Peroxide isozymes and Malondialdehyde content and ascorbate peroxidase activity in *Berberis integerrima* and *Cercis siliquastrum* under Cd-induced stress

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Abstract

ROS detoxification is an essential process in protecting plant cells and their organelles, which is caused by ROS generated in stress condition. Peroxidase (POD) isozyme, Malondialdehyde (MDA) content, and Ascorbate peroxidase (APX) activity are the indicators of plants for measuring pollution effects. The purpose of the present study was to investigate Cd-induced stress on POD isozymes, MDA content, and APX activity in seedlings of *Berberis integerrima* and *Cercis siliquastrum.* 100 seedlings were treated with cadmium chloride separately at concentrations of 0, 1000, 2000, 4000, 6000 mg/kg three times at intervals of 15 days. POD activity varied at different levels of Cd stress *Cercis siliquastrum*, while this was not the case with *Berberis integerrima*. MDA was slightly increased up to 6000 mg/kg from 5% to 27%, but a sharp increase (41%) was found at 6000 mg/kg compared with 4000 mg/kg Cd treatment in *Cercis siliquastrum*. The highest MDA content (10.5 µM/ g FW) was recorded at 6000 mg/kg trearment in *Berberis integerrima*, while the lowest value (2.3 µM/ g FW)was found in control *Cercis siliquastrum* plants. The maximum and minimum APX activity were found in the treatments of 6000 mg/kg (0.13 unit/mg protein) and control (0.02 unit/mg protein), respectively, for both *Cercis siliquastrum* and *Berberis integerrima*. In control *Berberis integerrima*, APX activity (0.02 unit/mg protein) was higher than that of *Cercis siliquastrum* (0.006 unit/mg protein) ($p < 0.05$). Overall, the findings suggest that *Berberis integerrima* is less tolerant than *Cercis siliquastrum* under Cd-induced stress.

Keywords*:* cadmium; Cercis siliquastrum; Malondialdehyde; peroxide isozyme

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Introduction

The exposure of plants to heavy metals increases the production of reactive oxygen species (ROS) such as, singlet oxygen ($1O_2$), superoxide (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH). ROS detoxification process in plants is essential in protecting plant cells and their organelles against the toxic effects of these species (Apel and Hirt 2004; Mittler 2002). Differences in subcellular localization and biochemical properties of antioxidant enzymes and the distinct responses in gene expression, in addition to the presence of non-enzymatic

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mechanisms, result in a versatile and flexible antioxidant system able to control the optimum ROS levels (Vranova et al. 2002). ROS detoxification systems consist of enzymatic and non-enzymatic antioxidant components (Scandalios 2005). Ascorbate (AsA) and glutathione (GSH), non-enzymatic antioxidants, are crucial for plant defense against oxidative stress, playing a significant role as antioxidant buffers (Foyer and Noctor, 2005; Mittler 2002). Other non-enzymatic antioxidants involved include flavonoids, phenolic compounds, alkaloids, and carotenoids (Gratão et al. 2005).

Malondialdehyde results from lipid peroxidation of polyunsaturated fatty acids (Davey et al*., 2005).* It is a prominent product in Thromboxane A_2 synthesis where cyclooxygenase 1 or cyclooxygenase 2 metabolize arachidonic acid to prostaglandin H_2 by platelets and a wide array of other cell types and tissues. This product is further metabolized by Thromboxane synthase to Thromboxane A_2, 12-Hydroxy heptadecatrienoic acid, and malonyldialdehyde *(*Davey et al., 2005*).* Alternatively, it may rearrange non-enzymatically to a mixture of 8-cis and 8-trans isomers of 12-hydroxyeicosaheptaenoic acid plus malonyldialdehyde (Pryor and Stanley, 1975*).* The degree of lipid peroxidation can be estimated by the amount of malondialdehyde in tissues *(*Davey et al., 2005*).* POD is an antioxidative enzyme which has a significant role in plant response to abiotic stresses such as heavy metal-induced stress (Shalini and Duey, 2003). In addition, POD has a key role in H_2O_2 detoxification, MDA removal, and maintaining the stability of cell wall (Hojati et al., 2011).

