

Molecular responses of Vetiver grass (*Chrysopogon zizanioides*) as phytoremediator to phenanthrene soil pollution stress

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ABSTRACT

Polycyclic Aromatic Hydrocarbons (PAHs) like phenanthrene are persistent environmental pollutants. This study evaluated the potential of Vetiver grass for the phytoremediation of phenanthrene-contaminated soil and investigated key detoxification mechanisms. Vetiver grass was exposed to various phenanthrene concentrations (0-4000 ppm), and its response was analyzed. The experiment included 7 treatment levels: a negative control (soil without pollutant or acetone) (0), and phenanthrene concentrations of 250, 500, 1000, 2000, and 4000 mg/kg (ppm) of dry soil, each with 3 replicates. The results demonstrated that Vetiver grass effectively removed phenanthrene from the soil, achieving a 68.9% reduction at the highest concentration (4000 ppm). This removal was accompanied by a significant upregulation of stress-related enzymes genes. Gene expression analysis of glutathione-S-transferase phi 2 (*GSTF2*), an important detoxification enzyme increased in both roots and shoots, with the highest levels observed at 4000 ppm. Similarly, the gene expression of phenylalanine ammonia-lyase 3 (*PAL3*) another important detoxification enzyme in roots was significantly enhanced at 250 and 4000 ppm. In conclusion, Vetiver grass shows high potential for phenanthrene phytoremediation. The enhanced expression of *GSTF2* and *PAL3* genes is a crucial molecular mechanism that mitigates phenanthrene toxicity and contributes to its successful removal from contaminated soil. This research has the novelty and significance of the molecular investigation.

Keywords: *GSTF2*; *PAL3*; Phytoremediation; Polycyclic Aromatic Hydrocarbons

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are widespread environmental pollutants characterized by two or more fused benzene rings. Phenanthrene, a common aromatic hydrocarbon, is often found at the highest concentrations among PAHs in contaminated soils [1]. Its chemical formula, C₁₄H₁₀, represents a structure of three fused benzene rings [2]. Two main approaches are used for PAH removal: biological and physical/chemical methods. Biological approaches include bioreactors, phytoremediation, and bioremediation [3]. Among these, phytoremediation has emerged as a cost-effective and sustainable strategy for cleaning soil and water resources. Over recent decades, many plant species have been tested for PAH

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removal, showing significant potential for remediating contaminated sites [4]. Glutathione transferases are multifunctional proteins involved in several diverse intracellular events such as primary and secondary metabolisms, signaling and stress metabolism [5, 6]. This study evaluated the phytoremediation potential of Vetiver grass (*Chrysopogon zizanioides*) for phenanthrene uptake and investigated the expression of two key enzymes: phenylalanine ammonia-lyase (*PAL3*) and glutathione-S-transferase phi 2 (*GSTF2*). While previous studies have examined Vetiver for wastewater and heavy metal remediation, and studied its uptake of aromatic hydrocarbons in field samples [7], no research has specifically addressed Vetiver phytoremediation of phenanthrene at varying concentrations or the molecular responses of Vetiver plants to phenanthrene contamination. The molecular response of vetiver grass to phenanthrene-induced stress and increased gene expression of *GSTF2* and *PAL3* in vetiver grass associated with the reduction of phenanthrene toxicity.

MATERIALS AND METHODS

Plant cultivation, Phenanthrene Treatment and determination: Initially, 100 Vetiver seedling pots were sourced from the Vetiver propagation center (Khormoj, Boshier province, Iran) and the voucher specimen of this material has been deposited in the Shiraz University Herbarium with the number 60181. Three Vetiver plants were cultivated in sandy silt soil in the greenhouse of the Sciences Faculty of Shiraz University with four replications. This soil was homogenized and sieved and the plants were maintained for six months. The final amount of soil used per treatment was seven kg. Greenhouse conditions were 14-hour light and 10-hour dark, and also 40 and 20 centigrade degree for day and night, respectively.

The experiment included 7 treatment levels: a negative control (soil without pollutant or acetone) (0), and phenanthrene (Merck, Germany) concentrations of 250, 500, 1000, 2000, and 4000 mg/kg (ppm) of dry soil, each with 3 replicates. For preparing the phenanthrene concentrations for soil treatments 6, 12, 24, 48 and 96 grams of Phenanthrene were dissolved in two liters of acetone under mild heating (approximately 40–45°C) with continuous magnetic stirring, which enhanced solubility and allowed complete dissolution of the required amount. Then, the solution was cooled to room temperature and waited for complete acetone evaporation before spraying (200 ml) on the soil. The soil was completely mixed after the spraying process to ensure homogeneous contamination. Vetiver plants were irrigated every one week (1.5 liters) according to the soil's field capacity over six months. At the end of this period, plant samples (leaves and roots) and soil samples were collected for analysis.

