

Nickel toxicity induced antioxidant enzyme and phenylalanine ammonia-lyase activities in *Jatropha curcas* L. cotyledons

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ABSTRACT

Jatropha curcas L. embryos were germinated and grown *in vitro* under nickel concentrations of 100, 200, 400 and 800 μmol to observe the effects of high nickel concentrations on seedling growth. Observed biological makers included biomass, and the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and phenylalanine ammonia-lyase (PAL) in the cotyledons. The fresh weight of cotyledons in all the tested nickel concentrations was lower than that of the control, but dry weight of cotyledons increased with increasing nickel concentrations up to 200 μmol . SOD activity increased significantly up to 400 μmol and then decreased at 800 μmol nickel. POD activities were induced remarkably at 100 and 200 μmol , but the activity decreased with increasing nickel concentrations. Similarly, a negative link between CAT activity and nickel concentrations was observed in this experiment. PAL activity had a positive correlation to nickel concentrations, and the highest activity was found at 400 μmol nickel. Electrophoresis analysis suggested that a significant correlation between nickel concentrations and isoenzyme patterns of SOD and POD was observed, and these results were consistent with the changes of the activity assayed in solutions.

Keywords: heavy metals; ROS-scavenging enzymes; defensive mechanism of plant; *in vitro* embryo culture

Nickel (Ni) is an essential micronutrient for plants since it is the active center of the enzyme urease required for nitrogen metabolism in higher plants. However, excess Ni is known to be toxic and many studies have been conducted concerning Ni toxicity of various plant species. The most common symptoms of nickel toxicity in plants are inhibition of growth, photosynthesis, mineral nutrition, sugar transport and water relations (Seregin and Kozhevnikova 2006). Heavy metal affects plants in two ways. First, it alters reaction rates and influences the kinetic properties of enzymes leading to changes in plant metabolism. Second, excessive heavy metals lead to oxidant stress. During the period of metal treatment, plants develop different resistance mechanisms to avoid or tolerate metal stress, including the changes of lipid composition, the profiles of isozymes and

enzyme activity, sugar or amino acid contents, and the level of soluble proteins and gene expressions. These adaptations entail qualitative and/or quantitative metabolic changes that often provide a competitive advantage, and affect plant survival (Schützendübel and Polle 2002). Therefore, plant cells contain protective and repair systems that, under normal circumstances, minimize the occurrence of oxidative damage.

It is known that excessive heavy metal exposure may increase the generation of reactive oxygen species (ROSs) in plants, and oxidative stress would arise if the balance between ROS generation and removal were broken. Oxidative stress is a part of general stress that arises when an organism experiences different external or internal factors changing its homeostasis. In response, an organism either aims to maintain the previous status by

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activation of corresponding protective mechanisms or goes to a new stable state (Mittler 2002). In several plants, the Ni-induced changes in activity of ROS-scavenging enzymes, including SOD, POD and CAT were detected (Schickler and Caspi 1999, Gajewska and Skłodowska 2007). Therefore, it is imperative to compare the ROS-scavenging enzyme activity among plant genotypes with different Ni tolerance, as it will tell us the intrinsic role of antioxidant enzymes against Ni tolerance.

Jatropha curcas L. is cultivated as a medicinal plant in many tropical and subtropical countries. It is suitable for preventing soil erosion and shifting of sand dunes. Various parts of the plant hold potential for use as a source of oil, animal feed or medicinal preparations. Recently, their seeds were investigated mainly as a potential source of oil that was recognized as an adequate substitute motor fuel (Openshaw 2000). As shown in the previous review of the literature, little information concerning the antioxidant defense responses of *Jatropha curcas* plant to nickel treatment is available. The present study developed an *in vitro* embryo process to understand the antioxidant defense responses in *Jatropha curcas* L. cotyledons subjected to nickel stress, with emphasis on the changes of SOD, POD, CAT and PAL.

MATERIAL AND METHODS

Mature *Jatropha curcas* L. seeds (physic nut or purging nut) were collected in August 2007 from more than 10 individual wild trees in Panzhihua, Sichuan, China. Seeds were oven dried, selected and stored in a plastic box and deposited at 4°C until processing. Methionine and nitro blue tetrazolium (NBT) were purchased from Sigma (St. Louis, MO, USA). Other reagents used were of reagent grade.

