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RESEARCH ARTICLE

Independent and Combined Nickel and Cadmium Induced Lipid Peroxidation of Biological Membranes and its Mitigation through Antioxidant Enzymes in *Grewia asiatica* L.

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ABSTRACT

Grewia (*G.*) *asiatica* L., family Tiliaceae is grown as minor fruit native to Pakistan, India, Cambodia and many other tropical countries. During the past few decades, rapid development of industrial sector and high anthropogenic pressures have resulted in contamination of soil by heavy metals. Among these metal contaminants, Nickel (Ni) and Cadmium (Cd) are rated as top pollutants near metropolitan cities. The *G. asiatica* plants were exposed to Ni and Cd singly by using 20, 40 and 60 mg of $NiSO_4$ and Cd $(NO_3)2.4H_2O$ per kg soil and in combination of both by using 20, 40, and 60 mg of each compound per kg of soil for 8 weeks. The extent of membrane damage via Malondialdehyde (MDA) (a product of lipid peroxidation) and mitigation of metal stress through antioxidant enzymes Superoxide Dismutases (SOD), Catalase (CAT) and Peroxidases (POD) were assessed in leaves. The extent of membrane damage was more profound when Ni and Cd were given collectively thus metal ions seemed to exert a non-antagonistic effect. The mitigation of metal stress appeared to be acquired by the enhanced production of antioxidant enzymes. SOD activity increased in a dose dependent manner profoundly for Cd and Ni+ Cd. The activity of CAT showed a consistent decline due to its possible transformation from tetrameric to monomeric subunits that is POD which then served as a potential stress mitigation factor which can break down the obnoxious compounds as well as cell wall strengthening factor. These findings contributed towards mechanistic explanation of antioxidant defense mechanism in *G. asiatica* L. leaves. Thus, it can serve as a model species for plants of arid and semi-arid regions and the biomarkers (MDA, SOD, CAT and POD) as useful tools to evaluate metal stress and its mitigation.

INTRODUCTION

Heavy metals are substantial ecological contaminants and their toxicity is of hazardous consequence for environment and human health (Mengoni et al., 2012). During the past few decades, uncontrolled anthropogenic activities caused rapid increase in the levels of heavy metal contamination in soil, water and air. The amount of these contaminants in the environment each year exceeds than all radioactive and organic wastes combined (Sharma and Dhiman, 2013). The major metal contaminants include Nickel (Ni), Lead (Pb), Copper (Cu), Cadmium (Cd), Zinc (Zn).

Like many other developing and third world countries, the agricultural soils of Pakistan have also been stated to contain exceeding levels of several metal pollutants (Yusuf et al., 2011). Among these pollutants, Ni is an essential element that plays a role in Nitrogen metabolism by acting as enzyme activator. It is found to be related with some metallo enzymes (urease) which are essential for various plant processes such as nitrogen assimilation (Lopez and Magnitskiy, 2011). It has been established that Ni in a concentration of 0.057 ppm is necessary for plant growth (Chen et al., 2009). However, optimum requirements differ for plant species ranging from 0.05 to 10 ppm (Teixeira et al., 2012). Elevated Ni levels can induce toxicity symptoms in plants because excessive amount leads to the production of nitric oxide, hydrogen peroxide, hydroxyl radicals and superoxide anions. Though, Ni is not directly involved in the generation of reactive oxygen species (ROS) due to its non-Redox activity but it interacts with antioxidant enzymes (Kaveriammal and Subramani, 2015). Like Ni, industrial operations, mining, phosphate fertilizers, sewage and different human activities also results in excessive Cd in the environment which is injurious to plant health through generation of free radicals (Stasinos and Zabetakis, 2013). Though Cd is categorized as a non-essential element, but the threshold of plant species differs considerably for Cd thus degree of resistance for its transport and subsequent translocation is quite variable (Pandey et al., 2017). The increased levels of Cd in soil can induce inhibition in the uptake of essential minerals by the plants and other metabolic disorders (Salgado et al., 2017). The generation of free radicals (ROS) can damage all types of biological molecules such as lipids proteins and nucleic acids and can cause irreparable tissue damage that ultimately results in cell death (Tripathi et al., 2017). Moreover, generation of free radicals stimulate production of a lipid peroxidation factor; Malondialdehyde (MDA) which is frequently being utilized in plants to evaluate membrane stability under metal stress (Dubey and Pandey, 2011).

