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Research Article

Differential gene expression associated with heavy metal resistance and altering polyphenolic profile in *Tamarix hispida* grown in two different habitats differing in heavy metal pollution

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Abstract

The issue of heavy metal pollution poses a significant environmental concern, exerting detrimental effects on the growth and viability of plant life. Plants have evolved various mechanisms to effectively manage heavy metal stress, including the ability to modify their gene expression patterns. This adaptive response allows plants to mitigate the detrimental effects caused by excessive heavy metal accumulation. By altering their gene expression, plants can regulate the production of specific proteins and enzymes that aid in heavy metal detoxification and tolerance. Also, the changes that the plant makes in the production rate of secondary metabolites, including polyphenols, can be a mechanism to deal with the toxicity of environments contaminated with heavy metals. This sophisticated adaptation enables plants to maintain their physiological functions and overall health in the presence of heavy metal stress. This study utilized real-time reverse transcription polymerase chain reaction (RT-PCR) to examine and compare the differential gene expression of the plant, *Tamarix hispida* in addition to using HPLC to identify the amount of polyphenols in this plant. The plant was harvested from areas with varying levels of heavy metal pollution, including both non-polluted and polluted environments. The findings of this study reveal a noteworthy increase in the all *NAC* studied genes in *T. hispida*, within the contaminated site when compared to the uncontaminated area. Also, the consistent and inconsistent changes in the amount of polyphenols in this plant show that some polyphenols such as tamarixtein, hesperidin, galic acid and protocatechuica acid increased and some decreased such as quercetin, rutin, carsnoic acid, naringin acid and apigenine in the polluted environment. These results suggest that these genes and expression of secondary metabolites may play a crucial role in the process of metal detoxification, which allows the plant to tolerate heavy metals. The findings of our study offer valuable insights into the intricate molecular mechanisms underlying the resistance of *T. hispida* plant to heavy metals. Additionally, we identify promising candidates that could be utilized in genetic engineering approaches for phytoremediation purposes.

Keywords: Environment contamination, Kashgar tamarisk, *NAC*, RT-PCR, Tamarixtein

Introduction

The term "heavy metals" is used to describe elements that possess a significantly high atomic weight and density (Abbasi *et al*., 2023). These elements can either occur naturally or become released into the environment as a result of human behaviors (Abbasi *et al*., 2023; Danielyan and Chailyan, 2019). The environmental distribution of these elements, which are caused by various human activities, can be classified as the discharge of industrial waste, the application of fertilizers, the disposal of sewage, mining operations, and smelting activities, among others (Zargari *et al*., 2020; Zeng *et al*., 2023). Heavy metals such as iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), and nickel (Ni) are essential micronutrients that play an essential function in supporting the growth and development of plants (Arif *et al*., 2016). However, the presence of excessive concentrations of these metals can have detrimental impacts on plants, exceeding the acceptable threshold as indicated by previous studies (Arif *et al*., 2016; Mohammadi *et al*., 2021). Non-essential heavy metals, such as cadmium (Cd), lead (Pb), chromium (Cr), mercury (Hg), and arsenic (As), were shown to have harmful influences on plants, even at low concentrations (Mousavi *et al*., 2022). The presence of heavy metals in the environment poses a significant threat to plant health, as it can result in various physiological and biochemical disorders. These

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disorders include oxidative stress, impairment of cellular membranes, disturbance of nutritional balance, suppression of enzyme activity, and even DNA mutation (Pourakbar *et al*., 2007a; Lawal *et al*., 2021).

Plants have evolved a variety of mechanisms to mitigate the detrimental effects of heavy metal stress (Pourakbar *et al*., 2007b). These mechanisms can be broadly classified into two distinct strategies: avoidance and tolerance (Pasricha *et al*., 2021). The primary objective of avoidance mechanisms is to lower the uptake or translocation of heavy metals in plants. Various mechanisms are involved in this procedure, including root exudation, cell wall binding, membrane transporters, and vacuolar sequestration. The tolerance mechanisms discovered in plants have been intricately designed to effectively counteract the adverse effects of heavy metals (Skuza *et al*., 2022). These mechanisms primarily aim to either minimize the harmful impact of heavy metals or aid in their detoxification process within the cellular framework of plants (Mousavi *et al*., 2021). Various mechanisms are involved in the response of organisms to stressors (Dalvi and Bhalerao, 2013). These mechanisms encompass processes such as chelation, activation of the antioxidant system, osmotic adjustment, and the synthesis of stress-related proteins. The regulation of these systems involves molecular networks that regulate the expression of various genes in response to signals from heavy metals (Ghori *et al*., 2019).

