Assessment of antioxidant enzymes in leaves and roots of *Phaseolus vulgaris* plants under cadmium stress

Evaluación de enzimas antioxidantes en hojas y raíces de plantas *Phaseolus vulgaris* bajo estrés de cadmio

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**ABSTRACT**

The aim of this work was to evaluate the response of *Phaseolus vulgaris* plants to oxidative stress by cadmium in leaves and roots at different concentrations (0 (control), 0.25, 0.50 and 1 μM). We assessed oxidative stress by the contents of hydrogen peroxide (H$_2$O$_2$) and malondialdehyde (MDA), as well as protein content. Likewise, we determined the antioxidant enzymatic activity of the superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) enzymes. The results demonstrated a decrease in protein content of roots and leaves, starting with the addition of 0.25 μM Cd, but the MDA content and H$_2$O$_2$ levels increased with the addition of 0.25, 0.50 and 1 μM Cd, this due to the formation of reactive oxygen species. The SOD, APX and GPX enzymatic activity increased in roots treated with 0.25 μM Cd, but these enzymes decreased to higher concentrations (0.50 and 1 μM). On the other hand, the activity of CAT increased in leaves exposed to 0.5 and 1 μM of Cd. These results indicate that these antioxidant enzymes can act simultaneously in the elimination of reactive oxygen species.

**Keywords:** cadmium, oxidative stress, antioxidant enzymes, *Phaseolus vulgaris*

**INTRODUCTION**

Cadmium is a highly toxic heavy metal that disrupts physiological processes in plants; this heavy metal induces oxidative stress and cause cellular damage to plants. Proliferation of radical superoxide (O$_{2}^{-}$) can produce the hydroxyl radical (•OH) and hydrogen peroxide (H$_2$O$_2$) that converts fatty acids into toxic lipid peroxides and degrade biological membranes (Weckx y Clijsters, 1996; Lu et al., 2010). Cadmium (Cd) is one of the most common heavy metal pollutants for humans, animals and plants (Wang et al., 2009). It enters the environment mainly from anthropogenic processes and agricultural soils (Januškaitienë, 2014), including sources as pesticides, mining waste and chemical fertilizers (Daud et al., 2013; Daud et al., 2015). In plants, Cd affects photosynthesis, while also damaging the light-harvesting complex and photosystems, reducing chlorophyll biosynthesis (Hu et al., 2009) and water status, decreasing the transpiration rate due to stomatal closure (Deng et al., 2014). Although Cd is not involved in cellular redox reactions, that is to say cannot produce reactive oxygen species (ROS) directly, it might impair the respiratory chain, inhibit antioxidant enzymes, and displace other ions in metalloproteins, which will eventually generate Fenton reactions (Romero-Puertas et al., 2019).

Fenton reaction consist in the decomposition of hydrogen peroxide (H$_2$O$_2$) to highly reactive hydroxyl radical...
tein content, lipid peroxidation and antioxidant enzymes in oxidative stress caused by cadmium, and its effects on plants to was to assess the response of Phaseolus vulgaris L.). Because of this, the aim of the present study was to evaluate the response of Phaseolus vulgaris L. to cadmium exposure, as it can result in a decrease in the production of some crops (Shahid et al., 2014). This research has shown that there is an increased production of H$_2$O$_2$ in plants exposed to different concentrations of Cd, such as in Solanum lycopersicum (Nogueiro et al., 2016), Pteria vittata (Balestri et al., 2014), Oriza sativa (Roychoudhury et al., 2012) and in Hygrophi/a scul/a (Mandal et al., 2015). These radicals affect the permeability of cell membranes and induce lipid peroxidation, due to the increased accumulation of ROS (Rellán-Álvarez et al., 2006; Chamseedine et al., 2009).

However, plants possess defense systems; this defense systems in plants include both enzymatic and non-enzymatic antioxidant defense systems, that work in concert to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage (Gill and Tuteja, 2010; Hasanuzzaman et al., 2012). Antioxidant enzymes are fundamental, they catalyze or participate directly in generation of ROS (Gill and Tuteja, 2010; Gill et al., 2013). Antioxidant enzymes in plants include superoxide dismutase (SOD, EC 1.15.1.9), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), and guaiacol peroxidase (GPX, EC 1.11.1.9). Plants use this defense systems to counteract the effects of oxidative stress caused by heavy metals (Sharma and Chakraverty, 2013). Because the O$_2^-$ radical is usually the first to be generated (Gill and Tuteja, 2010), the SOD enzyme is the first line of defense as it converts and eliminate radical O$_2^-$ to H$_2$O$_2$ (Muradoglu et al., 2015). However, H$_2$O$_2$ is also toxic to cells, so it is necessary to remove it from cells. The enzymes involved in this process (CAT, APX and GPX) convert H$_2$O$_2$ into water and oxygen. These biological processes entail maintaining a constant balance between the antioxidant systems and ROS content so that the radicals remain at levels compatible with cellular metabolism (Halliwell, 2006). In this sense, the equilibrium between a plant’s oxidative and anti-oxidative capacities determines its fate.