Plant samples were ground in a mortar using liquid nitrogen. One-gram of the powdered plant samples was mixed with a 200 ml solvent composed of acetone and dichloromethane in a 1:1 volume ratio and subjected to ultrasonic extraction for one hour to extract phenanthrene. The 70 ml of supernatant was separated and the extraction process was repeated three times. The resulting solutions were combined and the phenanthrene concentrations were analyzed three replicates using High-Performance Liquid Chromatography, HPLC (AZURA model, KNAUER company, Germany).

For soil samples collected from the rhizosphere around the roots of the plants, 0.5 grams of soil were homogenized and mixed with 10 ml of the acetone and dichloromethane solvent in a 1:1 volume ratio. This mixture was also placed in an ultrasonic device for one hour for phenanthrene extraction. The solution was separated and the extraction steps were repeated three times. The combined solutions were then analyzed to determine the phenanthrene concentrations using HPLC (AZURA model, KNAUER company, Germany).

Analytical validation of phenanthrene determination was performed to ensure accuracy and reliability. Anthracene was used as internal standard and phenanthrene-d10 as surrogate standard. Recovery tests were conducted by spiking uncontaminated soil and plant samples with known phenanthrene concentrations, yielding recoveries of 85–92%. Blank samples (solvent and soil without phenanthrene) confirmed absence of background contamination. Calibration

curves were prepared at five concentration levels with correlation coefficients ($R^2 > 0.99$). As no certified matrix reference material was available, homogenized clean soil was used as matrix reference for calibration and recovery assessment.

Bioaccumulation and translocation indices and gene expression assay: Bioaccumulation factor (BAF) was calculated as the ratio of phenanthrene concentration in roots to its concentration in rhizosphere soil of planted pots at harvest. Translocation factor (TF) was calculated as the ratio of phenanthrene in shoots to roots. Concentrations were expressed as ppm on a dry-weight basis.

Leaf and root samples from *C. zizanioides* plants, were frozen in liquid nitrogen and stored at -20°C for 7 days until RNA extraction using kit. To analyze the expression of the genes encoding GSTF2 and PAL3 the total RNA was extracted [6]. In this section, the phenanthrene treatments 0, 250, 4000 ppm were chosen for control and also low and high concentrations.

First-strand cDNA was synthesized from 1,000 ng of DNase-treated total RNA using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Cat. No. K1621), according to the manufacturer's protocol. Reverse transcription was primed with a mixture of 0.5 μL oligo(dT) primer and 0.5 μL random hexamers in a final volume of 20 μL . Synthesized cDNA was diluted 1:5 with nuclease-free water and stored at -20°C .

Gene-specific primers for *GSTF2*, *PAL3*, and the reference gene *TBP* were designed using AlleleID v7.8 (Premier Biosoft) to span exon–exon junctions, minimizing genomic DNA amplification risk (Table 1). All reactions were performed in triplicate using RealQ 2× Master Mix Green (Ampliqon, Germany) on a real-time PCR system. Thermal cycling conditions were: Initial denaturation: 95°C for 10 min, 40 cycles of: 95°C for 15 s (denaturation), 60°C for 60 s (annealing/extension), Melt-curve analysis: 60°C to 95°C (0.5°C increment, 5 s/step).

Table 1: Sequence of specific reverse primers for real-time PCR

Genes	Primer Sequences	Sizes (bp)
<i>TBP</i>	Forward: 5'- GACGCCTCAAGCTATCCTCC -3'	244
	Reverse: 5'- TTGACACGGATCGAGGGTTG -3'	
<i>GSTF2</i>	Forward: 5'- CTCGTCCGCAACCCGTTT -3'	124
	Reverse: 5'- TCTGGCTTGTTTTGCGAGC -3'	
<i>PAL3</i>	Forward: 5'- GGTCAAGATCGAGGGTTCCA -3'	89
	Reverse: 5'- GCGTTGAGATGCCTGAGC -3'	

Statistical analysis: The experiments were carried out with 4 replications. The Shapiro-Wilk normality test was performed on the data. It indicated a p -value of greater than 0.05 suggesting that data followed a normal distribution. The procedure of One-way analysis of variance was taken using (ANOVA), and using the statistical program of SPSS software version 26.0 for analysis of variance. The level of significance was checked at $p \leq 0.05$ using Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Phytoremediation is an effective approach for reducing contamination by polycyclic aromatic hydrocarbons (PAHs) when plants are given sufficient time to establish and degrade pollutants. In this study, Vetiver grass was used to remove phenanthrene from pot soil, and concentrations were measured after six months using HPLC. Phenanthrene levels decreased in both planted and unplanted soils, but the reduction was greater in the presence of Vetiver.

