrations included impaired radicle development, coarser hypocotyl, and cotyledon chlorosis (Figure 1B). Taken together, it seems that the reduction of growth parameters, and morphological changes in response to different cobalt concentrations might be considered as a direct consequence of a limitation of photosynthesis. Such visual symptoms could be ascribed to the loss of chlorophyll and accumulation of cobalt on one hand, and to the early senescence on the other hand. In many plant species, seed germination and early seedling growth are the most sensitive stages to heavy metal stress, and radicle is more sensitive to excessive cobalt than cotyledons and hypocotyls. Various authors have also reported inhibition of seedling growth when exposed to excessive cobalt levels (Karuppanapandian, Kim 2013, Seneviratne et al. 2019), but results of the present study indicated that lower tested Co concentrations increased the production of fresh weight in the seedlings (Figure 1). Negative effects of heavy metal application on *J. curcas* growth were well described in our previous papers, supporting the fact that Co has more negative influence due to strong redox-active properties leading to decrease of biomass accumulation. This might also be due to long-term adaptation of *J. curcas* to polluted environments, or even the development of a mechanism of heavy metal tolerance to adjust to change in the Panzhihua region (Gao et al. 2008, 2010). These results lend further support to those previous findings. Although results obtained with plants grown *in vitro* might not be directly compared to those in field conditions, they are important in detailing plant growth response under controlled conditions.

Effects of different cobalt levels on superoxide dismutase (SOD) activity

Generally, it is known that growth inhibition, noted in plants under the uptake of heavy metals is related to some physiological process alterations

owing to the generated oxidative stress. Oxidative stress is believed to occur when the capacity of antioxidative systems becomes lower than the amount of ROS generated (Wang et al. 2018). Plants have developed different mechanisms enabling them to cope with the ROS formation induced by metals presence. Among them, an antioxidant enzyme system including SOD, GPX, CAT, and PAL is one of the major metal detoxification mechanisms in plants (Seneviratne et al. 2019, Barros, Dixon 2020). As shown in Figure 2, in the

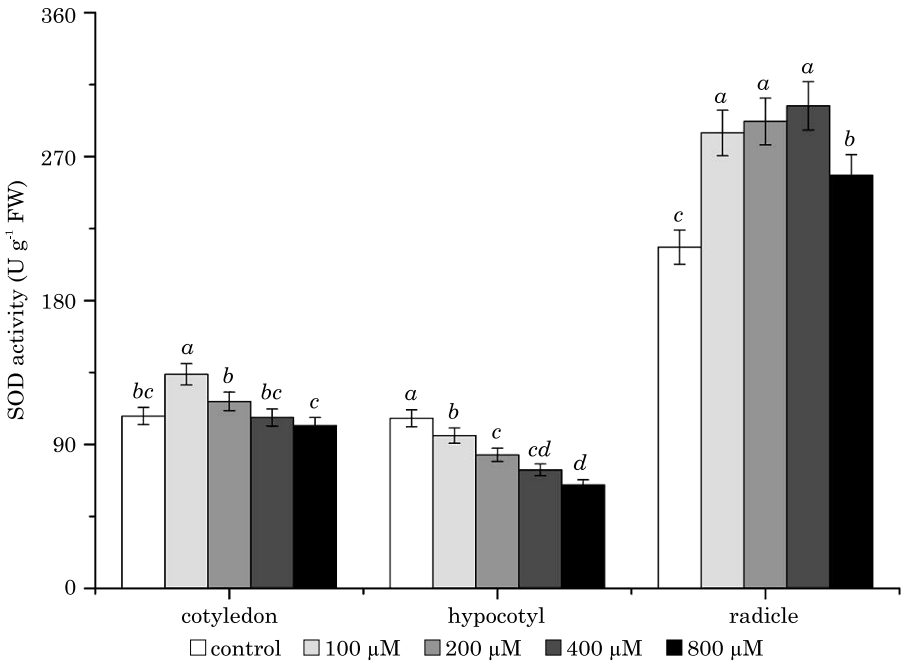

Fig. 2. Effects of cobalt on superoxide dismutase (SOD) activity of the cotyledons, hypocotyls and radicles in *Jatropha curcas* seedlings germinated and grown in MS medium containing 100, 200, 400 and 800 μM Co. Data points and error bars represent means ± SD of three replicates (n=3). Lowercase letters represent significant differences among different treatments, with a significance level of 0.05