Although, the toxicity of heavy metals depends mainly upon their oxidation states, but plants have an adequate capacity to alleviate the deleterious effects of toxic concentrations caused by redox reactivity of heavy metals. The innate ability of plants to combat metal toxicity is achieved by the production antioxidant enzymes. Both enzymatic and non-enzymatic defense become operational to scavenge excessive ROS thus maintain normal functioning of the plant. This is considered as a successful strategy for the mitigation of stress and a universal feature of plant defense to avoid oxidative damage (Pandey, 2017). Several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and non-enzymatic antioxidants such as tocopherols, ascorbic acid, carotenoids and glutathione have been well documented in plant species under heavy metal stress (Baxter et al., 2014). An imbalance between ROS products and detoxification factors (antioxidant enzymes) could result in oxidative damage. Moreover, when the stress is more profound than the degradation of antioxidants exceeds than their synthesis. Consequently, enhancement of lipid peroxidation that may ultimately results in cell death. Hence, the damage is compensated by the protection mechanism via antioxidants production by the plants (Ismail and Theodor, 2013). Superoxide dismutase (SOD) is the main O^{-2} scavenger and its activity results in the formation of H_2O_2 and O_2. SOD is essentially present in every cell and serves as the first line of defense against harmful effects of metals thus protect cellular injuries. Catalase (CAT) is an iron dependent enzyme, mainly located in peroxisomes and dismutates H_2O_2 into H_2O and O_2. Peroxidase (POD) is a stress predictor and enzymatic marker found in cell wall, extracellular spaces and vacuole. It utilizes H_2O_2 to produce phenoxy compounds that have the ability for polymerization to produce polysaccharides thus strengthens cell wall by stimulating lignin biosynthesis. Thus, robust cell wall itself poses a strong physical obstruction for toxic metal ions. Nevertheless, extent of production of these enzymes depends on several factors including state and concentration of metals, soil properties and plant species (Awasthi and Sinha, 2013). The *G. asitica* L., commonly known as “Phalsa” which is an Asiatic shrub. The species can be grown under wide range of soils and climatic conditions. Although, recent trends for phytoremediation focus on the potential of plant species from diverse groups but some native species have little been investigated so far. As such essential information about *G. asiatica* L.in relation to its growth on metal contaminated soils is also lacking. Therefore, it becomes imperative to identify a species that could serve as a model that can signify underlying mechanism of metal stress tolerance. We investigated the extent of membrane damage through lipid peroxidation and the mitigation by enzymatic antioxidants (SOD, CAT and POD) in *G. asiatica* L. to provide possible explanation of tolerance mechanism against essential (Ni) and non-essential (Cd) metals when present in excessive amount in the growth substratum.

MATERIALS AND METHODS

Growth experiment

Seeds of *G. asiatica* L. were obtained from Punjab Nursery, Multan, Punjab Pakistan. Air-dried sandy loam soil was mixed with $NiSO_4$ (Merck, Darmstadt, Germany) and Cd $(NO_3)_2.4H_2O$ (Sigma Aldrich, Germany): at concentrations 20, 40, 60 mg kg^{-1} of soil separately and in combination having same levels of both salts. The concentrations were selected based on the Ni and Cd content reported in agriculture soils of Pakistan present in the localities of large cities and industries (Yusuf et al., 2011).

