

Original Article

Response of the peroxisomal ascorbate peroxidase (*pAPX*) gene in aquatic plants, *Hydrilla verticillata* and *Lemna minor*, to cadmium, lead, and nickel stress

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Abstract: In plants, the *pAPX* gene is an important enzyme that breaks down hydrogen peroxide into hydrogen peroxide and oxygen, lowering the level of oxidative stress. This study aimed to determine how the *pAPX* gene in *Hydrilla verticillata* and *Limna minor* react to heavy metal stress. Different concentrations of cadmium (0.5, 2, and 4 ppm), lead (0.5, 5, and 10 ppm), and nickel (1, 5, and 10 ppm) were used in addition to the control. Genefold was measured. The treatment with Cadmium slowed plant growth significantly more than treatments with lead and nickel, and it also caused a greater accumulation of the *pAPX* gene. The nickel treatment was significant in increasing by a factor of more than one other metal (Cd and Pb). The fold change showed increased values in all cadmium, lead, and nickel treatments compared to the control. The recorded value of nickel was higher than that of lead, followed by cadmium. The conclusion was that nickel significantly enhanced the response to gene expression. These results can provide a deeper understanding of the role of the *pAPX* gene in protecting plants from oxidative stress caused by heavy metals. O₂ lowers the levels of oxidative stress.

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Introduction

Water pollution is a critical form of pollution that has threatened the environment and living organisms for many years. The availability of clean, sufficient drinking water is essential for enhancing health and reducing the environmental consequences of diseases. The goal of sustainable development is to ensure universal and fair access to clean and inexpensive drinking water by 2030 (Radfard et al., 2023; Al-Abboodi, 2023). Water contamination with heavy metals is a significant problem worldwide due to the increasing impact of anthropogenic activities (Gonçalves et al., 2017). For example, shifts in diatom communities in the Euphrates River reflect ecosystem responses to heavy metal fluctuations (Ali et al., 2023). Wastewater is immensely noxious for aquatic ecosystems and human health due to increasing concentrations of pollutants (Ahmed et al., 2017; Alzurfi et al., 2018; Kamel et al., 2022).

Heavy metals exist in different chemical forms, so

removing them from the wastewater is difficult. Spatial and temporal variation in heavy metal concentrations across aquatic systems further complicates pollution management (Al-Zurfi et al., 2024). Most metals easily pass through different trophic levels to persistently accumulate in the biota because they are not biodegradable (Zhu et al., 2016; Kamel et al., 2023). Even though plants do not move, they are always being affected by different environmental factors that quickly change how much reactive oxygen species like hydrogen peroxide (H₂O₂) they make and get rid of (Li, 2023; Fadhil et al., 2023; Gonçalves et al., 2017). Plant development and biotic and abiotic stresses regulate APX gene expression. The APX responses are directly involved in protecting plant cells from adverse environmental conditions (Caverzan et al., 2012). High levels of reactive oxygen species (ROS) can slow down the metabolism of individual cells, kill tissues, organs, and whole organisms, and cause oxidative stress (Gill

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and Tuteja, 2010). H_2O_2 is one of the more stable ROS compounds. It can move between different parts of cells using aquaporins, which are molecules that help cells talk to each other and control many biological processes, including responses to stress, growth, and development (Smirnov and Arnaud, 2018).

Recent identification of lipid compounds in *Hydrilla verticillata* supports its physiological plasticity under stress (Alzurfi et al., 2019). This multifunctionality is also reflected in medicinal plants that show therapeutic potential against viral infections (Alrashedi et al., 2021). To keep plants healthy, prevent oxidative damage, and respond to biotic stress, APX is essential for eliminating ROS. The importance of antioxidant enzymes such as APX has also been emphasized in broader biological contexts, including human disease studies (Lawi et al., 2021). This enzyme belongs to class I of the heme-containing peroxidase family. It breaks down H_2O_2 into water and monodehydroascorbate (MDHA), using ascorbate as a substrate. Additionally, studies on lipid composition in *Ceratophyllum demersum* highlight how biochemical responses in aquatic plants can shift under stress conditions (Abdali and Al-Haidarey, 2022).