Jatropha curcas L. seeds were subjected to 70% ethanol for 30 s, and then to 0.1% mercuric chloride for 8 min. The seeds were rinsed several times with sterile distilled water and soaked in sterile distilled water for 24–36 h at room temperature. Thus, each embryo was dissected from the seeds in clean bench. Embryos were separated into five lots. One lot was allowed to culture with semi-solid Murashige and Skoog (MS) medium containing 30 g/l sucrose, 6 g/l agar powder as control. The remaining four lots were exposed to four concentrations of nickel: 100, 200, 400 and 800 µmol, respectively. The pH of all the media was adjusted to 5.8 ± 0.1 prior to autoclaving

sterilization at $121 \pm 2^\circ\text{C}$ for 15 min. Nickel was supplied as NiCl_2 . Culture experiment was carried out at 30°C greenhouse. The treatments were superimposed on medium for 7 days of culture. When two cotyledons developed, they were selected and washed with distilled water. The experiments were arranged in a completely randomized design with three replicates and each replicate contained 15 embryos. Plant tissues were oven-dried at 80°C followed by the estimation of dry weights.

Fresh cotyledons were homogenized using a chilled pestle and mortar under liquid nitrogen, and then were extracted in 50 mmol sodium phosphate buffer (pH 7.0) including 1 mmol EDTA and 150 mmol NaCl. The crude extract was centrifuged at 12 000 rpm for 5 min and the supernatant was used to determine protein content and antioxidant enzyme activity. Protein content was measured by the Lowry method.

SOD assay was performed according to the Chen and Pan method (Chen and Pan 1996). The enzyme volume corresponding to 50% inhibition of the reaction (one unit) was calculated. POD activity was determined by measuring the increase in absorbance at 470 nm (Sakharov and Aridilla 1999). One unit of enzyme activity was defined as the amount of enzyme producing 1 absorbance change at 470 nm per min in the above assay conditions. CAT activity was measured following the Philippe method (Montavon et al. 2007). One unit of CAT is defined as the amount causing the decomposition of 1 µmol of H_2O_2 per min. SOD, POD and CAT activities were expressed in unit (U) per gram of fresh weight.

For PAL assay, crude enzyme extraction was performed according to an earlier report (Gao et al. 2008). PAL activity was determined by monitoring the reaction product *trans*-cinnamate at 290 nm (Hahlbrock and Ragg 1975). The reaction mixture contained 50 mmol Tris-HCl, pH 8.8, 20 mmol L-phenylalanine, and enzyme in a total volume of 3 ml. The reaction was allowed to proceed for 30 min at 30°C and was stopped by the addition of 0.5 ml of 10% trichloroacetic acid (TCA). One unit of enzyme activity was defined as the amount of enzyme that increased the absorbance by 0.01 per min under assay conditions.

PAGE for SOD and POD, CAT isoenzymes assay was performed with 7% (w/v) polyacrylamide gel. SOD isoenzymes were visualized in gels. The gel was equilibrated with 50 mmol phosphate buffer (pH 7.5) for 1 min and submerged in the same buffer containing 1 mg/ml MTT for 30 min in the absence of light. The gel was washed in

water and then stained in a solution containing 2.8×10^{-5} mol riboflavin, 28 mmol N,N,N,N-tetramethyl ethylenediamine (TEMED) for 30 min until the colorless bands appeared in a purple background. POD isoenzymes were detected by the Ros Barcelo method (Ros Barcelo 1987). The gels were rinsed in water and the gel was stained in a solution containing 0.06% (v/v) H_2O_2 , 0.1% (w/v) benzidine and 0.1% (v/v) acetic acid at room temperature till the brown colour. CAT isoenzymes were measured by the Woodbury method (Woodbury et al. 1971). Gels were incubated in 0.01% H_2O_2 for 10 min and developed in a 2% (m/v) $FeCl_3$ and 2% $K_3Fe(CN)_6$ (m/v) solution for 10 min until the colourless bands appear.

Data shown in this paper are reported as the mean \pm SD. We performed three independent experiments in duplicate for each condition. Statistical significance was considered to be significant when the P value was less than 0.05 using one way ANOVA.

