EFFECT OF Cr AND Pb ON THE ACTIVITY OF ANTIOXIDANT ENZYMES IN A CELL SUSPENSION CULTURE OF *Jatropha curcas*

EFECTO DEL Cr Y Pb EN LA ACTIVIDAD DE ENZIMAS ANTIOXIDANTES DE UN CULTIVO DE CÉLULAS EN SUSPENSIÓN DE *Jatropha curcas*

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Abstract

Jatropha curcas is a tolerant and accumulator plant of heavy metals (HMs). Little is known about the mechanisms behind this ability. It is suggested that antioxidant enzymes might participate; however, there are no studies reporting the relationship between the activities of antioxidant enzymes and the presence of HMs in an *in vitro* cell suspension culture of *J. curcas*. The aim of this study was to determine the effect of chromium (Cr) or lead (Pb) at 0.0 to 3.0 mM on the activity of three antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) through the growth of cell suspension cultures (CSC) of *J. curcas*. The activity displayed by those enzymes was statistically significant ($P \leq 0.05$) when Cr or Pb was used. The greatest enzymatic activity was noted at the first hour of culture for SOD and at five h for POX and CAT. After 192 h, the activity of these three enzymes decreased, which coincided with the exponential growth phase of the cell culture. The results indicated that there is a close relationship between the presence of Cr and Pb and SOD, CAT, and POX activities in a cell suspension culture of *J. curcas*, which can explain the plant's capability for tolerating and accumulating high concentrations of Cr and Pb.

Keywords: *Jatropha curcas*; cell cultures; heavy metal; chromium; lead; enzymatic activity.

Resumen

Jatropha curcas es una especie tolerante y acumuladora de metales pesados (HMs), pero poco se sabe sobre el mecanismo que le confiere esta habilidad. Se sugiere que las enzimas antioxidantes pueden ser partícipes; pero no hay reportes relacionando la actividad enzimática y HMs en cultivos de células en suspensión (CSC) de *J. curcas*. Se determinó el efecto del cromo (Cr) o plomo (Pb) (0.0 a 3.0 mM) sobre la actividad de las enzimas antioxidantes: superóxido dismutasa (SOD), catalasa (CAT) y peroxidasas (POX) en diferentes tiempos de crecimiento de CSC de *J. curcas*. Hubo diferencias significativas ($P \leq 0.05$) en la actividad de SOD, POD y CAT por la adición de Cr o Pb. La mayor actividad de SOD ocurrió durante la primera hora, y en POX o CAT se observó a las 5 h. Después de las 192 h, la actividad de todas las enzimas disminuyó, lo cual coincidió en el mayor crecimiento del cultivo celular Hubo una relación cercana entre el Cr o Pb y la actividad de SOD, POD y CAT, que podría relacionarse con la capacidad de *J. curcas* para acumular y tolerar altas concentraciones de Cr o Pb.

Palabras clave: *Jatropha curcas*; cultivo de células; metales pesados; cromo; plomo; actividad enzimática.

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1 Introduction

Plants can take up non-essential elements for nutrition such as heavy metals (HMs) like chromium (Cr) and lead (Pb), which are toxic to metabolic processes (Maksymiec, 1997; Siedlecka *et al.*, 2002). One of the main consequences associated with the incorporation of HMs is their toxicity; they promote an increase in the production and accumulation of superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl (OH) and singlet oxygen ('O_2) radicals, collectively known as reactive oxygen species (ROS). These ROS may lead to an unspecific oxidation of cell membranes, proteins, nucleic acids, and chloroplast pigments (Jain *et al.*, 2010; Weckx *et al.*, 1997; Tewari *et al.*, 2002).

The accumulation of ROS is caused by a disequilibrium in the balance between ROS production and the capacity of the antioxidant system to inactivate them, causing cellular oxidative stress and inducing cell death. A plant's antioxidant system comprises non-enzymatic and enzymatic processes. The former is associated with glutathione, carotenoids, and ascorbate synthesis, while the latter is associated with superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) activities (Noctor and Foyer., 1998; Srivastava *et al.*, 2004), which are induced by the exposure of high HMs concentrations (Lukatin *et al.*, 2104). SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2 and is crucial in plants to regulate ROS concentrations through its higher activity, which is related to *de novo* synthesis (Dazy *et al.*, 2009). Peroxidases, such as CAT and POX, catalyze the decomposition of H_2O_2, although their products and locations are different. CAT produces H_2O and O_2 and is found in the mitochondria and peroxisomes, while POX generates the oxidation of phenolic compounds and/or antioxidant compounds in the cytoplasm, vacuoles, membrane, and cell wall (Blokhina *et al.*, 2003). An important tool for understanding physiological and biochemical mechanisms in response to cellular oxidative stress is the plant cell and tissue culture (Errabii *et al.*, 2007).