At an initial concentration of 250 ppm, phenanthrene decreased by 96.31% in planted pots compared with 68.03% in unplanted pots. Similar trends were observed at higher concentrations, with reductions of 92.13, 82.77, 81.20, and 68.90% in planted soils versus

62.94, 57.59, 49.64, and 37.71% in unplanted soils at 500, 1000, 2000, and 4000 ppm, respectively. These results indicate that Vetiver enhanced natural degradation processes and contributed to pollutant uptake. The higher removal efficiency at lower concentrations suggests greater effectiveness under moderate contamination, whereas efficiency decreased at very high concentrations (e.g., 4000 ppm), possibly due to plant stress or saturation of uptake capacity. Nisa and co-workers reported that *C. zizanioides* facilitated the removal of aromatic hydrocarbons from contaminated soils [7]. Overall, the results confirm the significant role of Vetiver grass in enhancing phenanthrene reduction in soil through phytoremediation.

As soil phenanthrene concentration increased, its accumulation in Vetiver roots also increased, reaching the highest level at 4000 ppm. No phenanthrene was detected in the leaves indicating that uptake was restricted to the roots. BAF values ranged from 1.44 at 250 ppm to 0.43 at 4000 ppm, while TF remained zero across all treatments, demonstrating strong root sequestration and no upward translocation to shoot (Table 2). Roots of *Lolium perenne* accumulated phenanthrene and pyrene with limited translocation to aerial parts [8]. Similarly, the dense and deep root system of Vetiver provides a large surface area for pollutant absorption through transport pathways in root membranes, explaining the confinement of phenanthrene accumulation to roots and the increased uptake at higher soil concentrations. Overall, the results demonstrate a root-specific mechanism for phenanthrene uptake and sequestration, supporting the potential of Vetiver grass for phytoremediation of contaminated soils.

Table 2: Mean Phenanthrene soil, root and shoot concentration and their bioaccumulation (BAF) in Vetiver grass

Treatment (ppm)	Soil with plant (ppm)	Root (ppm)	BAF_root
250	9 ± 0.5	13 ± 0.4	1.44 ± 0.06
500	38 ± 0.7	31 ± 0.6	0.82 ± 0.09
1000	167 ± 0.8	84 ± 0.3	0.50 ± 0.04
2000	367 ± 0.4	234 ± 0.5	0.64 ± 0.03
4000	1212 ± 0.9	519 ± 0.7	0.43 ± 0.08

Note: All treatments showed significant difference with each other at 0.05.

GST are a family of enzymes with more than 25 variants that conjugate the tripeptide glutathione (γ -Glu-Cys-Gly) to pollutant molecules, facilitating their transport into the vacuole [9]. In this study, *GSTF2* gene expression in both roots and shoots of Vetiver grass significantly increased under phenanthrene stress at concentrations of 250 and 4000 ppm compared to the control, forming three distinct statistical groups. At 250 ppm, *GSTF2* expression increased by 50% in roots and 33.33% in shoots relative to the control. At 4000 ppm, expression rose by 80% in roots and 61.54% in shoots, indicating a strong enzymatic response to severe phenanthrene stress. Overall, *GSTF2* expression gene was consistently higher in roots than in shoots, suggesting that this enzyme plays a critical role in detoxification, particularly in roots directly exposed to the contaminant (Fig. 1A and 1B).

PAL is widely recognized as a marker of environmental stress in various plant species [10]. In this study, *PAL3* gene expression in Vetiver roots showed a significant increase under phenanthrene stress at concentrations of 250 and 4000 ppm compared to the control, with results statistically grouped into three distinct categories. At 250 ppm, *PAL3* expression in roots increased by 23%, while at 4000 ppm the increase reached 54.55%, nearly double the level observed at the lower concentration. In contrast, no significant increase in *PAL3* expression was detected in the shoots at either concentration (Fig. 1C and 1D). This indicates that the plant's response to phenanthrene stress was primarily localized in the roots, which are directly exposed to the contaminant. The molecular response enhances the resilience of Vetiver grass and contributes to its phytoremediation potential in phenanthrene-contaminated soils.