Aquatic plants have also been successfully employed in practical wastewater treatment due to their pollutant-absorbing properties (Ali et al., 2022). In this regard, it works similarly to GPx. MDHA naturally transforms into DHA through its peroxidase forms (Mohammadian et al., 2012). The APX enzyme's primary function is to recycle ascorbate and maintain the ascorbate-glutathione cycle within chloroplasts. It also cleans up ROS in the cytosol, mitochondria, and peroxisomes (Rizhsky et al., 2004). Our understanding of how aquatic plants survive in heavy metal-contaminated water remains incomplete. There is limited information on their long-term growth patterns, and there is a pressing need for environmentally sustainable methods to remediate water contaminated with metals such as cadmium, nickel, and lead. This is compounded by emerging pollutants like nanoplastics, which show toxic effects in microalgae such as *Chlorella vulgaris* (Qahtan and

Al-Zurfi, 2025). These problems call for the creation of environmentally sustainable strategies. The study's goal was to investigate how *Hydrilla verticillata* and *Lemna minor* respond to Cd, Ni, and Pb contamination, and how changes in gene expression related to the enzymatic antioxidant system affect seed growth rate.

Materials and Methods

Reagents: For each analytical grade, a stock solution of 1000 mg/L of pure Cd, Pb, and Ni was prepared in distilled water. The metal concentrations were determined using an atomic absorption spectrophotometer.

Design of experiment: The study was conducted in an Advanced Environment Laboratory, Ecology Department, Faculty of Science, University of Kufa, from January to November 2024, and the molecular study was conducted at Al-Ameen Foundation for Research and Studies, Najaf, Iraq. The aquatic plants of *H. verticillata* from Al-Abbasiyah Shat (center Abbasiyah city), and *L. minor* from Hawizeh Marshe (Um-Al-Naej Pond) were collected. The plants were brought to the laboratory in polyethylene bags and thoroughly washed with river water. They were thoroughly cleaned with river water and transported to the lab in plastic bags. After that, the samples were regularly cleaned with tap and distilled water to eliminate dirt or adhesions. Then, a sonicator cell (Ultrasonic-VCX130PB-USA) was used to remove any remaining bits of clinging plankton and tiny river animals.

The plants were planted for 14 days to help them adapt to living in the tap water enclosures. There were 30 plastic pots used in the experiment. Nine pots were used for Cd, nine for Pb, and nine for Ni treatments. As control, three replicates of each element concentrations were used: three treatments were exposed to different concentrations of Cd (0.5, 5, and 10 ppm), three treatments to varying concentrations of Pb (0.5, 5, and 10 ppm), and three treatments to different concentrations of Ni (1, 5, and 10 ppm). The *H. verticillata* terminal shoots were cut into 40 separate 10 cm sections, and the branches with flower

Table 1. The used primers in the current study (Cruz et al., 2021; Wu et al., 2016).

Gene	Direction	Sequence	Size (bp)	Annealing Temperature (°C)
pAPX	(5'-3')	AGAGGATCACGGGTCCATGCAC	152	62
	(3'-5')	GCAAGAACCACAGCCACGCCA		
Actin	(5'-3')	TGCTATCCTTCGGTTGGACC	160	62
		CGGACGATTCCCGTTCAG		

buds and roots were removed. *Lemna minor* was then taken (30 g) and planted in monocultures in plastic enclosures that were 30 cm long, 20 cm wide, and 18 cm high. Each container was filled with 8 L of tap water for storage, and the water level was the same throughout the experiment to compensate for any loss due to evaporation. The aquatic plants were identified before they were employed in the current experiments using proper references (Al-Saadi and Al-Mayah, 1983; Al-Mayah et al., 2016).

Quantitative real-time PCR methods: The TRIzol-up kit was used to extract the total RNA from the plant's tissues. After being frozen at -80°C, samples were allowed to thaw at room temperature. Once fully thawed, the RNA extraction procedure outlined in Pfaffl (2001) was followed.

Estimating total RNA purity: The extracted RNA purity was assessed using a UV/visible spectrophotometer based on its absorbance. Pure RNA typically exhibits an A260/280 ratio of 2.0.

Preparing the Primers: Lyophilized primers (Table 1) were reconstituted in nuclease-free water according to the manufacturer's instructions to obtain stock solutions with a final concentration of 100 pmol/μl. A 10 μl aliquot of the stock solution was then combined with 90 μl of nuclease-free water to achieve a final volume of 100 μl. This process resulted in a primer working suspension with a concentration of 10 pmol/μl. The solutions were stored at -20°C until required for use.

qPCR reaction: One-step RT-qPCR checks how much β-actin and pAPX are expressed in the samples. The GoTaq 1-Step RT-qPCR System comprises GoScrip Reverse Transcriptase and GoTaq qPCR master mix. It is a one-step real-time amplification method. The system includes Sybr Green Dye, a proprietary fluorescent DNA-binding dye tailored for RT-qPCR. The system makes it possible to determine

the amounts of RNA expression by employing the one-step RT-qPCR method, where the GoTaq® 1-Step RT-qPCR part, total RNA, primers, and nuclease-free water were put on ice to thaw, then mixed well. RT-qPCR experiments were conducted based on the Table 2 program.