cotyledons, the SOD activity increased by 24.2% and 8.49% at the Co levels of 100 and 200 μM, respectively, compared to the control. In the radicles, the SOD activity peaked at the tested Co concentration of 400 μM (41.4%), and an increase of 21.1% was recorded at the 800 μM Co treatment. However, the activity showed a slight decrease of 5.52% at the tested level of 800 μM with respect to the control. In the hypocotyls, the SOD activity decreased by about 10.2%, 21.5%, 30.3%, and 39.1% at the tested Co levels of 0, 100, 200, 400, and 800 μM, respectively. The activity in the radicles was significantly higher than in the cotyledons and hypocotyls. Both an increase and a decrease in the activity of SOD have been observed in heavy metal-treated *J. curcas* and other plants (Gao et al. 2008, 2010, Wang et al. 2018), suggesting that higher SOD activity has a role in imparting tolerance against any

type of heavy metal stresses. SODs are multimeric metalloproteins and have three isoforms on the basis of the metal cofactor in plant cells. Isozyme charts are molecular level types after gene expression. The enzyme band color can reflect the relative quantity of the isozyme activity (Hu et al. 2007, Borgstahl, Oberley-Deegan 2018). As shown in Figure 3, our results suggested that there are at least three isoforms visualized in the cotyledons, hypocotyls and radicles, and the staining intensities of these isoenzymes differ between the tested cobalt levels and plant tissues. According to the SOD isozyme chart, SOD isozyme bands were darker with lower Co concentrations, and it meant that the activity was weak. With the increase of Co, SOD isozyme

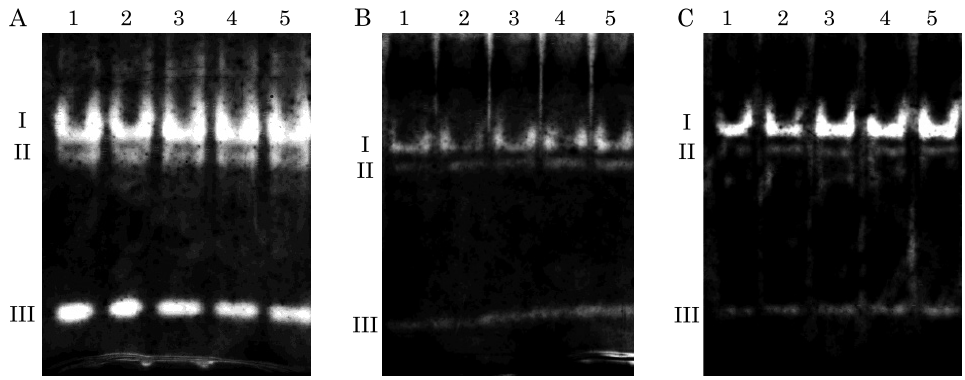

Fig. 3. Patterns of SOD isoenzymes present in the cotyledons, hypocotyls and radicles of *J. curcas* seedlings: A – patterns of SOD isoenzymes in the cotyledons, B – patterns of SOD isoenzymes in the hypocotyls, C – patterns of SOD isoenzymes in the radicles. Lanes from 1 to 5 were 0, 100, 200, 400 and 800 μM, respectively

bands became strong, which meant that the activity was enhanced, which coincided with the measured results of SOD activity. Various SOD isozymes are differentially compartmentalized, and depending on the tissue, are likely to respond differently to different Co concentrations in *J. curcas* seedlings. In our previous experiments, we cloned, expressed and characterized the isoforms of Cu/Zn-SOD, Fe-SOD, and Mn-SOD (unpublish) from *J. curcas* leaves (Ou-Yang et al. 2012, Chao et al. 2013). Based on the nucleic acid sequence and molecular weight of SOD, three isoforms visualized in the cotyledons, hypocotyls, and radicles may be identified as Fe-SOD (I), Mn-SOD (II), and Cu/Zn-SOD (III) – Figure 3. However, three isoforms of SOD differently contributing to the total activity were present in different organs, and it was important to further assess the contribution of each isoform to the total activity of SOD under different Co concentrations. Similar reports have been shown in *Vicia faba* subjected to Co and lead stress, respectively (Wang et al. 2010, Rancelis et al. 2015). Moreover, the changes in the staining intensities of these isoenzymes in different tissues were correlated with the quantitative changes of SOD activity assayed in solutions. It was also observed that the expression of Fe-SOD (I) and Cu/Zn-SOD (III) at all tested Co levels was higher than that of Mn-SOD (II) isoforms (Figures

2 and 3). These findings implicated that heavy metal stresses might result in a high SOD protein turnover and promote more and/or new SOD enzyme synthesis to maintain sufficient SOD levels. Through the effective work of SOD isozymes, the toxicity of ROS is relegated from O_2^- to H_2O_2, then the ROS scavenging enzymes come into play.