Likewise, Cd affects the morpho-physiological and biochemical processes of plants, such as germination, growth and the root/shoot ratio (Ashraf et al., 2015) and can causes a decrease in biomass production (Ammar et al., 2008). This can result in a decrease in the production of some crops to economic importance such as bean cultivation (Phaseolus vulgaris L.). Because of this, the aim of the present study was to assess the response of Phaseolus vulgaris plants to oxidative stress caused by cadmium, and its effects on protein content, lipid peroxidation and antioxidant enzymes in leaves and roots.

**MATERIALS AND METHODS**

**Plant material, growth conditions and experimental design**

Plants produced by seeds were grown in semi-hydroponic conditions in plastic pots with perlite and peat moss (3:1) as substrate. The pots were kept in a greenhouse under natural light conditions. The composition of the nutrient solution applied it was done according to Chaoui et al. (1997) at pH 5.5; pots were provided daily with 200 mL of the nutrient solution. Once the first trifoliate leaf appeared, Cd was added to the nutrient solution as Cd(NO$_3$)$_2$. The treatments were: 0 (control), 0.25 µM, 0.50 µM, and 1 µM of Cd. After 15 days of adding Cd, leaves were cut and roots were carefully separated from the substrate.

**Cadmium content determination**

Roots and leaves were washed with deionized water and dried in an oven at 80°C. Then, samples were ground and stored in polyethylene bags until used in acid digestion. Approximately 0.5 g (DW) of sample was taken and digested in 15 mL of concentrated HNO$_3$ in a microwave digester (MARS 5 CEM). Potency was 1,200 W, pressure 195 psi, and temperature 210°C. The ramp and hold times were set at 15 and 10 min, respectively. The solution was then filtered through 42 µm filters, and deionized water was added to a final volume of 100 mL. Cd content was determined using Microwave Plasma Atomic Emission Spectroscopy (MP-AES) (Model 4200, Agilent Technologies). All concentrations of Cd in leaves and roots were reported in mg kg$^{-1}$.

**H$_2$O$_2$ content, lipid peroxidation and enzyme assays**

H$_2$O$_2$ was estimated following the method of Jana and Choudhuri (1981) and lipid peroxidation was determined by measuring malondialdehyde (MDA) using the method of Gérard-Monnier et al. (1998). The MDA concentration was determined at 1,1,3,3-Tetramethoxypropane (0, 0.5, 1, 2, 3 and 4 µM) as a standard curve. For the determination of the enzymatic activity 0.5 g of fresh tissue of leaves and roots were taken and was homogenized in 1 mL of 50 mM Tris-HCl (pH 7.8) containing 1 mM of EDTA and 2% (w/v) polyvinyl pyrrolidone (PVP), using a chilled mortar and pestle, and then stored in an ice bath. The homogenate was centrifuged at 15,000 g at 4°C for 30 min (HERMELE Labnet, Z233 MK-2). The supernatant was used to determine SOD, CAT, APX and GPX activity, and soluble protein content. To measure APX (ascorbate peroxidase) activity, the tissue was ground separately and the other ingredients (Balestri et al., 2014) all assays were performed at 25°C. Superoxide dismutase (SOD) activity was determined following Beyer and Fridovich (1987); catalase (CAT) activity was measured following Aebi (1981); ascorbate peroxidase (APX) activity was assayed according to Nakano and Asada (1981) and guaiacol peroxidase (GPX) activity was determined according to Kato and Shimizu (1987). Protein content was determined using Direct Detect® Infrared Spec-
trometer equipment with Bovine Serum Albumin (BSA) as the standard.

**Statistical analysis**

Data were based from ten independent samples of each treatment (control, 0.25 µM, 0.50 µM and 1 µM); data was expressed as mean ± standard deviation. Normality was verified by the Shapiro-Wilk test, and the homogeneity of variance was tested using Levene’s test. Statistical analyses were carried out by a one-way analysis of variance (ANOVA) followed by Games–Howell test for multiple comparisons using SPSS 20.0 software. A significant difference was considered at level p < 0.05.