Jatropha curcas is a species widely distributed in tropical areas of Mexico and other places worldwide. This plant was found to be able to decrease Zn, Pb, Cr, Cd, and Cu concentrations in sewage sludge containing those HMs. Zn content of the sewage sludge was 366.23 mg kg^{-1} before planting and 117.97 mg kg^{-1} after harvesting; Pb after harvesting measured at 1.12 mg kg^{-1} while the initial level was 5.18 mg kg^{-1}; Cr after harvesting measured at 7.69 mg kg^{-1} while the initial level was 33.86 mg kg^{-1}; Cu after harvesting measured at 4.13 mg kg^{-1} while the initial level was 19.18 mg kg^{-1}; Cd content was initially 0.16 mg kg^{-1} and after harvesting measured at 0.04 mg kg^{-1}.

The quantification of HMs in the whole plant showed the highest accumulation of Zn (29.5 mg kg^{-1}), Cu (0.44 mg kg^{-1}), and Cd (8.35 mg kg^{-1}) in the roots, while Pb (4.63 mg kg^{-1}) and Cr (0.33 mg kg^{-1}) contents were higher in the aerial parts, suggesting that *J. curcas* might be a suitable plant for phytoremediation (Ahmadpour *et al.*, 2010). In fact, the highest biomass and growth performance of *J. curcas*, in terms of height, basal diameter, and number of leaves was found in 40% contaminated sawdust in combination with 60% soil. On the other hand, an increase in biomass production (from 10.4 to 15.1 g L^{-1}) of cell suspension cultures from *J. curcas* was displayed by adding Pb to the culture medium. Moreover, when adding Cr and Pb (3 mM), this species displayed the capability for tolerance and accumulation of significant amounts of these HMs, accounting for 6.37 mg kg^{-1} for Cr and 8.61 mg kg^{-1} for Pb respectively (Bernabé-Antonio *et al.*, 2015). Despite the convenience for using *J. curcas* as a Cr and Pb accumulator when the whole plant or a cell suspension culture is used, the mechanisms involved are still not well understood or described.

Thus, the aim of this work was to investigate the effect of different concentrations of Cr and Pb on the enzyme antioxidant system in a cell suspension culture of *J. curcas* as an approach to understanding the antioxidant defense mechanisms behind the plant's detoxification strategy in response to heavy metals.

2 Materials and methods

2.1 *Cr and Pb bioassays*

For conducting this work, a cell suspension culture of *Jatropha curcas* established by Bernabé-Antonio *et al.* (2015) was provided by the Universidad Autonoma Metropolitana Campus Iztapalapa (UAM-I). Fresh weight biomasses (2 g each) were inoculated in 125 mL Erlenmeyer flasks containing 25 mL of the culture medium described by Murashigue and Skoogs (1962). Sucrose (3% w/v) and 2,4-dichlorophenoxyacetic acid (2,4-D; 2.27 μM) were added to the culture medium. $K_2Cr_2O_7$ or $Pb(NO_3)_2$ salts (source: Baker Analyzed, 108 Phillipsburg, NJ) were added to the culture medium as a source of Cr and Pb, respectively, using

concentrations of 0.0, 0.5, 1.0, 2.0, and 3.0 mM, according to Bernabé-Antonio *et al.* (2015). Cultures were incubated for 15 days on an orbital shaker at 110 rpm and 25±2 °C, and maintained at a 16 h photoperiod with white fluorescent light 60 μmol m^2 s^{-1}.