Ascorbate peroxidase (APX) is a hydrogen peroxide-scavenging enzyme that is specific to plants and is indispensable to protect chloroplasts and other cell constituents from damage by hydrogen peroxide and hydroxyl radicals produced from it.

Berberis integerrima and *Cercis siliquastrum* are important plant species which have been planted in different parts of Iran in afforestation and green space. On the other hand, Cd is a heavy metal which is widely found in most types of soil particularly in urban area. Hence, the aim of the present study was to investigate Cd-induced stress on POD isozymes and MDA in *Berberis integerrima* and *Cercis siliquastrum.*

Materials and Methods

Experimental treatments

Three-year-old seedlings of *Berberis integerrima* and *Cercis siliquastrum* in Alborz Nursery belonging to the Research Institute of Forests and Rangelands in Karaj, Iran, were cultivated in plastic pots containing approximately 8 kg soil. Afterwards, the plants were treated with cadmium chloride separately at concentrations of 0, 1000, 2000, 4000, and 6000 ppm, and 100 cc per plant three times at intervals of 15 days. Subsequently, the leaves of both spices were sampled in four directions of crown one month after the last treatment.

POD isozymes

The POD was assessed by PAGE technique. For this purpose, proteins and enzymes were separated based on the method of Krzakowa and Dunajski (2007) using electrophoresis.

MDA

MDA was measured based on its accumulation using TBA. In this regard, 0.2 g leaf was homogenized in 2 mg 1% TCA and centrifuged at 1300 rpm for 15 min. After that, 1 mg of this solution was mixed with 2 ml TBA (5 %) including TCA (20 %) and put in the benmary for 30 min. Subsequently, samples were put in ice and centrifuged at 10000 rpm for 10 min. MAD concentration (μMg^{-1} FW) was recorded at the wavelength of 532 nm in spectrophotometer.

Ascorbate peroxidase

Ascorbate peroxidase relative activity was measured using the method described by the United States Environmental Protection Agency (USEPA, 1994). A reaction mixture consisting of 100 µl supernatant, 17 mM H_2O_2 (450 µl; Fisher Scientific), and 25 mM Ascorbate (450 µl; Fisher Scientific) was then assayed for 3 min at 290 nm. Activity was measured as

disappearance of Ascorbate. One unit of enzyme activity was defined as a decrease in absorbance of 0.001 min–1 at 290 nm.

Statistical analysis

Data were submitted to SAS 9.3 for MDA and APX and Duncan multiple range test was applied to mean comparison.

Results

In this study, new isozymes were found in stress condition and named based on band distance at the bottom of the gel. Figure (I) shows the POD isozymes pattern in *Berberis integerrima* and *Cercis siliquastrum.* Different band patterns were observed for POD in *Cercis siliquastrum.*

As seen in Figs. (I) and (II) with an increase in Cd stress, more gens were expressed and subsequently the new bands appeared at different intensity. POD5 appeared in the beginning of stress at all levels. POD7 was present at 2000 mg/kg and also appeared with high intensity at 6000 mg/kg. POD4 only was found at 6000 mg/kg and is considered as an isozyme which can be present at high intensity of stress. In addition, at 6000 mg/kg, POD1 had less intensity compared with other levels while POD7 had more intensity and this shows high activity of this isozymes in high stress condition. POD activity varied at different levels in *Cercis siliquastrum* (Fig. I) while this was not found for *Berberis integerrima* (Fig. II). A significant increase was found in MDA with increasing Cd concentration in both *Berberis integerrima* and *Cercis siliquastrum* plants (Fig. III).The highest MDA content (10.5 µM/g FW) was observed at 6000 mg/kg in *Berberis integerrima* while the lowest value (2.3 µM/ g FW) was found in control *Cercis siliquastrum* plants. As is seen in Fig. (IV), The highest and lowest APX activities were found