Gallic acid, protocatechuic acid, tamarixtein, rosmaric acid, caffeic acid, and hesperidin are examples of plant polyphenols (Li *et al*., 2003). Polyphenols are a heterogeneous collection of phytochemicals present in plants, characterized by a fundamental monomer structure consisting of a phenolic ring with at least one hydroxyl group linked to it. They are categorized into various classes, such as tannins, lignans, phenolic acids, phenolic alcohols, flavonoids, stilbenes, coumarins, and chalcones. Flavonoids are the predominant group of polyphenols, encompassing several subclasses including flavanones, flavanols, flavonols, isoflavonoids, flavones, and anthocyanidins. Polyphenols are present in various plant sources, such as grapes, apples, berries, oranges, pomegranate, tomatoes, coffee, tea, wine, and olive oil. These substances are recognized for their ability to prevent oxidation and have been extensively researched for their potential to promote good health and treat different illnesses (Mousavi *et al*., 2021; Singla *et al*., 2019).

Differential gene expression analysis is a highly effective technique employed to identify the specific genes involved in plant responses to stress induced by heavy metals (Nosek *et al*., 2020; Peng *et al*., 2021; Soni *et al*., 2021; Xie *et al*., 2015). Reverse transcription polymerase chain reaction (RT-PCR) is a frequently used conventional technique that plays a crucial role in the validation of gene expression. The proposed methodology entails the utilization of primers and probes for the purpose of selectively amplifying and detecting the expression of specific target genes. Several studies have utilized these methodologies to investigate the specific patterns of gene expression associated with the emergence of heavy metal tolerance in various plant species (Liu *et al*., 2009; Santos *et al*., 2018; Srivastava *et al*., 2007; Zhang *et al*., 2020).

The *NAC* genes are accountable for the encoding of transcription factors that pertain to the *NAM*, *ATAF*, and *CUC* (*NAC*) family. The family under consideration is recognized as a prominent and very heterogeneous group of transcription factors that are exclusive to plants (Song *et al*., 2022; Wang *et al*., 2013). It is noteworthy for its substantial size, consisting of over 100 members in numerous plant species. The hypothesis put forth in our study suggests that the expression of these genes would undergo differential regulation when exposed to heavy metal stress in the two plant species.

A number of *NAC* genes, specifically *NAC5*, *NAC6*, *NAC7*, *NAC9*, and *NAC17*, have been extensively studied for their involvement in the response to heavy metal stress (Xue *et al*., 2023). Recent research has revealed that the *NAC5* gene, present in rice, exhibits a remarkable ability to enhance resistance against cadmium and oxidative stress. This enhanced resistance is achieved through the up regulation of two crucial genes: Glutathione S-transferase and metallothionein. The findings of this study shed light on the potential of the *NAC5* gene in improving the tolerance of rice plants to harmful environmental factors (Singh *et al*., 2021). The *NAC6* gene, identified in the model plant species Arabidopsis, has been discovered to confer enhanced tolerance to heavy metals such as cadmium and copper (Zhu *et al*., 2021). The *NAC7* gene has been recognized as a key player in improving the tolerance of cotton plants to zinc and copper (Zhang *et al*., 2019). Recent research has revealed a significant breakthrough in the field of plant biology, specifically in relation to the *NAC9* gene found in tomatoes. This gene has demonstrated a remarkable capacity to enhance the plants' resilience against the harmful effects of lead and cadmium toxicity. The mechanism behind this phenomenon lies in the *NAC9* gene's ability to regulate the expression of two crucial genes: Glutathione peroxidase and phytochelatin synthase (Xue *et al*., 2023). These findings shed light on the intricate molecular pathways that enable tomatoes to combat heavy metal toxicity, offering valuable insights for future agricultural practices and environmental remediation strategies. The recent discovery of the *NAC17* gene in grapevines has unveiled its potential to enhance the plant's capacity to withstand and adapt to drought conditions. The regulation of jasmonic acid production is responsible for this achievement. Furthermore, it has been suggested by Jensen and Skriver (Jensen and Skriver, 2014) that the *NAC17* gene could potentially have implications in the cellular response to heavy metal stress.