Eight pre-germinated seeds (2-4 leaves) after two weeks were shifted into each of 36 plastic pots (height 42 cm, internal diameter 30 cm), which were filled with 9.0 kg soil containing Ni and Cd. The experiment was organized in a Complete Randomized Design (CRD). To simulate field conditions, plants were grown in a wire netting house in botanic garden under natural conditions (28 ± 5 °C, 8h day light, and 38% RH). Seedlings were allowed to grow for further two weeks then thinned out to four in each pot. Watering was done by gentle sprinkling using a spray gun to avoid leaching. Plants were harvested after eight weeks of growth. The leaves from the plants were separated and kept frozen for biochemical analyses.

Estimation of lipid peroxidation (MDA) and antioxidant activity

Lipid peroxidation (MDA content) was estimated using the protocol of Cakmak and Horst (1991). Superoxide dismutase (SOD) was analyzed by Giannopolitis and Ries (1977). Catalase (CAT) Peroxidase (POD) were determined by method of Chance and Maehly (1995).

Statistical analysis

The statistical analysis of the data was carried out by using COSTAT (2007 Cohort Software). Each parameter was subjected to a one-way ANOVA to determine significant effect of different Cd and Ni levels. Multiple range test was employed to find significant difference (Snedecor and Cochran1989).

RESULTS

MDA

Lipid peroxidation (MDA) increased up to 40% at 20 mg kg^{-1}, 79 % and 119 % for 40 mg kg^{-1} and 60 mg kg^{-1} respectively when plants were treated with Ni. Similarly, rise was noticed in MDA content when plants were treated with Cd. Increase was 11% at 20 mg kg^{-1} and 61% was noticed at 60 mg kg^{-1} compared to its respective control. While 26% decrease was observed when plants were given 40 mg kg^{-1} Cd. A consistent increase was noted in dose dependent manner when both metals were given in combination. 101%, 117% and 144% rise were observed for 20, 40 and 60 mg kg^{-1} Ni + Cd as shown in Fig. 1.

Activities of Antioxidant enzymes (SOD, POD, CAT)

The SOD activity decreased in a concentration dependent manner i.e., 17%, 27.46% and 38.63% at 20, 40 and 60 mg kg-^{1} respectively when Ni was applied separately. A consistent increase was noticed when Cd was given to plants as 30.07 % rise in SOD was observed at 20 mg kg^{-1} whereas 47.82% and 73% was noticed at 40 and 60 mg kg^{-1}of Cd, respectively. There has been observed a profound increase in SOD activity when plants were treated with both metals. 73% rise was observed at 20 mg kg^{-1} Ni+ Cd and up to 110% and 150% rise was observed at 40 mg kg^{-1} and 60 mg kg^{-1} Ni +Cd.

As far as CAT activity is concerned, there has been observed a progressive decline. Reduction in CAT activity was observed when Ni was applied singly to the plants. 49%, 45% and 56% decrease were noted for 20 mg kg^{-1}, 40 mg kg^{-1} and 60 mg kg^{-1} Ni. 57%, 60% and 66% decrease were observed for 20, 40 and 60 mg kg^{-1} for Cd. A steady decline was observed when Ni + Cd was given in combination as shown in Fig 2.