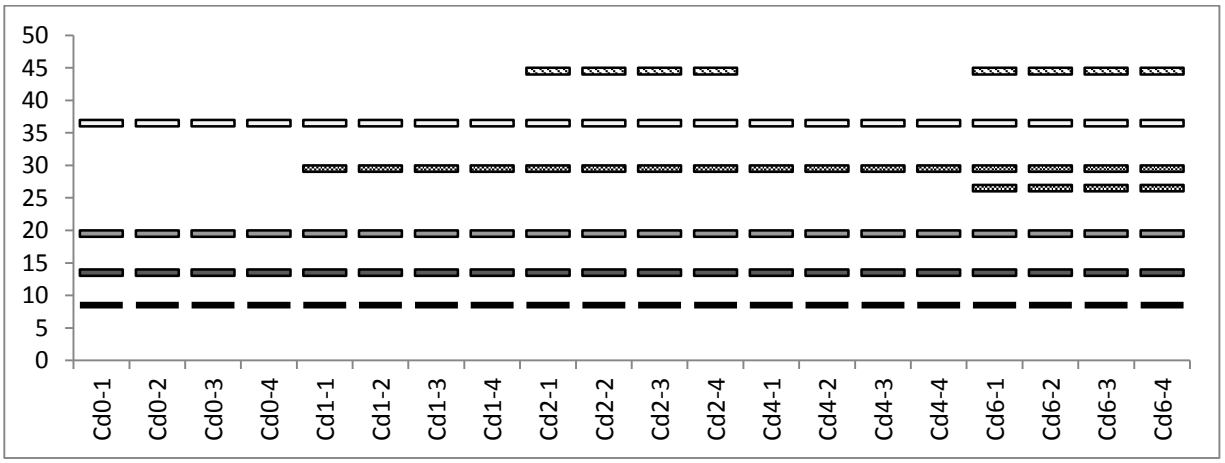

Fig. I. Acrylamide gel electrophoresis pattern in *Cercis siliquastrum* under Cd stress in control and 1000 mg/kg (Cd1), 2000 mg/kg (Cd2), 4000 mg/kg (Cd4), and 6000 mg/kg (Cd6); the intensity of bands was displayed in the order of black, gray, hachured, and white.

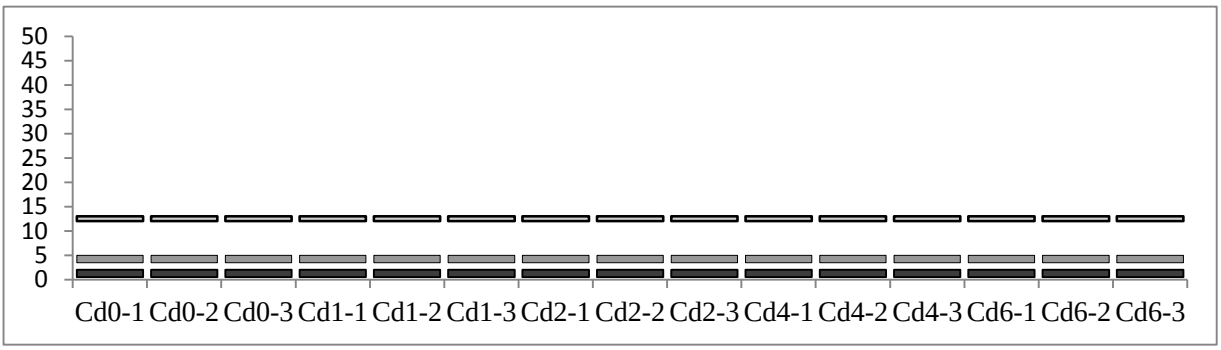

Fig. II. Acrylamide gel electrophoresis pattern in *Berberis integerrima* in control and 1000 mg/kg (Cd1), 2000 mg/kg (Cd2), 4000 mg/kg (Cd4), and 6000 mg/kg (Cd6) Cd stress; the intensity of bands was displayed in order of black, gray, hachured, and white.

in the treatments of 6000 mg/kg (0.13 unit/mg protein) and control plants (0.02 unit/mg protein), respectively, for both *Cercis siliquastrum* and *Berberis integerrima*. In control, APX activity of *Berberis integerrima* (0.02 unit/mg protein) was higher than that of *Cercis siliquastrum* (0.006 unit/mg protein) ($p < 0.05$).