Effects of different cobalt levels on guaiacol peroxidase (GPX) activity

GPX is considered as one of the stress indicators of plants, and increases in GPX expression in response to various metals have been reported. It is suggested that the increases may be a kind of defensive response for scavenging the ROS generated due to metal toxicity (Kidwai et al. 2020). As shown in Figure 4, in the cotyledons, all tested cobalt levels led to an increase in the GPX activity, and the values reached 145.6%, 167.7%, 154.4%, and 132.6% at Co levels of 100, 200, 400, and 800 μM with respect to the control, respectively. In the hypocotyls, the GPX activity increased by 45.9%, 45.7%, and 19.8% at Co levels of 100, 200, and 400 μM compared to the control, respectively. However, there was no significant change in the GPX activity at the tested level of 800 μM in comparison to the control. In the radicles, the GPX activity was increased obviously by 25% in 100 μM

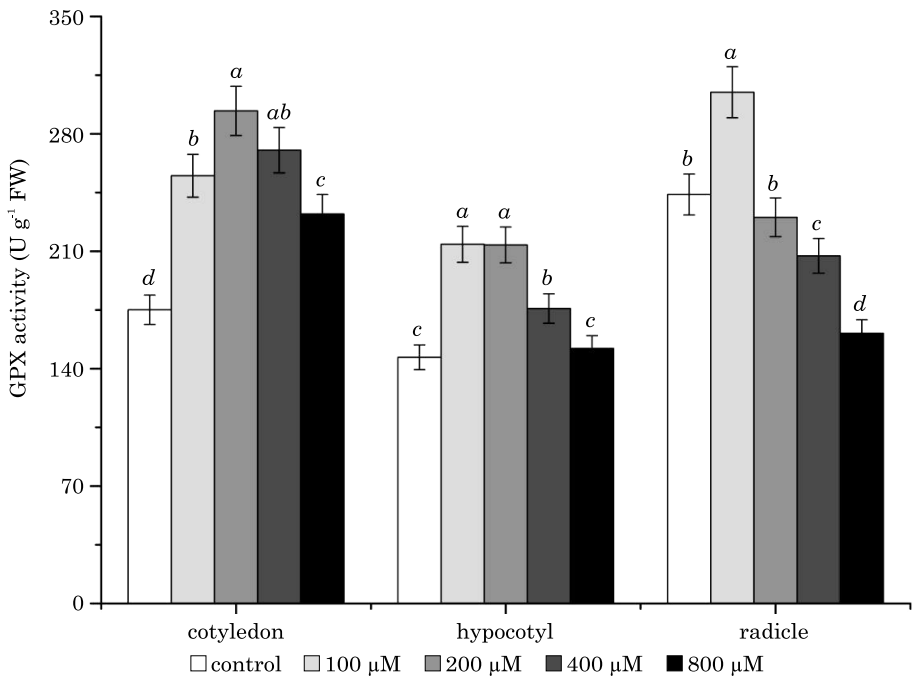

Fig. 4. Effects of cobalt on guiacol peroxidase (GPX) activity of the cotyledons, hypocotyls and radicles in *Jatropha curcas* seedlings germinated and grown in MS medium containing 100, 200, 400 and 800 μM Co. Data points and error bars represent means ± SD of three replicates (n=3). Lowercase letters represent significant differences among different treatments, with a significance level of 0.05

Co treated plants compared to the control. However, the GPX activity gradually decreased with increasing cobalt levels treatments, and the activity was decreased by 5.59%, 15%, and 33.9%, respectively, in 200, 400, and 800 μM cobalt treated plants as compared to the control. The plant POD family consists of multiple isoenzymes with distinct sub-cellular locations in vacuoles, the cell wall and the cytosol, which exhibit different tissue-specific expression patterns and environmental stress responses (Kidwai et al. 2020). To determine whether there were Co stress-mediated differences among individual GPX isoenzymes, GPX activity assays were also performed by activity staining. As shown in Figure 5, on the activity gels, at least six GPX isoenzyme bands were visualized in the cotyledons (Figure 5A), whereas five GPX isoenzyme bands were observed in the hypocotyls and radicles (Figures 5B and C),