Tamarix hispida, a perennial plant species, exhibits a remarkable capacity to flourish in ecosystems

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characterized by elevated levels of salinity and alkalinity (Yang *et al*., 2017). In a recent study, it was discovered that *T. hispida* possesses a remarkable capability to accumulate substantial amounts of heavy metals within its tissues, all while displaying no visible indications of toxicity (Yang *et al*., 2016). The primary aim of this research endeavor was to perform a comprehensive comparative analysis of differential gene expression in *T. hispida*. This plant was harvested from two distinct habitats with varying levels of heavy metal contamination. The main objective of this study was to ascertain the particular genes that might be involved in the resistance mechanisms of the respective subjects.

Materials and methods

Plant sampling and pollution measurement: Plant sample of *T. hispida* was collected from two distinct habitats in Iraq, namely the Rumaitha Water Project (RWP) and Al-Khdher District (AKD) (Figure 1). The RWP site has been identified as a location affected by pollution, primarily due to the release of industrial wastewater. In contrast, the AKD site has been designated as a non-polluted area and is currently utilized for agricultural activities. The collection of samples took place in September 2022, adhering to the ethical guidelines set forth by the local authorities. In order to ensure the preservation of plant species, a total of three replicates were collected from each location. These replicates were carefully stored in liquid nitrogen, a widely recognized method for maintaining the integrity of biological samples. This meticulous approach guarantees the long-term viability and accessibility of these plant specimens for future research and conservation efforts. Samples of soil and water were collected from each designated site and carefully retained in glass containers.

Heavy metals measurement: The concentrations of lead (Pb), chromium (Cr), and mercury (Hg) in the soil and water samples along with plant samples were determined utilizing atomic absorption spectroscopy (AAS) with the PerkinElmer AAnalyst 800 instrument. In accordance with established protocols, Jindy *et al*. (2020) adhered to the standard procedures for sample preparation, calibration, and analysis.

The heavy metals were detected using the following wavelengths and had the following detection limits: Lead (Pb) at 217.0 nm with a detection limit of 0.01 mg/L; chromium (Cr) at 357.9 nm with a detection limit of 0.005 mg/L; mercury (Hg) at 253.7 nm with a detection limit of 0.001 mg/L; and cadmium (Cd) at 228.8 nm with a detection limit of 0.002 mg/L. The calibration curves were generated by utilizing standard solutions containing heavy metals. Quality control was ensured through the utilization of blanks, duplicates, and certified reference materials. In this study, three repetitions were done to ensure the reliability of the results.

Polyphenol content measurement: High-performance liquid chromatography (HPLC) was used to assess the polyphenol content and composition of the plant samples (Karimi *et al*., 2022; Cui *et al*., 1999). A C18 column (250 mm × 4.6 mm, 5 µm) was used for the separation, and it was run at 25°C with a mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The following was the gradient elution: 5 minutes, 5 percent B; 15 minutes, 15 percent B; 25 minutes, 25 percent B; 35 minutes, 35 percent B; 45 minutes, 45 percent B; 55 minutes, 100% B; 65 minutes, 100 percent B; and 70 minutes, 5% B. The injection volume was 20 µL, and the flow rate was 1 mL/min. Gallic acid, protocatechuic acid, tamarixtein, rosmaric acid, caffeic acid, hesperidin, chlorogenic acid, p-coumaric acid, carsnoic acid, and vanillic acid displayed a detection wavelength of 280 nm; naringin, rutin, quercetin, kaempferol, quercetin-3-O-rutinoside, apigenin-7-O-glucoside, and naringin-O-glucoside were detected at 330 nm. By comparing the retention periods and peak areas with the standards, the polyphenols were identified and quantified. The sum of the individual polyphenols was used to determine the overall polyphenol content.

RNA extraction and RT-PCR: In this study, the extraction of total RNA from whole parts of plant samples, including the root and shoot, was performed using the TRIzol reagent (Invitrogen), following the guidelines provided by the manufacturer. The quantification and assessment of RNA quality were performed using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and 1% agarose gel electrophoresis. A total of 1 gram of RNA was employed in the process of reverse transcription, utilizing the SuperScript III First-Strand Synthesis System (Invitrogen) and following the guidelines provided by the manufacturer. The RT-PCR experiment was conducted utilizing the Step One Plus Real-Time PCR system, produced by applied biosystems. Real time PCRs were performed in a volume of 12.5 µl using Maxima SYBER Green/Fluorescein qPCR Master Mix (Fermentas, Germany) according to the manufacturer's recommendations. The study utilized specific primers for each gene, as outlined in Table 1. In the present study, *α-tubulin* and *β-tubulin* were employed as internal reference genes for normalization in *T. hispida*. The thermal cycling conditions employed in this study were as follows: The temperature was adjusted to 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. The relative expression level of each gene was calculated using the Expression Fold Change $2^{-\Delta\Delta CT}$ method (Amraee *et al*., 2019).