2.2 Extraction of enzymes

Cell culture samples (1 mL) were collected in aseptic conditions at 1, 5, 10, 24, 192, and 360 h after the beginning of incubation. The maximum sampling time (360 h) corresponded with the end of the exponential growth phase, established according to growth kinetics (Fig. 1) of a *J. curcas* cell suspension culture (CSC) as reported by Bernabé-Antonio *et al.* (2015). The cell samples were transferred into 2 mL vials and immediately frozen in liquid nitrogen. To obtain the crude enzyme extracts (CEE), the cells were filtered and then rinsed with EDTA (10 mM) to remove extracellular Cr or Pb. Subsequently 100 mg of cells (fresh weight) were re-suspended in a phosphate buffer (100 mM, pH 6.8), sonicated for 15 min, and then centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant containing the CEE was used to determine the SOD, POX, and CAT activity.

2.3 Determination of enzymatic activities

2.3.1 Superoxide dismutase (SOD)

Each SOD assay was conducted by the method of nitroblue tetrazolium (NBT) described by Fryer *et al.* (1998). The purple color developed was measured at 560 nm in a spectrophotometer (CECIL 3000 series). The assay buffer (100 mM K$_2$HPO$_4$) without SOD was used as a blank. An inhibition curve at 560 nm contrary to an increasing volume of the sample was performed.
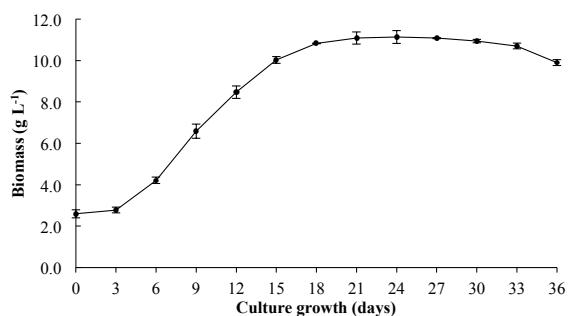

Fig. 1. Growth kinetics of a cell suspension cultures

of *J. curcas* during 36 days on MS medium with 2.27 mM 2,4-D. (Taken of Bernabé-Antonio *et al.*, 2015). One unit of SOD was defined as that contained in the extract volume that caused 50% inhibition of the SOD inhibitable fraction of the NBT reduction (Beyer and Fridovich 1987). Each unit of enzymatic activity was expressed as SOD units per gram of fresh biomass (USOD g^{-1}).

2.3.2 Peroxidase (POX)

POX activity was determined according to Kar and Mishra (1976). The amount of purpurogallin was determined at 420 nm in a spectrophotometer (CECIL 3000 series). One unit of peroxidase was defined as the amount of enzyme causing an increase of 0.1 absorbance units at 420 nm. Each unit of enzymatic activity was expressed as POX units per gram of fresh biomass (UPOX g^{-1}).

2.3.3 Catalase (CAT)

Catalase activity was determined according to Aebi (1984). Catalase activity was estimated by the decrease in absorbance of H$_2$O$_2$ at 240 nm in a spectrophotometer (CECIL 3000 series). One unit of catalase was defined as the amount of enzyme that breaks 1 μmol of H$_2$O$_2$ per minute at room temperature and pH 7. Each unit of enzymatic activity was expressed as CAT units per gram of fresh biomass (UCAT g^{-1}).

2.4 Statistical analysis

The obtained data of the enzymatic units from HMs (Cr and Pb) treatments were subjected to variance analysis (ANOVA) followed by a Tukey's multiple range test ($P \leq 0.05$). SAS 9.0 software (SAS Institute Inc., 2002) was used for statistical analysis. Each treatment consisted of three flasks and three samples per flask were obtained. The experiments were repeated twice.