**RESULTS AND DISCUSSION**

**Cadmium content**

Cd concentrations in roots increased significantly (p < 0.001) at higher concentrations of this contaminant in the nutrient solution; when adding 0.25 µM of Cd, the content in roots was 2.96 ± 0.54 mg kg⁻¹; at 0.50 µM, it was 6.52 ± 0.66 mg kg⁻¹; and at 1 µM, 21.08 ± 2.84 mg kg⁻¹. However, no Cd was detected in leaves at any of the Cd concentrations after two weeks of contamination (Table 1). On the other hand, the length of root was reduced with the addition of 0.25 and 0.50 µM of Cd in the nutrient solution, but at 1 µM, the length was greater (Fig. 1). In leaves symptoms of phytotoxicity were observed caused by Cd although Cd was not detected, also leaves showed symptoms like chlorosis and necrosis, so the possibility of a very low concentration of Cd in leaves is not discarded (Fig. 2).

Nevertheless, it has been reported that Cd influences the absorption and transport of nutrients and compete with their transport. This is because nutrient transporters show a wide specificity with divalent metals, including Fe²⁺, Zn²⁺, Mn²⁺ and Cd²⁺ (Liu et al., 2017). The mechanism by which Cd inhibits the uptake of essential nutrients is not completely clear (Hédiji et al., 2015). It is assumed that Cd may interfere with nutrient uptake by affecting the permeability of plasma membrane and modify the activity of nutrient transporters, leads to changes in its concentration and composition (Bouilla-Zoghlami et al., 2006; López-Millán et al., 2009; Hédiji et al., 2015). These interactions can cause serious nutritional deficiencies and the symptoms of chlorosis and necrosis, it can not only be due to the phytotoxicity produced by Cd, being as the nutritional balance in *P. vulgaris* leaves and roots could be altered.

**Oxidative stress**

Exposure to Cd resulted in a significant increase (p < 0.001) in H₂O₂ content, which was greater at higher Cd concentrations. The highest production of H₂O₂ in roots and leaves was in plants treated with 1 µM Cd; however, H₂O₂ concentrations were always higher in roots than in leaves under all treatments (Fig. 3). The results obtained show that Cd significantly increased (p < 0.001) MDA content in roots and leaves of *P. vulgaris* (Fig. 4A). In roots, MDA content increased 2.0, 3.1 and 5.38 times more than in control (0.25, 0.50 and 1 µM Cd, respectively), while the increases in leaves were 1.6, 4.1 and 7.7, compared to control. Contrary to H₂O₂ content, lipid peroxidation was more notable in leaves under all treatments. Finally, protein content decreased significantly (p < 0.001) in leaves when adding 0.50 and 1 µM of Cd, with respect to the control (Fig. 4B). However, no significant dif-

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**Table 1.** Cadmium content (mg kg⁻¹) in roots and leaves of *Phaseolus vulgaris* plants.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control</th>
<th>0.25 µM</th>
<th>0.50 µM</th>
<th>1 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roots</td>
<td>ND</td>
<td>2.96 ± 0.54°</td>
<td>6.52 ± 0.66°</td>
<td>21.08 ± 2.84°</td>
</tr>
<tr>
<td>Leaves</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = Not detectable
Data presented as means ± SD (n = 10); different letters indicate significant differences in each treatment.
En las hojas y raíces de *Phaseolus vulgaris*, la concentración de Cd aumentó significativamente la producción de 
H$_2$O$_2$ y causó la peroxidación lipídica con el aumento de MDA. En las hojas se observó una mayor inducción de MDA y en raíces, de H$_2$O$_2$. Se ha informado que la toxicidad del Cd toma la forma de estrés oxidativo causado por la producción de ROS (Sanità di Toppi y Gabbrielli, 1999; Gao et al, 2010). El Cd no está implicado en las reacciones redox celulares y no produce ROS directamente. Sin embargo, estudios bioquímicos y transcriptómicos muestran que el estrés oxidativo es uno de los primeros consecuencias de la toxicidad del Cd en plantas y otros organismos (Romero-Puertas et al, 2019). Aunque Cd no es un metal de transición, comúnmente causa estrés oxidativo en plantas, pero el modo de producir daño celular es aún enmascarado (Gallego et al, 1996; Groppa et al, 2007; Lin et al, 2007; Iannone et al, 2010).

En plantas, H$_2$O$_2$ es continuamente producido como un producto de varias reacciones metabólicas. Por otro lado, es importante recordar que Cd en hojas no fue detectado en ninguno de los tratamientos, pero a pesar de esto, se observaron aumentos en el contenido de MDA, H$_2$O$_2$ y actividad enzimática. Esto podría explicarse por el hecho de que H$_2$O$_2$ actúa como un agente oxidante.