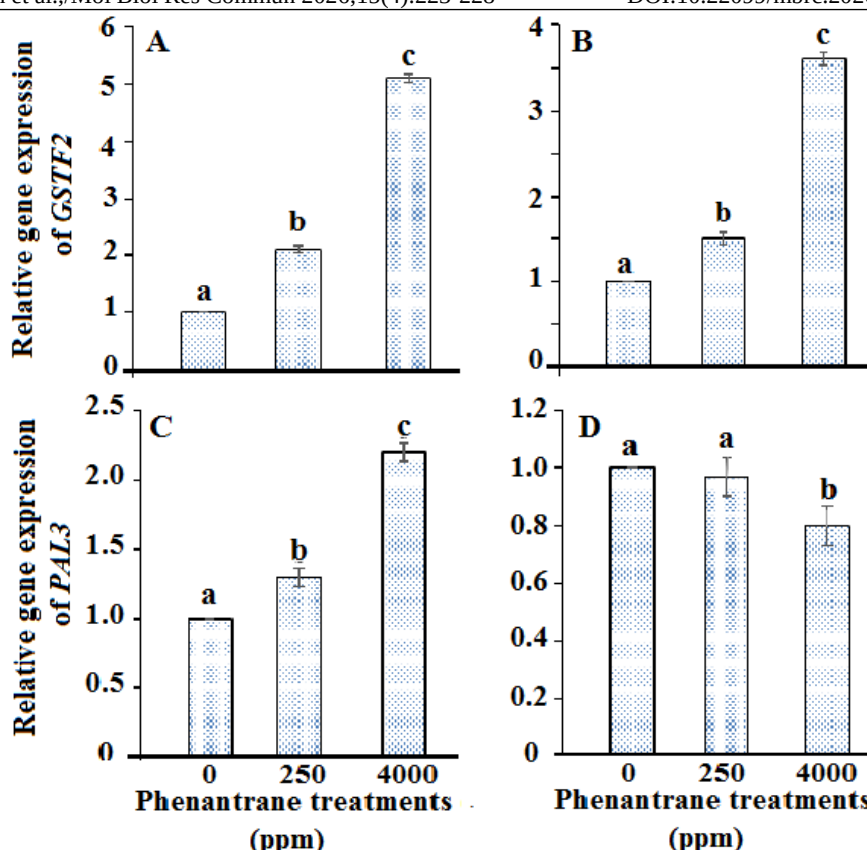


Figure 1: Means comparison of phenanthrene impact on the expression level of glutathione S-transferase F2 (GSTF2) (A and B) and phenylalanine ammonia-lyase 3 (PAL3) (C and D) of *C. zizanioides* roots (A) and shoots (B) in soil. Identical letters indicate the absence of a significant difference at the 0.05 level. Vertical bars indicate the standard error of the mean.

Conflict of Interest: None

Authors' Contribution: This research was accomplished with the collaboration of all authors. NN performed the experiments, methodology, analysis and interpretation of the data. SM designed and supervised the study, validation and investigation. ZJ interpretation of data and wrote the manuscript. MZ contributed to the conceptualization, review and editing. MN performed the experiments, methodology of the data. All authors read and approved the final manuscript.

REFERENCES

1. Wise SA, Sander LC, Schantz MM. Analytical methods for determination of polycyclic aromatic hydrocarbons (PAHs)—a historical perspective on the 16 US EPA priority pollutant PAHs. *Polycycl Aromat Compd* 2015;35(2-4):187-247.
2. Boström CE, Gerde P, Hanberg A, Jernström B, Johansson C, Kyrklund T, Rannug A, Tornqvist M, Victorin K, Westerholm R. Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air. *Environ Health Perspect* 2002; 110:451-488.
3. Singh SK, Singh RK. An overview on remediation technologies for polycyclic aromatic hydrocarbons in contaminated lands: a critical approach. *Environ Dev Sustain* 2025;27:2753-2787.

4. Jia H, Li J, Li Y, Lu H, Liu J, Yan C. The remediation of PAH contaminated sediment with *mangrove* plant and its derived biochar. J Environ Manage 2020;268:110410.
5. Mohsenzadeh S, Esmaili M, Mohabatkar H. Isolation and characterization of Phi class glutathione transferase partial gene of Iranian barley. Mol Biol Res Commun 2012;1:21-26.
6. Mohsenzadeh S, Malboobi MA, Razavi K, Farrahi-Aschtiani S. Physiological and molecular responses of *Aeluropus lagopoides* (Poaceae) to water deficit. Environ Exp Bot 2006;56: 314-322.
7. Nisa WU, Rashid A. Potential of vetiver (*Vetiveria zizanioides* L.) grass in removing selected PAHs from diesel contaminated soil. Pak J Bot 2015;47:291-296.
8. Yu XZ, Wu SC, Wu F, Wong MH. Enhanced dissipation of PAHs from soil using mycorrhizal ryegrass and PAH-degrading bacteria. J Hazard Mater 2011;186(2-3):1206-1217.
9. Kang F, Chen D, Gao Y, Zhang Y. Distribution of polycyclic aromatic hydrocarbons in subcellular root tissues of ryegrass (*Lolium multiflorum* Lam.). BMC Plant Biol 2010;10:210.
10. Valifard M, Mohsenzadeh S, Niazi A, Moghadam A. Phenylalanine ammonia lyase isolation and functional analysis of phenylpropanoid pathway under salinity stress in *Salvia* species. Aust J Crop Sci 2015;9:656-665.