3 Results

3.1 Effect of Cr and Pb on SOD activity

In this work, the concentration and type of HMs (Cr or Pb) affected significantly ($P \leq 0.05$) the catalytic activity of SOD, CAT, and POX enzymes in a cell suspension culture of *J. curcas*. In the control treatment (HMs-free), SOD activity did not present
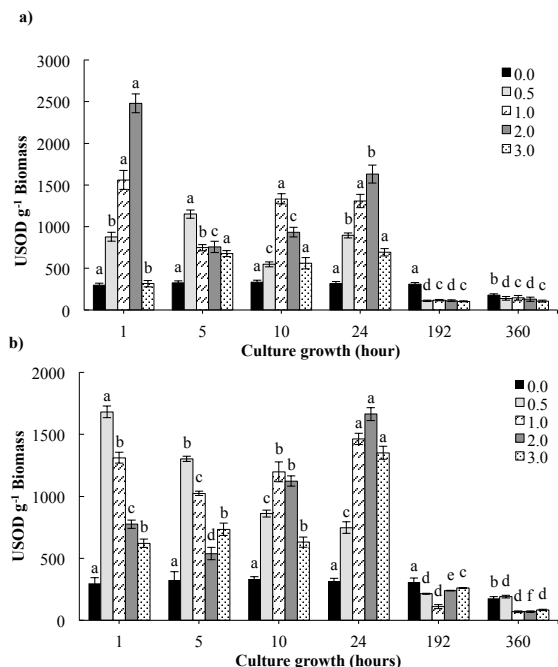
a)

b)

Fig. 2. Effect of different concentration (mM) of Cr (a) and Pb (b) in SOD activity during 360 hour in a cell suspension culture of *J. curcas*. Error bars represent standard deviation, columns with the same treatment at different times followed by the same letter are not statistically different ($P \leq 0.05$).
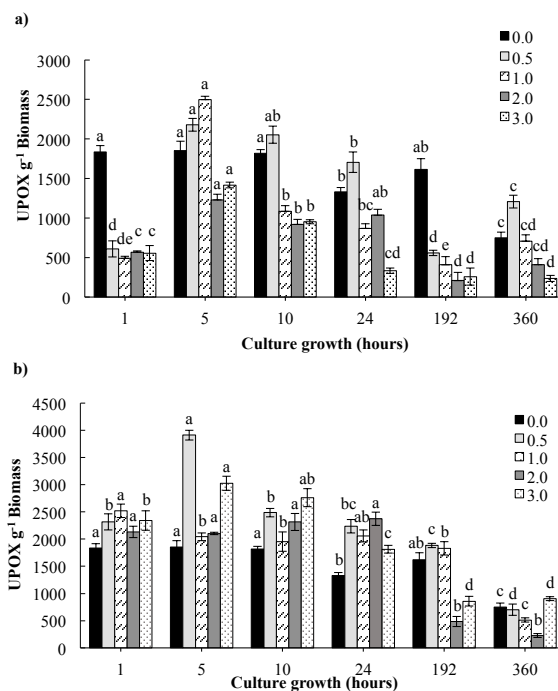
a)

b)

Fig. 3. Effect of different concentration (mM) of Cr (a) and Pb (b) in POX activity during 360 hour in a cell suspension culture of *J. curcas*. Error bars represent standard deviation, columns with the same treatment at different times followed by the same letter are not statistically different ($P \leq 0.05$).

significant changes from 1 to 192 h (297.4 - 333.5 USOD g^{-1}), but at the end of the exponential growth stage (360 h) (Fig. 2a, 2b) a decrease was observed (178.7 USOD g^{-1}). Cr or Pb treatments (0.5 - 3.0 mM) significantly increased the activity of SOD from 1 to 24 h in comparison to the control sample. In contrast, an opposite effect occurred at 192 and 360 h, respectively (Fig. 2a, 2b). In fact, the activity of SOD 1 h after the addition of the HM was increased when Cr concentrations from 0.5 to 2.0 mM were used. An opposite effect was observed when Pb was used from 0.5 to 3.0 mM. Moreover, the highest SOD activity observed for both HMs was assessed at 1 h, corresponding to 2.0 mM of Cr (2479.39 USOD g^{-1}) (Fig. 2a) and 0.5 mM of Pb (1681.0 USOD g^{-1}); a concentration of 2.0 mM of Pb at 24 h also showed high SOD activity (1663.8 USOD g^{-1}) (Fig. 2b). These results indicate that a cell suspension of *J. curcas* displays the ability to respond efficiently in a relatively short time (from 1 to 24 h) to scavenge the superoxide anion radicals produced after the addition of Cr or Pb by increasing SOD activity.