![Fig. 2](image1.png)

**Fig. 2.** Efectos fitotóxicos causados por la acumulación de cadmio en las raíces de *Phaseolus vulgaris*. Control (A), 0.25 µM (B), 0.50 µM (C) y 1 µM (D).

![Fig. 3](image2.png)

**Fig. 3.** Contenido de peróxido de hidrógeno en las hojas y raíces de las plantas *Phaseolus vulgaris* expuestas al cadmio. Datos presentados como medias ± DE (n = 10); letras diferentes indican diferencias significativas entre tratamientos.

![Fig. 4](image3.png)

**Fig. 4.** Contenido de malondialdehído (A) y proteína (B) en las hojas y raíces de plantas de *Phaseolus vulgaris* expuestas al cadmio. Los datos son presentados como medias ± DE (n = 10); letras diferentes indican diferencias significativas entre tratamientos.
systemic signaling molecule against oxidative stress caused by exposure to heavy metals (Wang et al., 2006; Zayneb et al., 2015). ROS can also serve as signaling molecules, and a possible mitigating effect of H$_2$O$_2$ derived from Cd stressors has been proposed (Gill and Tuteja, 2010). In recent years, several studies have focused on the role of H$_2$O$_2$ in response to tolerance for a wide range of stress conditions, so it is interesting to see what happens in response to internal and external stimuli to improve stress tolerance (Cuypers et al., 2016). This seems to be used positively in plants to activate multiple stress-sensitive genes, so it is widely accepted that H$_2$O$_2$ is one of the signaling molecules due to its high stability and mobility (Christou et al., 2014). However, in high concentrations, H$_2$O$_2$ is harmful to plants and leads to programmed cell death (Gill and Tuteja, 2010).

**Antioxidant enzymes**

The effect on SOD activity (Fig. 5A) in roots were significant with the application of 0.25 μM of Cd (p < 0.001), as it increased 9.1 times more than in the control; whereas at 0.50 and 1 μM Cd, activity was 8.5 and 5.9 times greater than in the control, respectively. In leaves, the activity of this enzyme increased at higher concentrations, and significant differences were found compared to the control in all Cd treatments (p < 0.001). CAT activity (Fig. 5B) in roots was significantly higher (p < 0.001) in the treatment with 0.25 μM of Cd (6.9 times more than in the control) but decreased with the application of 0.50 and 1 μM Cd. No significant differences were observed between these two treatments (p = 0.340). In contrast, the greatest activity of this enzyme in leaves occurred in treatments with 0.50 and 1 μM of Cd (11.6 and 12.1 times more, respectively), compared to the control (p < 0.001). However,
and 1 μM. The decrease in CAT activity in roots of Cd, in contrast to leaves where the highest activity was at 0.50 μM of Cd, it was inhibited in roots with the addition of 0.50 μM of Cd. In leaves, this enzyme’s activity was lower than in roots under all treatments. However, in the treatment with 1 μM of Cd it was 8.1 times higher than in the control (p < 0.001). Finally, APX activity (Fig. 5D) was significantly higher in roots (p < 0.01) at the concentration of 0.25 μM of Cd, compared to all other treatments. In treatments with 0.50 and 1 μM of Cd, this enzyme’s activity was inhibited; as shown by the finding that concentrations increased. In terms of leaves, APX activity increased at concentrations. The highest activity was with 1 μM of Cd (11 times more than the control).

The enzymes considered most important to eliminate intracellular H₂O₂ levels in plants are CAT, APX and GPX (Al-fadul and Al-Fredan, 2013). Like SOD, these enzymes showed a different behavior between leaves and roots of P. vulgaris. The APX and GPX activity was higher in roots when adding 0.25 μM of Cd, since at 0.50 μM this activity decreased, although it was always greater than in the control. Regarding CAT, it was inhibited in roots with the addition of 0.50 μM of Cd, in contrast to leaves where the highest activity was at 0.50 and 1 μM. The decrease in CAT activity in roots of P. vulgaris can be compensated with a higher APX and GPX activity (0.25 μM Cd), indicating that these two enzymes can act simultaneously. Therefore, the decrease in CAT with the increase in H₂O₂ in roots suggests the sensitivity of this enzyme to Cd (Roychoudhury et al., 2012). A higher H₂O₂ concentration was observed in roots than in leaves of P. vulgaris. In addition, the sensitivity of CAT to O₂⁻ radicals and Cd levels can cause its inactivation (Nouairi et al., 2009).