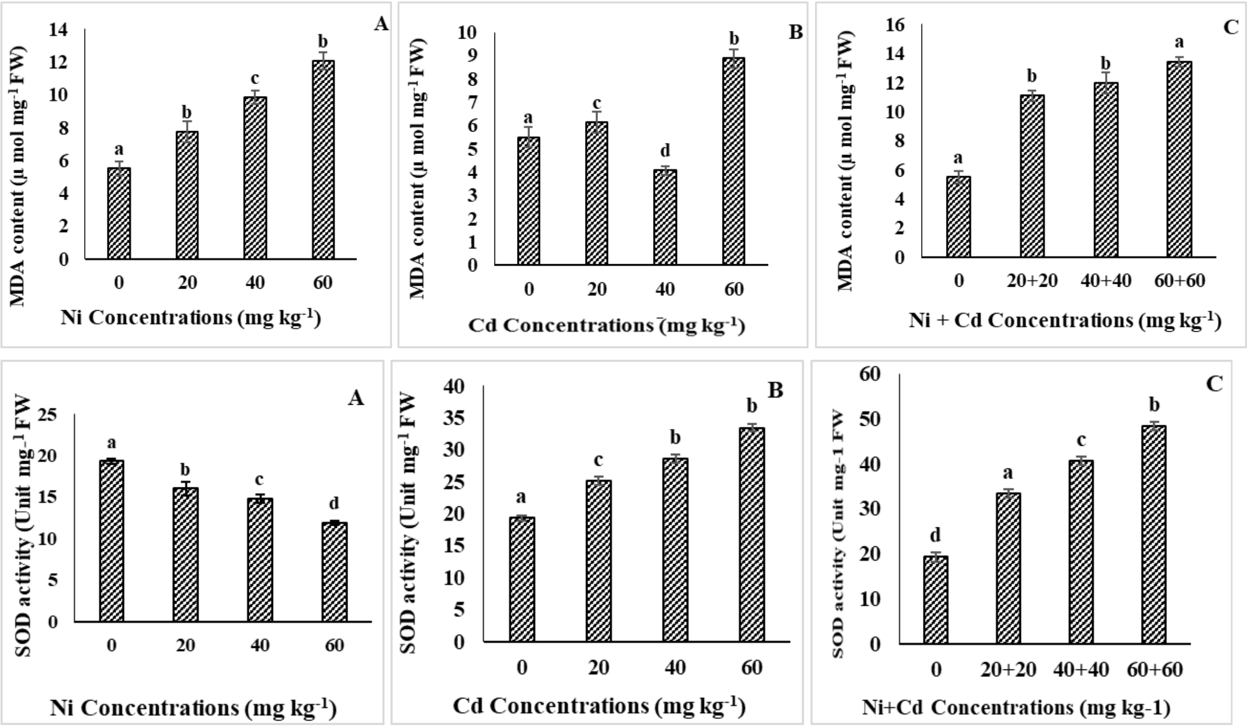

Fig. 1: Malondialdehyde (MDA) content and Superoxide Dismutase (SOD) activities in leaves of *Grewia asiatica L.* in response to Nickel (Ni), Cadmium (Cd) and Ni+Cd treatments, respectively. Values presented are means across three replicates ± S.E.