3.2 Effect of Cr and Pb on POX activity

POX induction is a general response of higher plants to abiotic factors and this activity is correlated to elevated concentrations of HMs such as Zn, Cd, Cu, Ni, and Pb (Assche and Clijsters, 1990). In this work, significant differences ($P \leq 0.05$) were found in POX activity in the cell suspension culture of *J. curcas*. In cultures treated without HMs (Fig. 3a, 3b) the POX activity was almost constant during the first 192 h of culture (1617.0 - 1852.5 UPOX g^{-1}), and decreased significantly at 360 h. Cr and Pb caused significantly different patterns in the POX activity in comparison with the control sample. In general, 0.5 mM of Cr from 5 to 24 h and at 360 h showed higher POX activity compared to the control sample, while the other Cr concentrations showed lowering POX activity (Fig. 3a). The highest POX activity was observed with Cr 1.0 mM at 5 h (2497.0 UPOX g^{-1}); meanwhile all tested concentrations of Pb induced an increase of POX activity from 1 to 24 h (Fig. 3b), and the highest POX activity (3911.7 UPOX g^{-1}) was obtained
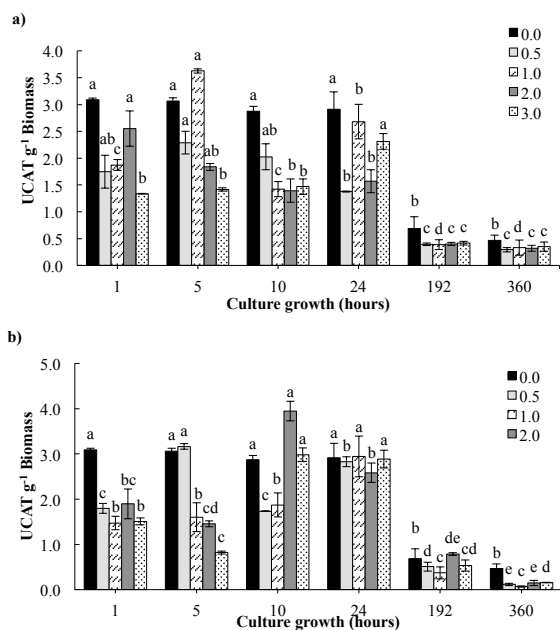
Fig. 4. Effect of different concentration (mM) of Cr (a) and Pb (b) in CAT activity during 360 hour in a cell suspension culture of *J. curcas*. Error bars represent standard deviation, columns with the same treatment at different times followed by the same letter are not statistically different ($P \leq 0.05$).

at 0.5 mM and 5 h (Fig. 3b). The highest activity of POX from a cell suspension culture of *J. curcas* was determined at 5 h for both HMs. This indicates that there is a relationship with SOD activity which generates H_2O_2, the substrate of POX at 1 h of culture, and consequently leads to greater activity of POX at 5 h.

3.3 Effect of Cr and Pb on CAT activity

In the same way to the other enzymes, statistically significant differences ($P \leq 0.05$) were found in CAT activity as a result of the Cr or Pb heavy metals. In both cases, CAT activity in cell suspension cultures of *J. curcas* was lower than that displayed by the control treatment (Fig. 4a, 4b). CAT activity was from 0.8 - 3.63 UCAT g^{-1} during the first 24 h, and decreased significantly at 192 and 360 h. The highest CAT activity induced by Cr was with 1.0 mM at 5 h (3.63 UCAT g^{-1}) (Fig. 4a); the highest CAT activity induced by Pb was with 2.0 mM until 10 h (3.95 UCAT g^{-1}) (Fig. 4b). These results suggest that the CAT enzyme in a cell suspension culture of *J. curcas* had low effectiveness in detoxifying H_2O_2 due to high amounts of Cr or Pb. This behavior could be due to the structure of the enzyme that contains a heme
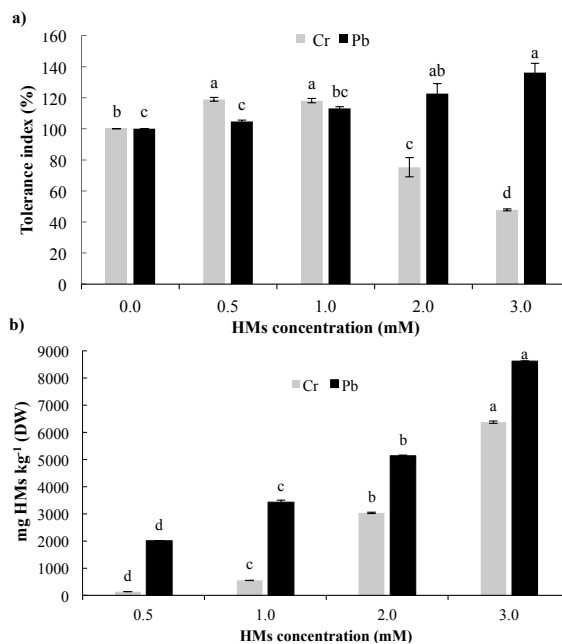