On the other hand, APX plays an important role in the elimination of H₂O₂, but its activity depends on the concentration of metal, and its main function is to quickly eliminate H₂O₂ at the site where it is generated (Asada, 1992; Gill et al., 2013). GPX can be induced by toxicity generated by heavy metals and is more efficient than CAT in the elimination of H₂O₂ (Wang et al., 2010; Nadgórska-Socha et al., 2013). However, it was observed that the CAT enzyme proved to be more efficient in removing H₂O₂ in leaves than in roots of P. vulgaris. Depending on Cd concentration and plant species, Cd may inhibit or stimulate the activity of several antioxidative enzymes before any visible symptoms of toxicity appear (Anjum et al., 2011; Gill et al., 2012; Xu et al., 2014). Excessive levels of H₂O₂ could be minimized through the activities of APX and GPX; it may be due to the damage to enzymatic proteins due to the excess of radicals (Lou et al., 2011). This could explain the fact that in roots after the addition of 0.50 μM Cd, the excess of free radicals inhibited the enzymatic activity of APX and GPX.

Other studies have also observed increases or decreases in enzymatic activities at different concentrations of metals. In the case of Brassica juncea, the activity of CAT and GPX progressively decreased in leaves exposed to 30 μM of Cd, but APX activity was induced (Markovska et al., 2009). Bankaji et al. (2015) showed that the excess of Cd or Cu mainly decreased the enzymatic activity of CAT, GPX and APX in the leaves of Suaeda fruticosa, suggesting that antioxidant systems are altered by the action of heavy metals. Likewise, the patterns produced in response to the stress caused by heavy metals vary in the different organs of the plants, as was seen in the case of P. vulgaris.

These differences in enzymatic activity can be explained by the fact that roots are the first organ to come into direct contact, so a quick and effective response is important in response to oxidative stress caused by this type of contaminants (Souza et al., 2015). Some studies have shown that SOD activity increases due to the stress produced by Cd, which was observed in P. vulgaris leaves; however, this enzyme was inhibited in roots by adding 0.50 and 1 μM of Cd. This reduction may be due to the inactivation of SOD by the action of H₂O₂ (Namdjoyan et al., 2011). Therefore, the H₂O₂ produced by SOD must be rapidly metabolized, since it is still toxic and needs to be converted into H₂O and O₂ by other enzymes (Rahoui et al., 2014).

In the other hand, it is also important to note that no Cd was detected in the leaves in any treatment, though increases in MDA and H₂O₂ content and enzymatic activity were observed. This could be explained by the fact that H₂O₂ can act as a local and systemic signaling molecule against oxidative stress caused by exposure to heavy metals (Wang et al., 2006; Zayneb et al., 2015). ROS can also serve as signaling molecules, and a possible mitigating effect of H₂O₂ derived from the stress factors of Cd has been proposed (Gill and Tuteja, 2010). In recent years, several studies have focused on the role of H₂O₂ as a response to tolerance for a wide range of stress conditions, so it is interesting to see what is produced in response to internal and external stimuli in order to improve tolerance of stress (Cuypers et al., 2016). This seems to be used positively in plants to activate multiple stress-sensitive genes, which is why it is widely-accepted that H₂O₂ is one of the signaling molecules due to its high stability and mobility (Chistou et al., 2014). However, at high concentrations, H₂O₂ is actually harmful to plants, and leads to programmed cell death (Gill and Tuteja, 2010).

The results of this study show that although cadmium was deposited in roots but not detected in leaves, an enzymatic antioxidant response was observed in both roots and leaves of P. vulgaris. This could be due to the induction of systematic signaling mechanisms due to oxidative stress that occurs at roots as a means to protect the leaves. However, it is possible that Cd accumulates at such low concentrations in the roots, so it was not detected.

**CONCLUSIONS**

Our results suggest the generation of oxidative stress caused by Cd by the accumulation of MDA and H₂O₂ in leaves and roots. However, the antioxidant defense system in the roots of P. vulgaris was not effective enough in the elimina-
tion of free radicals generated with the addition of 0.50 and 1 µM of Cd.

Regarding the enzymatic activity in leaves, there was an increase in all concentrations with Cd, although this contaminant was not detected, could be due to the induction of systematic signaling mechanisms caused by oxidative stress as a means to protect the leaves. Therefore, it is necessary to carry out further studies on oxidative stress and the antioxidant response at low concentrations of Cd and gain a better understanding of the connection between ROS production, antioxidant defense mechanisms and signaling mechanisms in plants.

REFERENCES