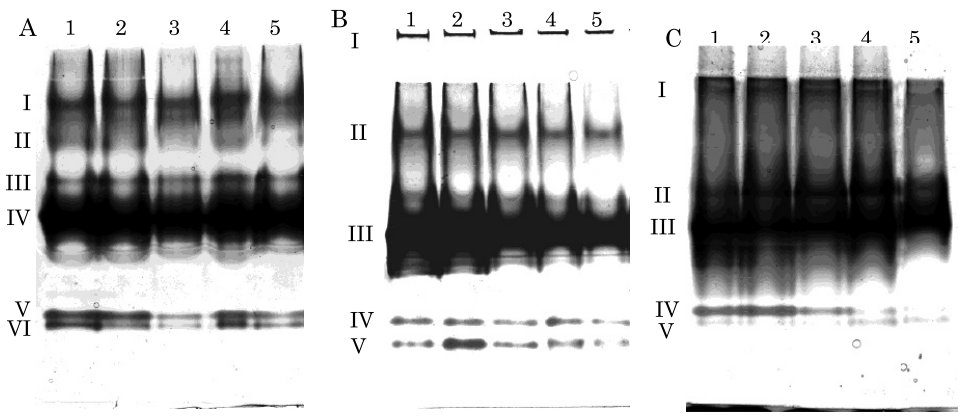

Fig. 5. Patterns of guiacol peroxidase (GPX) isoenzymes present in the cotyledons, hypocotyls and radicles of *Jatropha curcas* seedlings: A – patterns of GPX isoenzymes in the cotyledons, B – patterns of GPX isoenzymes in the hypocotyls, C – patterns of GPX isoenzymes in the radicles. Lanes from 1 to 5 were 0, 100, 200, 400 and 800 μM, respectively

respectively. According to the GPX isozyme chart, GPX isozyme bands in the cotyledons and hypocotyls were gradually strong with the increasing Co concentrations up to 200 and 200 μM, respectively, which meant that the activity was enhanced. With the increase of Co concentrations, GPX isozyme bands became dark, and it meant that the activity decreased, which coincided with the measured results of GPX activity. In the radicles, the color of GPX isozyme bands was increasingly lighter except for 100 μM, and it meant that the activity was decreasing gradually, which coincided with the measured results of GPX activity (Figure 4). These findings indicated that *J. curcas* seedlings possess a strong GPX system to deal with the stress induced by higher Co concentrations. However, with the rising concentrations of Co, the seedlings were required to maintain higher GPX activity and isozyme expression levels. Karuppanapandian and Kim (2013) found that three isoforms of POD (POD I, II, and III) were detected in Indian mustard leaves, which showed differential responses to Co treatment. Hu et al (2019)

also showed that the enhanced activity of POD activity in *L. minor* plants was related to ROS-scavenging upon exposure to Co, which may explain the lack of significant ROS accumulation recorded after 7 days of exposure to Co. Moreover, the effects of cobalt stresses on GPX activity depend on the ion concentration, plant species and organ type (Kabeya et al. 2018, Kidwai et al. 2020). Our present observations were consistent with previous reports and supported the view that GPX can be the key functional antioxidant enzyme contributing to *J. curcas* tolerance to Co stress and might endow the plant with the capacity to reduce Co-induced oxidative stress.

Effects of different cobalt levels on catalase (CAT) activity

CAT is a powerful antioxidant metalloenzyme that catalyzes the dismutation of H_2O_2 into H_2O and O_2, and which plays a central role in plant growth, development and stress conditions (Tehrani, Moosavi-Movahedi 2018). As shown in Figure 6, in the cotyledons, CAT activity significantly increased at 100 and 200 cobalt treatments, and the values reached 166.5% and 106.7% with respect to control, respectively. However, with the increasing Co levels, the activity significantly decreases at Co levels of 400 and 800 μM compared to the control, and the values only represent 72.9%