Fig. 5. Tolerance index (a) and bioaccumulation of HMs (b) of *J. curcas* cell suspension culture treated with Cr and Pb. Taken of Bernabé-Antonio *et al*. (2015).

group where Fe is a key element in the transference of electrons. The absorption of non-essential metals in plant nutrition occurs due to competition of nutrient transport proteins with analogous metals that are essential.

4 Discussion

Metal tolerance in plant cells is related to an increased catalytic activity of antioxidant enzymes (Srivastava *et al*., 2004; Szollosi, 2014). The enhanced activity of SOD, POX, and CAT enzymes in cells of *J. curcas* under stress from Cr and Pb was related to higher levels of ROS. This activity contributes to the plant's toleration and accumulation of high concentrations of HMs. For instance, Bernabé-Antonio *et al*. (2015) reported a higher tolerance index in the cells of *J. curcas* treated with Cr (0.5 and 1.0 mM), but with higher concentrations of Cr (2.0 and 3.0 mM) it was significantly decreased (Fig. 5a). However, using 0.5 and 1.0 mM of Pb the tolerance index was not statistically different compared to the control, and it was higher with 2.0 and 3.0 mM of Pb (Fig 5a). In addition, the accumulation of both Pb and Cr in *J. curcas* cells was increased as the concentrations of HMs were increased (Fig. 5b).

These results indicated that Cr was more toxic than Pb, which could be associated with the differences observed between the activities of the antioxidant enzymes, in which SOD and POX were crucially important (Fig. 2-4). If the concentration of HMs is associated with the generation of ROS, the fact that higher SOD activity is observed in the presence of Cr rather than Pb indicates that higher levels of superoxide anion radicals are produced in the presence of Cr. Thus, SOD could be used as a biomarker of environmental stress (Dazy *et al*., 2009; Lozano *et al*., 1996). Moreover, the activity of other antioxidant enzymes such as glutathione S-transferase could also be associated with the toleration and accumulation of high levels of Cr and Pb in *J. curcas* (Mani and Kumar, 2014; Yadav *et al*., 2009).

In other HMs tolerant species such as *Atriplex hortensis* and *A. rosea*, the leaves from plants grown in polluted soil in the presence of Pb, Ni, Cu, and Zn (1333.5, 1673.7, 501, and 3587.9 ppm, respectively) showed a lower SOD activity (0.06 USOD g^{-1}) compared with *J. curcas* cells (Kachout *et al*., 2009). Also, root cells of *Pisum sativum* treated with 1 mM $Pb(NO_3)_2$ had an increased activity of SOD and CAT which was reported to contribute to the accumulation of excessive amounts of Pb (Malecka *et al*., 2001). From these types of studies, the catalytic activity of antioxidant enzymes seems to be potentiated for HMs tolerant plants as a response to oxidative cell stress (Srivastava *et al*., 2004; Szollosi, 2014).

On the other hand, there are reports indicating that Pb, a non-essential element, is an analogue for Fe and Zn nutrients (Maldonado-Magaña *et al*., 2013; Peer *et al*., 2005). Therefore, high concentrations of Pb could be transported within the *J. curcas* cells, limiting the transport of Fe and consequently decreasing the activity of CAT. A reduction of the activity of CAT in *in vitro* cultures of *Prunus cerasifera* and *Borage officinalis* was observed in the presence of Fe (Lombardi *et al*., 2003; Mohamed and Aly, 2004). The lower Cu accumulation in the leaves of *Zea mays*, and higher antioxidant enzyme (SOD, POX, ascorbate peroxidase, and glutathione reductase) activities in this cultivar suggested an enhanced tolerance capacity of this cultivar to protect the plant from oxidative damage (Tanyolac *et al*., 2007). In addition, CAT plays a key role in preventing cellular oxidative damage by degradation of H_2O_2 to H_2O and O_2 (McKersie and Leshem, 1994). The increase in CAT activity can be crucial for plant survival under moderate stress caused by HMs (Youssef and Azooz, 2013). Nonetheless, other non-enzymatic molecules

as phenolic compounds may play an important role in antioxidant activity (Sánchez-Rangel *et al*., 2014).