Discussion

Peroxidase isozymes

In this study, a large number of new bands were present in Cercis siliquastrum, which may be due to other physiological activities of this enzyme in plant or relative adaptation of this species to stress condition. POD, SOD, and CAT are metalliferous enzymes which protect cell against oxidative stresses. As POD activity is influenced by low temperature, air pollution, heavy metals, pests etc., it can be applied as an appropriate indicator for environmental stresses (Passardi et al., 2005). High POD activity under metal stress is due to its role in the construction of the physical barrier against toxic metals entering the cell and decomposing H_2O_2 (Passardi et al., 2005).Hence, POD is considered as a parameter of metabolism activity which can be used against Cd toxicity. The change in activity of antioxidative enzymes may be because of new isozymes synthesis (Kang et al., 1999). Induction of the new isozymes and change in its profile shows the important role of cellular defense against oxidative stress. Typically, because of the multiple role of POD in plants, this enzyme has different isozymes. Expression pattern of POD isozymes in different tissues are different and it can be adjusted by various types of biotic and

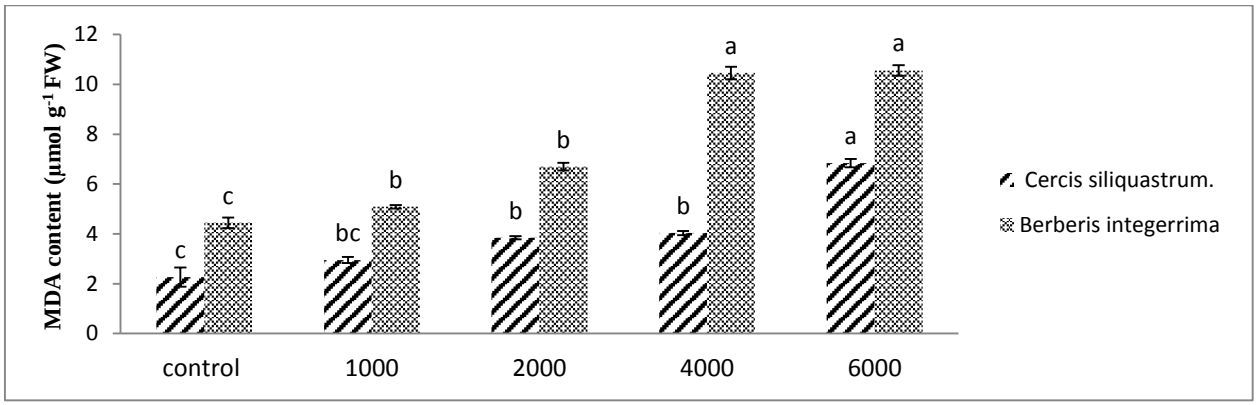

Fig. III. MAD content in *Berberis integerrima* and *Cercis siliquastrum* under Cd stress in control and 1000 mg/kg, 2000 mg/kg, 4000 mg/kg and 6000 mg/kg

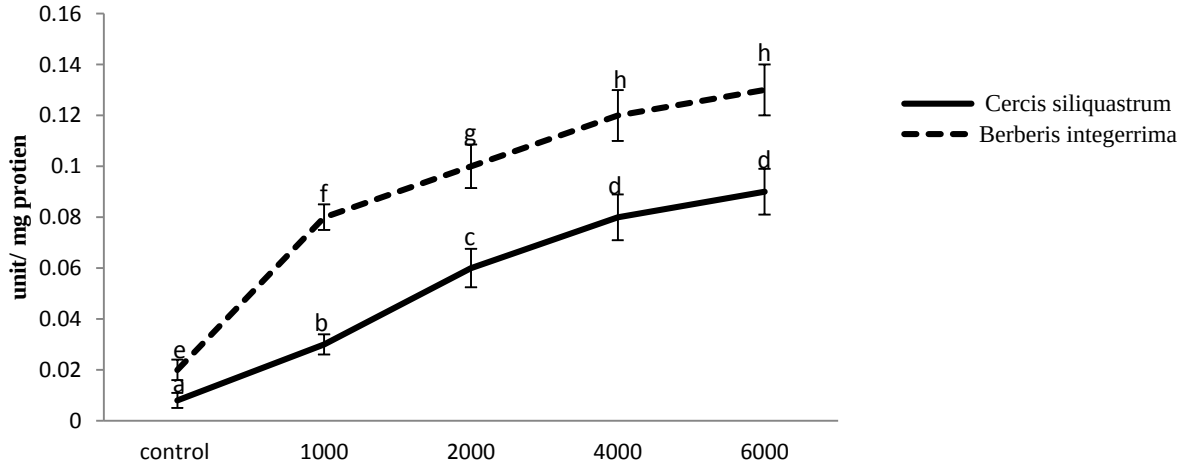

Fig. IV. Ascorbate peroxidase activity in *Berberis integerrima* and *Cercis siliquastrum* under Cd stress in control and 1000 mg/kg, 2000 mg/kg, 4000 mg/kg, and 6000 mg/kg.; Same letters indicate no significant difference at 5% level.