RESULTS AND DISCUSSION

The changes of fresh weight and dry weight in cotyledons exposed to nickel stress are shown in Figure 1. The fresh weight of cotyledons decreased by 3.9, 5.8, 30 and 47.2% at the concentrations of 100, 200, 400 and 800 μ mol Ni compared to the control, respectively. However, the dry weight of cotyledons increased with rising nickel concentrations up to 200 μ mol, the highest value

increasing by 13.2%. It is generally regarded that heavy metals stresses usually lead to decreasing cotyledon ratios of biomass. A similar result was observed in *Trigonella corniculata* L. (Parida et al. 2003) and *Triticum aestivum* L. (Gajewska and Skłodowska 2007). Our findings suggested that different changes in the fresh weight and dry weight of cotyledons are related to the effects of nickel on water content of the seedlings. In the present study, no root formation was observed in nickel concentrations higher than 400 μ mol. From these results it is concluded that nickel has an adverse effect on embryo germination and seedlings development, and that 400 μ mol nickel is the end point for embryo development. Such an observation suggested that the critical toxic threshold of exogenous nickel in MS medium to *Jatropha curcas* L. based on the aggravating oxidative stress is between 400 and 800 μ mol. Therefore, changes in biochemical parameters would occur before any visible symptom of toxicity appears, and the endpoint based on these parameters might be more sensitive or indicative than morphological observations in revealing eco-toxicity of nickel.

The changes of SOD activity in cotyledons exposed to nickel stress are shown in Figure 2. There was an increase in SOD activity with increasing nickel concentrations, and the highest activity increase by 107.8% at 400 μ mol nickel concentration. Induction of SOD activity in plant cells was correlated with development of increased tolerance to a variety of chemical compounds and physical stresses (Mittler 2002). Increased SOD

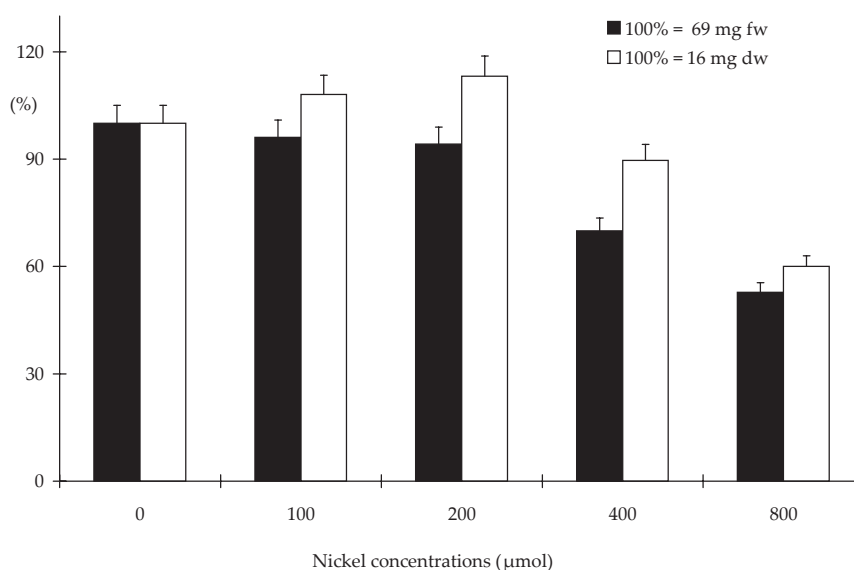


Figure 1. The changes of biomass in *Jatropha curcas* L. cotyledons. Data points and error bars represent means \pm SD of three replicates ($n = 3$)