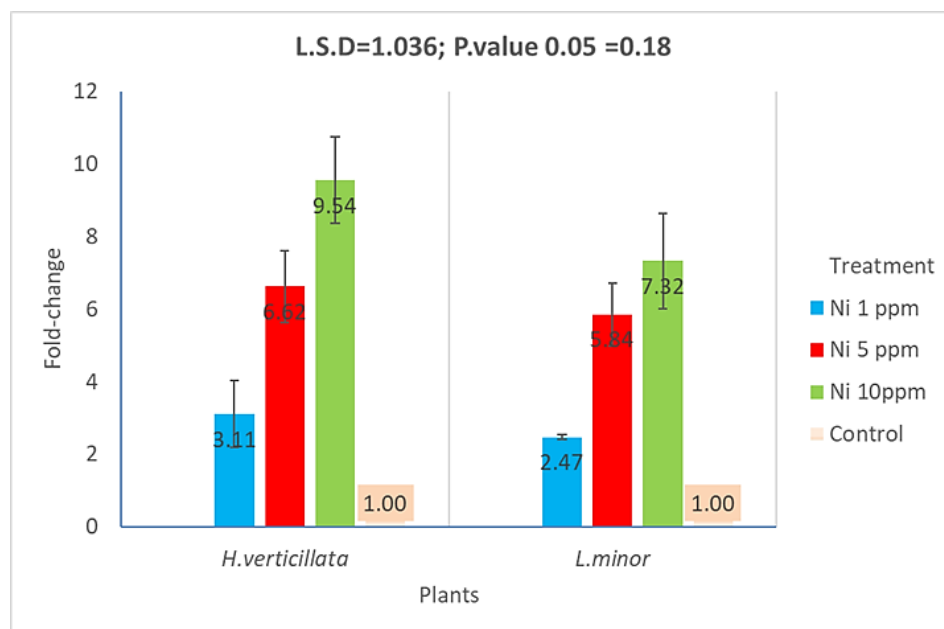
Expression level (fold change) calculation: The two techniques used to examine qPCR findings are absolute and relative quantification. Using a standard curve developed by Livak and Schmittgen (2001), the absolute quantification determined the amount of the input gene. Based on Pfaffl (2001), relative quantification is used to find changes in gene expression compared to a reference gene sample. One can also avoid standard dilution mistakes while creating a standard curve. Furthermore, the exact amounts of DNA/RNA molecules are not always as relevant as the relative gene abundance between the two treatment groups. Relative quantification is, therefore, commonly employed. The gene expression, gene fold, or RQ (relative quantification) value was calculated using the Pfaffl (2001) equation, using the formula of $RQ = 2^{-\Delta\Delta CT}$. The following approach was used to find the ΔCT value for each sample. After calculating the gene fold change, the average cycle threshold (CT) value for each triplicate sample was obtained using real-time PCR.

$$\Delta CT = CT(\text{gene of interest}) - CT(\text{reference gene})$$

The difference between the CT values of the reference gene and the gene of interest for a particular sample is known as ΔCT . Normalizing the gene of interest to one unaffected by the experiment is crucial. The following is the result of calculating the $\Delta\Delta CT$ value: $\Delta\Delta CT$ is equal to ΔCT (treated sample) minus ΔCT (control, untreated sample). After computing the CT for each sample, the equation of *Fold gene expression* $RQ = 2^{-\Delta\Delta CT}$ was used to calculate the gene expression (fold change).

Table 2. GoTaq® 1-Step RT-qPCR reaction mix.

Component	Volume Final	Concentration
GoTaq® qPCR Master Mix, 2X	10 µl	1X
GoScript™ RT Mix for 1-Step RTqPCR (50X)	0.4 µl	1X
Forward primer	0.6 µl	300 nM
Reverse primer	0.6 µl	300 nM
MgCl ₂	1.6 µl	25 mM
RNA template	5 µl	100 ng
Nuclease-free water	1.8 µl	-

Figure 2. Mean fold change pAPX gene of *Hydrilla verticillata* and *Lemna minor* at different concentrations of nickel.