abiotic stress factors temporally and spatially (Passardi et al., 2005). Foyer et al. (1994) indicated that the change in subcellular distribution of antioxidative enzymes with changes in isozymes suggests a protective mechanism in response to increasing the enzyme activity. Particular activity and antioxidative defense of enzymes during the stress depend on plant genotype (Abedi and Pakniat, 2010). It is reported that lignification of cell wall due to induction of POD isozymes is the mechanism which protects plants against Pb-induced stresses (El-Beltagi and Mohamed, 2010). Isozyme variation depends on plant species, tissue, developmental stage, and type and concentration of metal (Rastgoo and Alemzadeh, 2011).

Malondialdehyde

MDA increased with increasing Cd concentration in both *Berberis integerrima* and *Cercis siliquastrum*. The maximum MDA in Cd-induced stress was recorded at 6000 and 4000 mg/kg treatments which indicates the high production of ROS and lipid peroxidation at high intensity of stress. MDA slightly increased up to 6000 mg/kg from 5% to 27%, but an intense increase (41%) at 6000 was found compared with 4000 mg/kg treatment in *Cercis siliquastrum*. The highest MDA was observed at 4000 mg/kg in *Berberis integerrima*. Previous studies have shown cell membrane is the first part in plant which can be injured by stresses. Lipid peroxidation is an oxidative damage which affects cell membranes, lipoproteins, and other lipid-containing molecules under conditions of oxidative stress. Lipid peroxidation occurs due to the reaction of free radicals with polyunsaturated fatty acids of cell membranes. This process reduces the fluidity of biological membranes, resulting in increased permeability to uni- and divalent ions and disables membrane enzymes (Jovanović et al., 2013). MDA is the final product of lipid membrane oxidation accumulated as plants face oxidative stress. MDA concentration is considered as an indicator of lipid peroxidation (Chaoui et al., 1997; Ding et al., 2004). Plant cell membranes commonly were presented as primary location of injuries resulted from metals (Belkhadi et al., 2010). In this study, MDA was increased with increasing stress, which represents more oxidative stress at high concentrations of Cd. Similar results have been reported in Lonicera japonica under Cd stress (Jia et al., 2013). MDA concentration is less in *Cercis siliquastrum* compared with *Berberis integerrima*. This shows that *Cercis siliquastrum* is more able to deal with ROS produced in stress conditions. This is supported by maintaining the cell membranes in *Cercis siliquastrum*.

Ascorbate peroxidase

APX as an antioxidant enzyme has a significant role in plant damages induced by heavy metals. In our study, APX activity increased with increasing the cd concentration. As is shown in Fig. (IV), there is a sharp increase from control to 1000 mg/kg Cd treatment in both species especially for *Berberis integerrima*. This means that when the trees are influenced by Cd stress, they rapidly react and increase their APX activity to alleviate the ROS generated under pollution condition. The APX activity in *Berberis integerrima* is higher than that in *Cercis siliquastrum*. This suggests that *Berberis integerrima* is more sensitive to Cd stress and responds to it with high activity of APX. An increase in APX activity was reported by Beak and Woo (2010) in response to cadmium. There is no significant difference between 4000 and 6000 mg/kg treatments for these species. The reason may be due to the fact that the high level activity of APX is 4000 mg/kg, after this, with increasing cd content there is no significant increase in APX activity or 4000 mg/kg is enough for optimal APX activity in order to catalyses the hydrogen peroxide. These results emphasize the important role of APX in H_2O_2 scavenging under toxic metals in the soil. To sum up, according to the results obtained in our study, *Berberis integerrima* is less tolerant than *Cercis siliquastrum* under Cd-induced stress. Hence, in soils with high level of Cd, planting *Cercis siliquastrum* is suggested rather than Berberis integerrima.

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