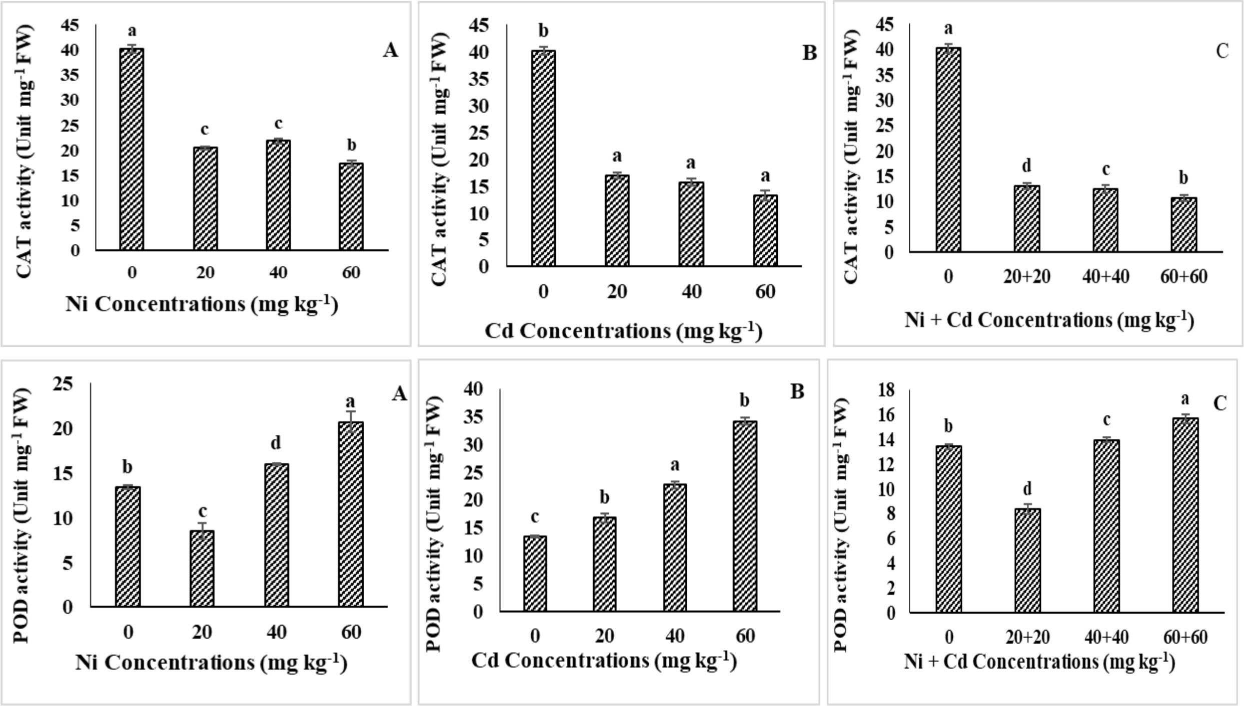

Fig. 2: Catalase (CAT) and Peroxidase (POD) activities in leaves of *Grewia asiatica* L. in response to Nickel (Ni), Cadmium (Cd) and Ni+Cd treatments respectively. Values presented are means across three replicates with ± S.E.

POD activity was decreased at 20 mg kg^{-1} i.e. 36% when plants were treated with only Ni. Increase was observed when concentration of Ni was increased. A total of 19% and 54% rise was noted for 40 and 60 mg kg^{-1} respectively. A consistent rise of 25%, 69%, and 155% was noted in POD activity at 20, 40 and 60 mg kg^{-1} when Cd was applied to the plants.

Under combined treatment POD activity was reduced at 20 mg kg^{-1} i.e. 37%. While there was a small increase in peroxidase at 40 and 60 mg kg^{-1} i.e 4.03% and 16.94% respectively. (Fig. 2).

DISCUSSION

Heavy metals enter the plants and further translocate to different tissues and organs. Under unfavorable abiotic conditions, production of free radicals such as thiyl and hydroxyl radicals induce damage to membrane proteins, DNA, cellular integrity and other physical strengthening mechanisms.

Nevertheless, plants have an innate selective mechanism to endure whatever environment offers to them. The mitigation of stress in plants is achieved by the production of special chemical substances which include enzymatic and non-enzymatic constituents. For this study we have reported lipid peroxidation in response to elevated levels of Ni and subsequent enzymatic defense in *G asiatica*.

The extent of membrane damage was evaluated through the production of MDA which is a measure of lipid peroxidation. Several studies have demonstrated an affirmative relationship between the amount of MDA and destruction of biological membranes that is more accumulation of MDA, higher the lipid peroxidation and greater extent of the damage (Sreekanth et al., 2013).

The degree of lipid peroxidation increased in response to both metals however, the application of combined metal stress (Ni+ Cd) resulted in greater accumulation than in the presence of single metal. The highest level of metal concentration induced more damage to cellular membrane when MDA content were used as predictors. These findings are in close conformity of many other workers who reported ROS generation such as O^{-}_{2}, $^{1}O_{2}$, -OH, which can initiate lipid peroxidation in response to exceeding metal levels (Soares et al., 2016). However, metals in the lowest concentrations resulted in moderate reactivity of ROS thus lesser damage to biological membranes (Vranova et al., 2002). The result of this study clearly indicated that combination of both metals increased lipid peroxidation (Fig.1) that led to enhanced MDA content in plants tissues (Pandey et al., 2009).