The statistical analysis was conducted using SPSS software, version 26. The pollution levels and gene expression levels among the two sites were compared using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. In statistical analysis, a p-value below the threshold of 0.05 is widely accepted

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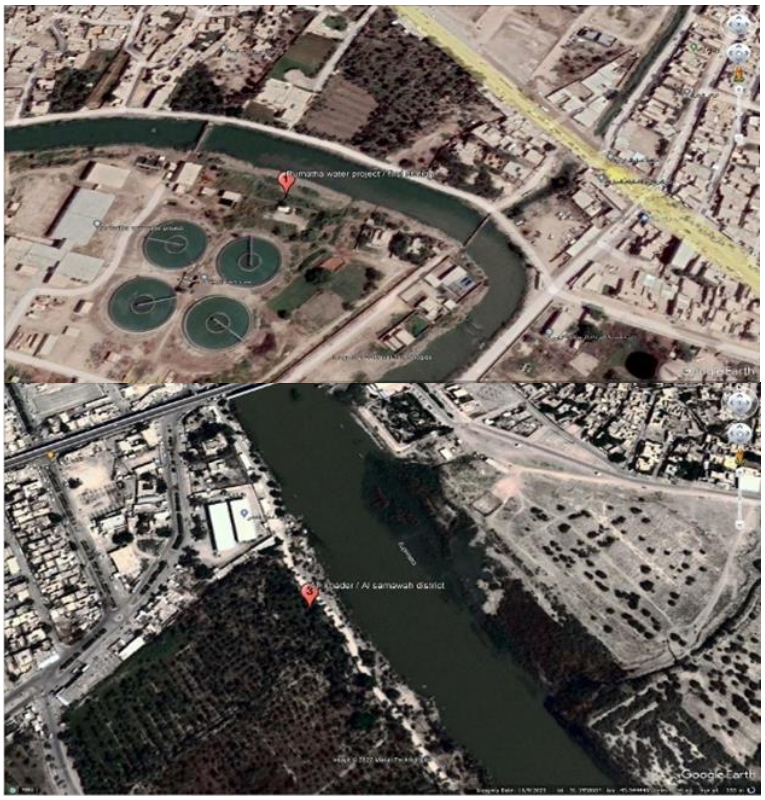


Figure 1. Sampling locations in Iraq. Upper: Rumatha water project, Lower: Samawah-Al-khadhir district.

Table 1. List of primers employed in Real-Time PCR for RT-PCR

Primers	Forward (5'-3')	Reverse (5'-3')	Gene Bank Accession number	Reference
ThNAC5	AAAGACAGGAGGGTGATG	CAACGCAAGAAGATGAAGT	JQ974959	(Wang *et al*., 2014)
ThNAC6	CAAGACTGGCAGAACTAAG	GTACGAGGTCCTGGAATCAC	JQ974960	Same
ThNAC7	TGGACGAGCAGAATGACAG	AACAGACAATCGCAAACA	JQ974961	Same
ThNAC9	TTCCTGGGCAGTGAACAA	AGAAAGACGCAATAGACC	JQ974963	Same
ThNAC17	GGTTCTGGAAGGCTACTG	TCTTCTTGAAGGCTCGGC	JQ974971	Same
Thβ-tubulin	CAACAAATGTGGGATGCT	GGAAGCCATAGAAAGACC	FJ618519	Same
Thα-tubulin	CACCCACCGTTGTTCCAG	ACCGTCGTCATCTTCACC	FJ618518	Same

as an indicator of statistical significance. The graphs and tables were generated using MS Excel.