Results and Discussions

Heavy metals (HM) have detrimental effects on plants, including poor photosynthesis, chlorosis, reduced biomass production, water imbalance problems, and poor nutrient absorption (Angulo-Bejarano et al., 2021). Over time, oxidative stress can damage cell structures and stop enzymes from working in the cytoplasm (Jadia and Fulekar, 2009). An often-observed result of HM toxicity is the overabundance of ROS and methylglyoxal (MG), both of which can lead to lipid peroxidation, protein oxidation, enzyme deactivation, DNA damage, disruption of ionic balance in plant cells, and/or interaction with other essential components of plant cells (Hossain et al., 2012; Jomova et al., 2023). According to Malik and Ashraf (Malik and Ashraf, 2012), ascorbic acid functions as a non-enzymatic antioxidant and is implicated in communication within the plant defense system.

Based on the results, *H. verticillata* fold change was highest at 10 ppm of Ni treatment (9.49) compared to control group (1) (Fig. 1). The Pb treatment (7.59 ppm) had the largest fold change compared to the control one in the Pb treatment (Fig. 2). In Cd treatment, the fold change showed the highest value at 4 ppm (3.7), compared to the control group (1) (Fig. 3). The fold change of *L. minor* was highest in the 10 ppm Ni treatment (7.24) compared to the control (1) (Fig. 1). The fold change of *L. minor* in the Pb treatment was highest at 10 ppm (5.33) compared to the control (2) (Fig. 2). *Lemna minor* showed a fold change at 4 ppm, and Cd treatment had the highest value in this plant (Fig. 3). Experiments with *L. gibba* confirm its efficacy in absorbing heavy metals in vitro (Al-Zurfi et al., 2018a). Similarly, *Cyperus papyrus* demonstrates significant metal bioaccumulation potential in Iraqi aquatic systems (Al-Enazi et al., 2022). The role of *Hydrilla*

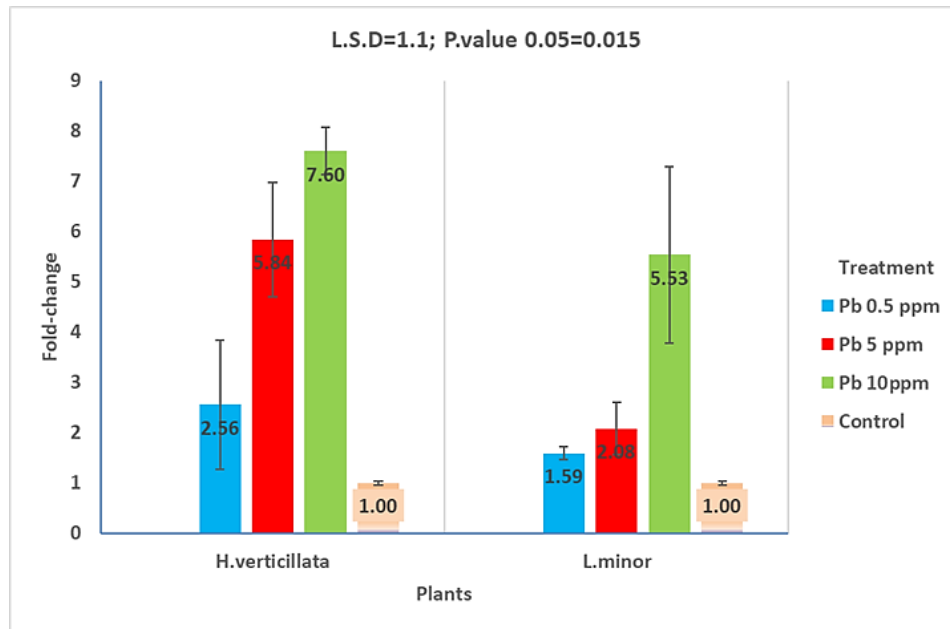


Figure 2. Mean fold change *pAPX* gene of *Hydrilla verticillata* and *Lemna minor* at different concentrations of lead.