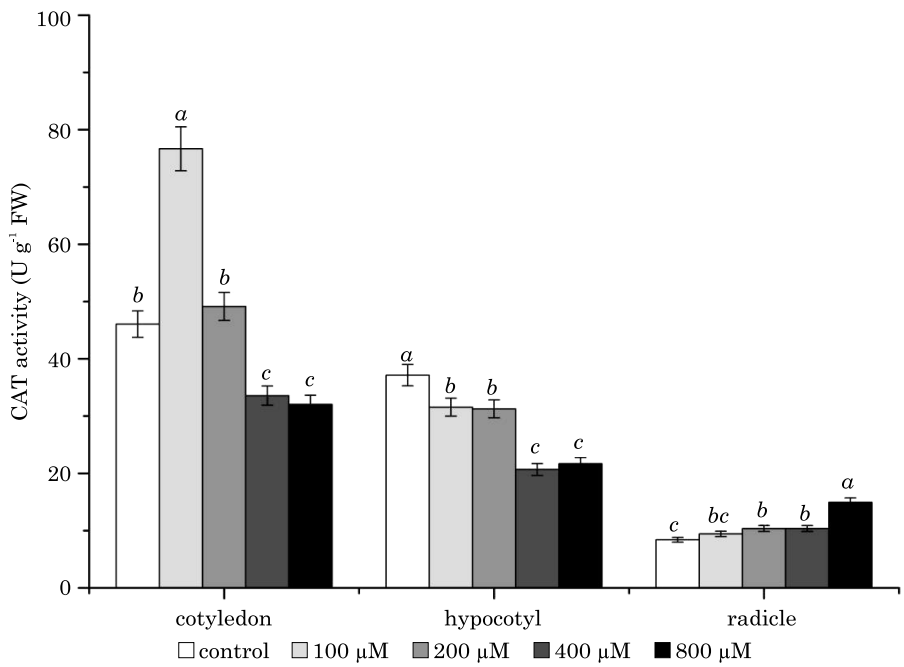

Fig. 6. Effects of cobalt on catalase (CAT) activity of the cotyledons, hypocotyls and radicles in *Jatropha curcas* seedlings germinated and grown in MS medium containing 100, 200, 400 and 800 μM Co. Data points and error bars represent means ± SD of three replicates (n=3). Lowercase letters represent significant differences among different treatments, with a significance level of 0.05

and 69.6%, respectively. In the hypocotyls, all tested Co levels led to the reduction of CAT activity, and the activity decreased by 15%, 15.8%, 44.3%, and 41.6%, respectively, in 100, 200, 400, and 800 μM cobalt treated plants as compared to control. In the radicles, the CAT activity gradually increased by 12.1-77.8% with the increasing Co levels up to 800 μM. Tewari et al. (2002) found that at lower cobalt concentrations, the activity of CAT increased, while decreasing at higher concentrations in plants (Karuppanapandian, Kim 2013). Studies have already reported that tested Co toxicity also enhances CAT in mustard and French bean plants (Chatterjee et al. 2006, Pratima et al. 2012, Karuppanapandian, Kim 2013). In this study, the increases in CAT activity indicated the accumulation of hydrogen peroxide and other oxygen radicals. More importantly, increased SOD and POD activities further suggested the induction of oxyradicals in cobalt-treated seedlings. In fact, it has been proposed that a complete set of antioxidant defense systems including SOD, GPX, CAT, etc., rather than a single antioxidant, is responsible for protection in *J. curcas* plants exposed to cobalt toxicity (Figures 2 and 4). According to the above, the changes in CAT activity in *J. curcas* seedlings confirmed that the plants exposed to stress induced by excessive Co stresses initiate a series of acclimatization responses that may provide increased protection against more severe stress.

Effects of different cobalt levels on phenylalanine ammonia-lyase (PAL) activity

PAL is a key enzyme of the steps within the biosynthesis of secondary metabolites which play an important role in the tolerance mechanisms against metal stress. PAL activity is stimulated in a variety of biotic and abiotic stresses, including pathogenic attacks, tissue wounding, UV irradiation, exposure to heavy metals, low temperatures, and low levels of nitrogen, phosphate, or ions (Barros, Dixon 2020). The on and off, up and down the activity of PAL in developmental processes and in stress responses mirrors its sophisticated regulatory control. As shown in Figure 7, in the cotyledons, the PAL activity significantly increased at 100, 200, and 400 μM cobalt treatments, and the values reached 188.9%, 181.8%, and 158.7% with respect to control, respectively. With the increasing Co levels, the activity showed no significant change. In the hypocotyls, 100 μM Co treatments resulted in an increase of 12% with respect to control. However, Co stress had inhibitory effects on the PAL activity, which decreased by 16.7% and 22.2% at 400 and 800 μM Co treatments, respectively. In the radicles, PAL activity enhanced concomitantly with increasing cobalt levels up to 400 μM, and the peak activity increase was by 47.9% compared to the control. Different types of stress, such as salt and heavy metal ones, cause the enhancement of PAL activity in *J. curcas* and *Lotus japonicus* plants (Gao et al. 2010, Mrázová et al. 2017). Similar induction in PAL activity was recorded in seedlings of soybean, *Brassica napus*, and *Ocimum basilicum* under cobalt stress (Chmielowska-Bąk et al. 2014, Azarakhsh et al. 2015, Ali et al. 2018). As can