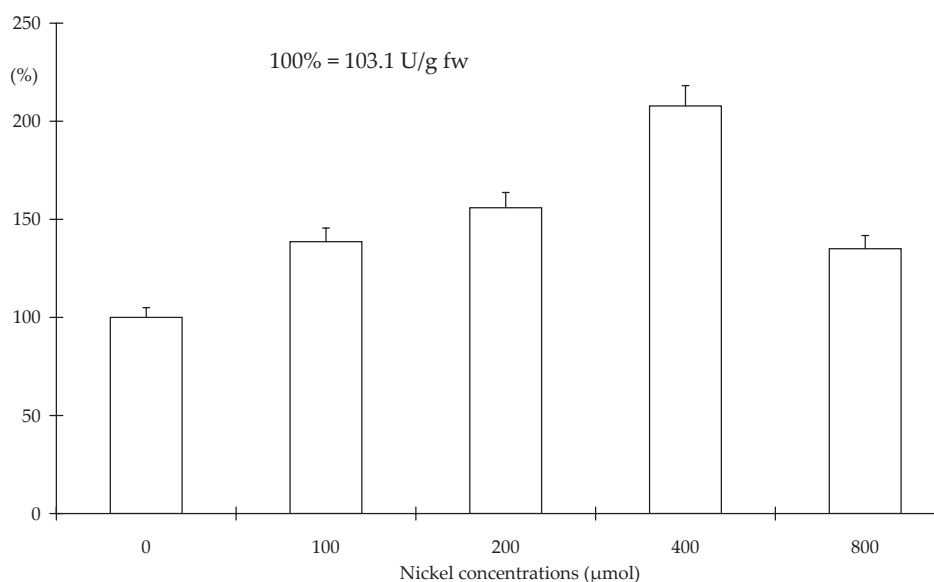


Figure 2. The changes of SOD activity in *Jatropha curcas* L. cotyledons exposed to nickel stress. Data points and error bars represent means \pm SD of three replicates ($n = 3$)

activity as observed in our studies was either due to increased production of reactive oxygen species or could be a protective measure adopted by *Jatropha curcas* L. plants against oxidative damage. Electrophoresis analysis suggested that three bands of SOD isoforms were observed under different nickel concentrations (Figure 3). These isoenzymes are induced in response to different nickel concentrations; however, the enzymes required for eliciting the response and the level of induction differ under different nickel stress. Increased activity of SOD isozymes in different organelles was suggested to be due to increased requirement of each organelle to combat the stressful conditions (Schützendübel and Polle 2002). These results can be used as a basis for elucidating the mechanisms

by which such compartment-specific regulation is controlled. In addition, the staining intensities of SOD isoform bands are correlated with the activity of quantitative changes (Figure 2). Our findings indicated that increased SOD activity might be involved in the tolerance mechanism of *Jatropha curcas* against nickel stresses.

POD, along with SOD and CAT, are based on redox metalloenzymes involved in cell defense against oxidative stress. POD can be also considered useful markers for environmental stresses since their activity is affected by low temperature, air pollution, ozone, heavy metals, wounding, salts, pathogen attack and UV radiation (Passardi et al. 2005). The changes of POD activity in cotyledons exposed to nickel stress are shown in Figure 4. POD activities increase significantly up to 200 μmol nickel compared to the control, and the highest value represents a 209% increment. A similar change is observed in CAT activities (Figure 6). Induction of POD activity after Ni treatment of plants was reported previously (Gomes-Junior et al. 2006). Enhancement of POD activity under metal stress was explained by its role in building up physical barrier against toxic metals entering the cell as well as in scavenging H_2O_2 (Passardi et al. 2005). Therefore, these PODs serve as a parameter of metabolism activity against nickel toxicity. Pattern of soluble POD isoenzymes from the cotyledons extract was obtained by non-denaturing PAGE (Figure 5). As shown in Figure 5, at least five POD isoforms were detected, but different patterns were found. The staining intensities of three isoform

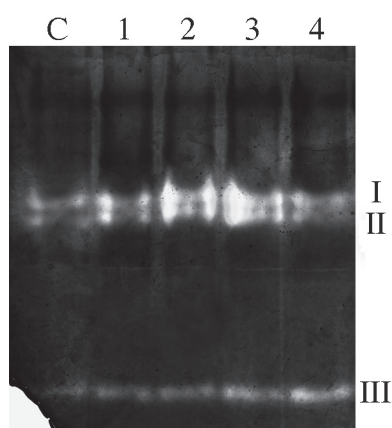


Figure 3. Patterns of SOD isoenzymes in *Jatropha curcas* L. cotyledons. Lanes from left to right were 0, 100, 200, 400 and 800 μmol