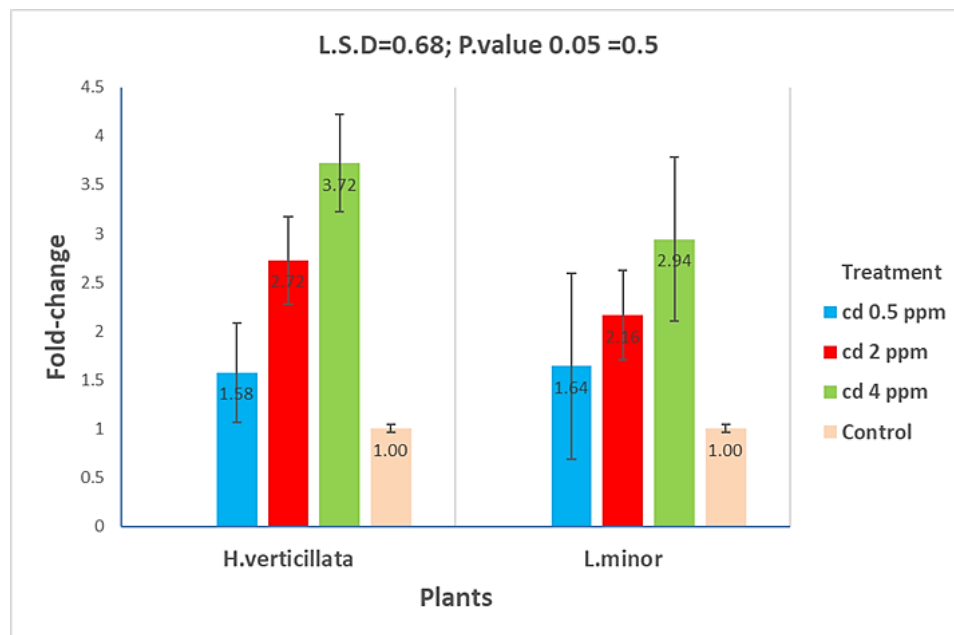


Figure 3. Mean fold change *pAPX* gene of *Hydrilla verticillata* and *Lemna minor* at different concentrations of cadmium.

verticillata in absorbing heavy metals from polluted waters has been established (Al-Zurfi and Al-Tabatabai, 2020). Similar findings show that Cd modifies this species' anatomical and physiological features (Al-Zurfi et al., 2021).

The results also showed significant differences between treatments for all heavy metals. However, there were significant differences between treatments and plants only in the Pb treatment. The study found

no significant difference between the two plants studied at the Cd treatment, but a significant difference was found between the two studied plants in the Ni and Pb treatments. This aligns with the findings in *H. verticillata* under Cd stress (Al-Zurfi et al., 2018b). Seasonal studies on Tigris River aquatic plants reveal peak metal uptake at different times of the year (Mohammed et al., 2023).

An increase in gene expression fold was observed

in the Ni-treated samples. Ni is an essential element, but its higher value can hurt plants. Plant resistance leads to higher gene expression because the pPAX gene and the peroxidase enzyme, which release excess reactive oxygen species (ROS), become more active (Cruz et al., 2021). Because of this, parts of cells cannot work as they are supposed to, which causes lipid peroxidation, oxidative protein degradation, nucleic acid and DNA disintegration, and blocking several enzymes (Dumanović et al., 2021). Metal stress significantly impacts sustainable agricultural production, plant growth, and development (Hasanuzzaman et al., 2019). Plants naturally produce various ROS due to cellular metabolism. There are non-radical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen (1O_2) as well as free radicals like superoxide anion ($O_2^{\bullet-}$), hydroperoxyl radical (HO_2), alkoxy radical ($RO\bullet$), and hydroxyl radical ($\bullet OH$) (Khan et al., 2017; Hasanuzzaman et al., 2019; Hasanuzzaman et al., 2020). On the other hand, stressed plant cells produce excessive ROS, which are very reactive chemicals that mess up plants' metabolism and damage proteins, lipids, carbohydrates, and DNA (Raja et al., 2017). According to Hasanuzzaman et al. (2019), this disruption disturbs the balance between antioxidant activity and typical ROS production, leading to plant oxidative stress.

A small amount of Ni (5 mg/l) made ROS-scavenging enzymes work better by protecting them. ROS production goes down when APX activity goes up. This makes oxidative stress and plant nanotoxicity less likely (Krishnaraj et al., 2012; El-Esawi and Alayafi, 2019). Research demonstrates that plants enhance their antioxidant systems in response to heavy metal stress. This facilitates the rapid removal of H_2O_2 and sustains growth (Mansoor et al., 2023). Our findings are consistent with those of Soliman et al. (2020) in this regard. Complementary ecotechnologies such as diatomaceous earth coagulants may enhance water treatment alongside phytoremediation (Benouis et al., 2022). Advanced AI-based biodiversity tools can aid in monitoring ecological health and heavy metal impacts in riverine

environments (Salman et al., 2025).

Conclusion

The pAPX gene plays a vital role in plants, purifying hydrogen peroxide by breaking down H_2O_2 to H_2O . Based on the results, the pAPX gene in *H. verticillata* and *L. minor* helps plants deal with oxidative stress. When the plant is exposed to Cd, plant growth is reduced compared to other metals, and the accumulation of the pAPX gene increases. As for treating the plant with NI, there was a significant increase in the response to gene expression. The current results indicate that all treatments involving the heavy elements under investigation increased the expression of the pAPX gene compared to the control groups. Ni showed the highest expression, followed by Pb and Cd. We concluded that Ni had a significant impact on enhancing the response to gene expression.

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