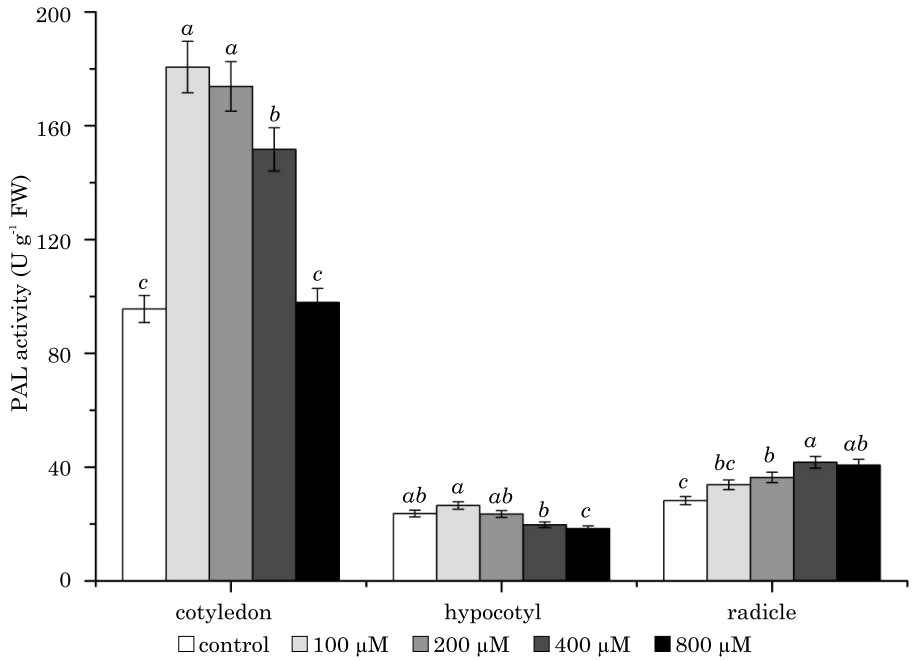

Fig. 7. Effects of cobalt on phenylalanine ammonia-lyase (PAL) activity of the cotyledons, hypocotyls and radicles in *J. curcas* seedlings germinated and grown in MS medium containing 100, 200, 400 and 800 μM Co. Data points and error bars represent means ± SD of three replicates (n=3). Lowercase letters represent significant differences among different treatments, with a significance level of 0.05

be seen from the findings, excessive Co applications can affect PAL activities, which means that the tested Co applications created stress on the plant. In some plant species, the enzymatic expression and induction of PAL have been reported to vary depending on the stress types, organs, and plant species, although the significance of such induction was not clear under heavy metals stress (Barros, Dixon 2020). In the present study, the responses of the PAL activity of *J. curcas* seedlings cultured in different Co concentrations are also known. Increased PAL activity might be a response to the cellular damage provoked by higher Co concentrations. Moreover, there was a significant positive pair-wise correlation among PAL activity, tested Co concentrations and organ types, suggesting that the changes in PAL activity were associated with the cobalt concentrations and plant organs (Barros, Dixon 2020). The present results indicated that increased PAL activity, at least here, appears to be an effective scavenger of ROS under excessive Co stress. These findings suggest that PAL may also be involved in modulating the resistance of *J. curcas* plants exposed to cobalt toxicity and their biological roles are more complex than expected.

CONCLUSIONS

In summary, the present study was designed to investigate the influence of growth and chemical changes based on *in vitro* embryo germination and culture. The present work demonstrated that Co-induced oxidative stress affects growth indices and physiological attributes of cotyledons, hypocotyls, and radicles in *J. curcas* seedlings differently. However, the inhibition was more dominant for hypocotyls and radicles than for cotyledons. Moreover, our results showed that *J. curcas* seedlings cope with high levels of Co *via* the antioxidant system by synthesizing and increasing the activities of critical enzymes, SOD, GPX, CAT, and PAL, which appear to play key roles in the antioxidative defense mechanism under excessive Co levels. The isoenzyme profile analysis of SOD and GPX revealed that Co-induced enhancement of SOD and GPX activity was accompanied by a change in the isoenzyme pattern, and these isoenzymes play major roles in combating oxidative damage. However, the biochemical networks involved in cellular responses to oxidative stress during the processes of *in vitro* embryo germination and culture exposed to Co toxicity are unclear and should be further investigated in field condition.

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