The deleterious effects of ROS are scavenged by the productions of several antioxidant enzymes comprising of SOD, CAT and POD (Sharma et al., 2012). The results showed that more SOD activity has been noted when Ni + Cd are applied together, while insufficient response of SOD to scavenge oxidative stress in leaves of *G. asiatica* was observed when Ni was applied separately. The enhanced production of POD was observed in response of Ni and Cd than in combination of both metals. Thus, lower production of SOD seems to be compensated by the enhancement in the activity of POD to avoid oxidative damage in leaves (Fig. 2). The results depicted a decrease in SOD in the leaves of *Helianthus annuus* when given only Cadmium stress (Mihailovic and Drazic, 2011) and the same trend was observed in *Pisum sativum* L. for Ni (Sandalio et al., 2001). The stimulation of SOD activity can be attributed to the generation of ROS or by switching on of the genes responsible for SOD production (Nadgorska-Socha et al., 2013).

A decrease in SOD activity was noticed when Ni was applied separately to *G. asiatica* plants and these findings are in close conformity with Lu et al. (2010). The activity of enzyme as estimated by their concentration was more pronounced under higher dose of Cd and thus protective mechanisms adapted by plants appears to be operated under elevated Cd levels through increased antioxidant enzyme production (Awasthi and Sinha, 2013). An increased SOD activity was observed when Ni and Cd treatments were given in combination to plants that can be attributed to greater extent of stress being imposed by the two metals together resulting in more superoxide production (Hussain et al., 2013).

CAT activity was decreased when plants were given Ni and Cd individually. Though many researchers reported an elevated CAT activity to protect plants from oxidative damage (Foroozesh, 2012). A decreasing trend in CAT generation in plants that were treated with both Cd and Ni in combination could be explained in the light of findings of Pandey et al., 2009 who reported reduction in CAT in response to combination of metal stress where the monomeric CAT subunits were broken down to tetrameric molecules that might behaved as POD. There are some other reports on the effect of Ni toxicity on CAT activity. Madhava Rao and Sresty (2000) showed a decreased CAT activity in pigeon pea at elevated Ni levels. CAT activity also decreased when Cd was applied individually. The results of this study are in accordance with Lombardi and Sebastiani, (2005) and Cho and Seo (2005).

Gajewska and Skłodowska (2010) described that peroxidase-catalyzed reaction involves lignification of cell wall. POD itself is utilized in consuming H_2O_2 to produce phenoxy compounds that are polymerized to generate cell wall components thus resulting in strengthening of the cells (Kovacik et al., 2009). In this study POD activity was also found enhanced in response to both Ni and Cd separately as compared to combined stress of the two metals. Xu et al. (2011) also reported similar findings for POD activity in maize seedlings.

Conclusion

Based on the results, it can be concluded that metal stress induced lipid peroxidation in *G. asiatica.* plants but the extent of membrane damage was more profound when Ni and Cd were given collectively that exerted non-antagonistic effects. The mitigation of metal stress seems to be acquired by the production of antioxidant enzymes. The enhanced activity Superoxide dismutase in a dose dependent manner signified antioxidant response of the species. The alleviation of metal stress via SOD was more profound for Cd than Ni. The activity of catalase showed a consistent decline due to its transformation from tetrameric to into monomeric subunits that is POD.

The POD then served as a significant stress mitigation factor that can break down obnoxious compounds by converting them into phenoxy substances thus thereby strengthen the cell wall. These findings contributed mechanistic approach for antioxidant defense in *G. asiatica* L. leaves. Therefore, the biomarkers MDA, SOD, CAT and POD served as useful predicators in scavenging metal stress.

Authors' contribution

SZ performed the experiments and collected data: SN and AA helped in improving the draft language, provided the technical comments while SM, being supervisor conceived the idea and designed the whole project.

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