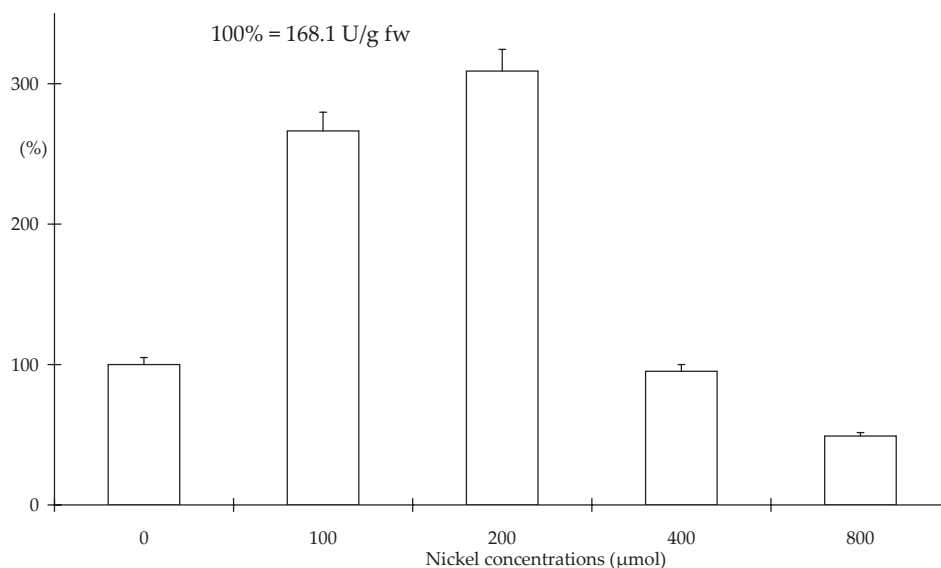


Figure 4. The changes of POD activity in *Jatropha curcas* L. cotyledons exposed to nickel stress. Data points and error bars represent means \pm SD of three replicates ($n = 3$)

bands (III, IV and V) are stimulated with increasing Ni concentrations up to 400 μmol and then decrease. POD are commonly found as several isoenzymes in plants because of its multiple functions. The pattern of expression of isoform varies in the different tissues of healthy plants and is regulated at different times and places by various kinds of biotic and abiotic stressors (Passardi et al. 2005). Therefore, our results suggest that in *Jatropha curcas* L. cotyledons biomass reduction might be related to the increased POD and CAT activity.

The changes of CAT activity in cotyledons under different nickel concentrations were shown in Figure 6. A similar tendency was found as in the case of POD activities; the peak activity increased by 93.2% compared to the control. Accumulating evidence indicates that CAT plays an important role in the protection against oxidative damage by breaking down hydrogen peroxide (Mittler 2002). Changes in CAT activities occur in a developmental and tissue specific manner, and differential regulation in response to nickel stress was reported (Schickler and Caspi 1999, Gajewska and Skłodowska 2007). Patterns of CAT isoenzymes suggest that only one band is measured in *Jatropha curcas* L. cotyledons extract, and the intensities of CAT isoform were similar to the quantitative result in assay solutions (pattern not shown). Many plants encode CAT as multigene families, which may reflect the multiple and diverse roles of these enzymes. The differential expression of these isozymes in response to different

tissues, developmental phase and environmental conditions were studied in detail (Willekens et al. 1995). Our findings indicate that increased CAT activities at lower nickel concentrations are involved in the defensive mechanisms of seedling against nickel stress.

The changes of PAL activities in cotyledons exposed to nickel stress are shown in Figure 7. PAL activities continuously increased with increasing nickel treatments up to 400 μmol , which caused 49.1% increment in comparison to the controls. PAL plays a key role in linking primary metabolism to phenylpropanoid metabolism by converting L-phenylalanine to *trans*-cinnamic acid. This reac-

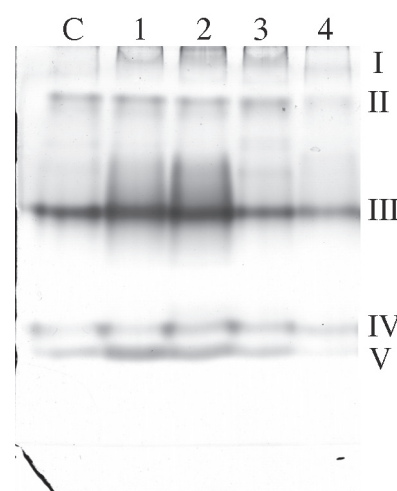


Figure 5. Patterns of POD isoenzymes in *Jatropha curcas* L. cotyledons. Lanes from left to right were 0, 100, 200, 400 and 800 μmol