Various studies evaluating the effects of some HMs on *in vitro* plantlets and cell suspension cultures have been investigated from the point of view of environmental biology and toxicology, but no attempts have been made to elucidate the activity of antioxidant enzymes. For instance, plantlets of *Prosopis laevigata* had the capacity to uptake Cd, Cr, Pb, and Ni, but their ability to accumulate these heavy metals was different; Cd and Cr were more toxic (Buendía-González *et al*., 2012). Additionally, cell suspension cultures of *P. laevigata* showed significant tolerance and accumulation of Pb under different concentrations of this HM (Maldonado-Magaña *et al*., 2013), although cells accumulate lower amounts than seedlings. A study carried out by Lizhong and Cullen (1995) in a cell suspension culture of *Catharanthus roseus* reported that the toxicity of HMs (Hg, Cu, Cd, Pb, Zn, and Cr) was directly proportional to the concentration of HMs in the culture media.

The present work establishes an approach of the role of SOD, POX, and CAT antioxidant enzymes during the growth of *J. curcas* cells in which the time of 192 h and 360 h corresponded to the middle and the end of the exponential stage, respectively (Bernabé-Antonio *et al*., 2015). The greatest activities for SOD and POX were seen from 1 to 192 h, while for CAT were seen from 1 to 24 h. This pattern indicates a higher function of SOD and POX during the exponential stage, where possibly high quantities of ROS were generated due to the high metabolic activity required to achieve cell growth (Klavina and Ievinsh, 2008); CAT could play an essential role in the *lag* phase of the culture. SOD activity was different through time in comparison to the growth of *Cardiospermum halicacabum* L. (Jahan *et al*., 2014). A study carried out by Klavina *et al*. (2008) using plant tissue cultures of *Sorbus* cultivars reported an increase in the activity of POX during the early hours of growth. The decrease in activity occurred during the exponential growth phase of the culture. Greater activity of CAT was also found in *in vitro* shoot cultures and acclimatized plants of *C. halicacabum* L. (Jahan *et al*., 2014).

The presence of enzymes that catalyze the degradation of H_2O_2 in *J. curcas* cells and follow different mechanisms of action or have different cell locations provides *J. curcas* cells an advantage for managing ROS in order to avoid their accumulation. The activity of CAT is reported to be increased in some species treated with HMs (Hassan and Mansoor,

2014), while the activity of POX has been reported to have an essential function to reduce stress caused by HMs (Tanyolac *et al*., 2007). ROS can suppress the activity of enzymes belonging to the antioxidant enzymatic system, usually through the depletion of glutathione and binding with sulfhydryl groups of enzymes (Juknys *et al*., 2012).

Conclusion

The activity of SOD, POX, and CAT enzymes from the antioxidant system of a cell suspension culture of *J. curcas* was significantly affected by Cr and Pb treatments. High SOD (538.4 - 2479.4 USOD g^{-1}) and POX (2042.7 - 3911.7 UPOX g^{-1}) activity was displayed from 1 to 24 hours under Cr or Pb (0.5 to 3.0 mM) treatments. The highest activity of SOD was found at 1 h of culture while in POX this behavior occurred at 5 h for both HMs (2 mM or 1 mM Cr for SOD and POX, respectively, and 0.5 mM Pb for both enzymes). It is likely that the SOD and POX enzymes are a key to the elimination of ROS induced by those HMs in cultures of *J. curcas*, allowing the cells to tolerate and accumulate Cr or Pb. This is the first study reporting the effect of HMs on the activity of SOD, POX, and CAT in a cell suspension culture of *J. curcas*. Further studies of secondary metabolism are required to evaluate other antioxidative enzymatic activities during all culture growth periods.

Acknowledgements

Notation

CAT	catalase
CEE	crude enzyme extracts
CSC	cell suspension culture
HMs	heavy metals
POX	peroxidase
ROS	reactive oxygen species
SOD	superoxide dismutase

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