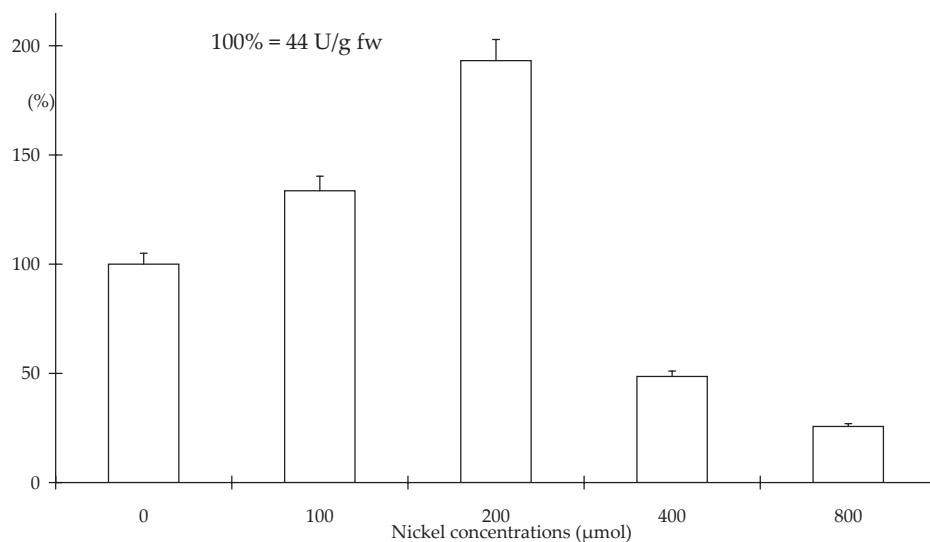


Figure 6. The changes of CAT activity in *Jatropa curcas* L. cotyledons exposed to nickel stress. Data points and error bars represent means \pm SD of three replicates ($n = 3$)

tion provides an entry point for the biosynthesis of a large number of defense related functions products, and PAL is considered a part of defense mechanism. The regulation of PAL activity in plants is made more complex in many species due to the existence of multiple PAL-encoding genes, some of which are expressed only in specific tissues or only under certain environmental conditions (MacDonald and D'Cunda 2007). The enhancement of PAL activity in this study may be related to the implication of this enzyme in the plant response to nickel stress, especially at the concentration of 400 µmol Ni. It was shown

that PAL is generally stimulated in plant tissues exposed to heavy metals stresses (Santiago et al. 2002). Thus, these findings suggest that PAL plays an essential role in modulating the resistance of plant tissues against nickel toxicity.

Our results enabled us to show a correlation between responses of antioxidant enzymes as well as PAL activities and nickel concentrations in *Jatropa curcas* L. cotyledons. The lower nickel concentrations and higher SOD, POD, CAT and PAL activities suggest the tolerance capacity to protect the plant from oxidative damage. Our findings clearly support the hypothesis that there

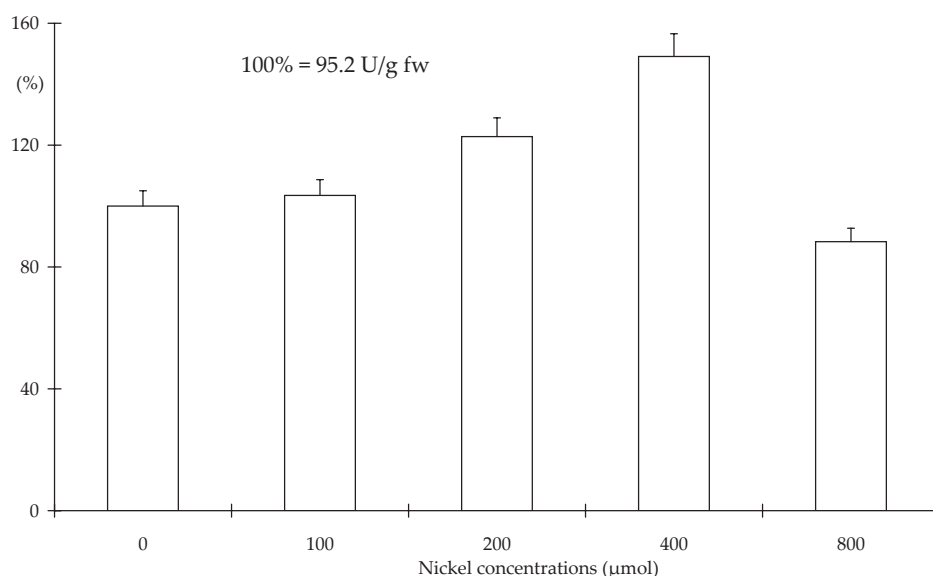


Figure 7. The changes of PAL activity in *Jatropa curcas* L. cotyledons exposed to nickel stress. Data points and error bars represent means \pm SD of three replicates ($n = 3$)

is an imbalance between ROS-generating and scavenging enzymes under nickel stress; SOD, POD, CAT and PAL seem to play a prime role in regulation of ROS level upon excessive nickel. Further investigation on a cellular or molecular level is necessary to understand the mechanism that causes these positive effects at low concentrations of nickel, as well as the changes in free radicals induced by nickel stress.

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