Results

Pollution levels of Pb, Cr, and Hg in soil and water samples: The levels of pollution pertaining to lead (Pb), chromium (Cr), and mercury (Hg) in soil and water are visually represented in Tables 1 and 2. The amount of each heavy metal in RWP versus AKD was measured at 22.5 v 13 for Pb, 25 v 24.5 for Cr, and 0.009 v 0.008 for Hg in soil samples (All the units are mg.kg) (Table 1). The water samples measured 0.05 v 0.06 for Pb, 0.12 v 0.13 for Cr, and 0.0002 v 0.0003 for Hg concentration (mg.kg) (Table 2). The analysis of variance (ANOVA) yielded noteworthy findings regarding the pollution levels observed at the two sites in relation to various metals present in soil samples ($P<0.01$), but the concentration of heavy metals in the control area (AKD) of the water samples surpasses that of the contaminated area (RWP). This occurred as a result of collecting water samples in the polluted region near the plant's roots, which was necessitated by the prevailing environmental conditions. Naturally, the presence of the plant led to a decrease in contamination. However, in the control area, water samples were collected at a location that was far away from any vegetation.

Levels of Pb, Cr, and Hg in *T. hispida*: The concentrations of Pb, Cr, and Hg heavy metals in the *T. hispida* plant have been documented and presented in Table 3. The measurement shows that in *T. hispida*, levels of Pb (16.86 v 0.01 mg.kg), Cr (10.54 v 0.69 mg.kg) and Hg (0.62 v 0.007 mg.kg) are different in the contaminated and non-contaminated sites. The analysis of variance (ANOVA) revealed significant variations in the concentrations of these metals across the two habitats for the plant ($P< 0.01$). The results also indicate that *T. hispida* grown in RWP habitat contained higher levels of Pb, Cr, and Hg compared to AKD ($P < 0.01$).

Concentration of different phenols in *T. hispida*: The polyphenol content in both plants was quantified

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Table 2. Pollution levels of Pb (Lead), Cr (Chromium), and Hg (Mercury) (mg/kg) in soil and water collected from two different habitats, Rumatha Water Project (RWP), the contaminated region and Al-Khadhir District (AKD), the non-contaminated

Sample	Site of study	Pb (mg/kg)	Cr (mg/kg)	Hg (mg/kg)
Soil	RWP	22.5 ± 1.09 [a]	25 ± 1.12 [a]	0.009 ± 0.0001 [a]
	AKD	13 ± 0.91 [b]	24.5 ± 0.98 [a]	0.008 ± 0.0002 [a]
Water	RWP	0.05 ± 0.001 [c]	0.12 ± 0.003 [b]	0.0002 ± 0.0001 [b]
	AKD	0.06 ± 0.004 [c]	0.13 ± 0.002 [b]	0.0003 ± 0.0001 [b]

Different letters show significance at the P ≤ 0.0 levels

Table 3. Levels of Pb, Cr, and Hg in *T. hispida* collected from the Al-khadhir district (AKD), non-contaminated and Rumatha Water Project (RWP), the contaminated habitats.

Sample	Site of study	Pb (mg/kg)	Cr (mg/kg)	Hg (mg/kg)
Soil	RWP	16/89 ± 1.23 [a]	10/54 ± 0.78 [a]	0/62 ± 0.009 [a]
	AKD	0/01 ± 0.002 [b]	0/69 ± 0.01 [b]	0/007 ± 0.001 [b]

Different letters show significance at the P ≤ 0.0 levels

using High-Performance Liquid Chromatography (HPLC). The obtained data demonstrate substantial and considerable alterations in certain polyphenols.

The levels of polyphenols, including gallic acid, protocatechuic acid, tamarixtein, caffeic acid, hesperidin, p-coumaric acid, and naringin, have been found to increase in *T. hispida* plants under conditions of heavy metal pollution stress. Among these polyphenols, hesperidin (in AKD vs RWP = 2824.49 vs 4117.68 µg/ml) and tamarixtein (2257.98 vs 4059.7 µg/ml) exhibit the highest concentrations in the contaminated site. The most significant increase is observed in protocatechuic acid, which had an undetectable concentration under normal conditions but was identified at a concentration of over 2004 micrograms per milliliter of plant concentrate in the contaminated site.

However, in the interim, quercetin-3-O-rutinoside apigenin-7-O-glucoside naringin-O-glucoside exhibits a minor decline in the contaminated environment, while quercetin (1755.42 vs 1457.73 µg/ml) and carsnoic acid (1664.88 vs 1064.94 µg/ml) demonstrate a substantial and noteworthy decrease. Rutin, on the other hand, experiences a severe decline to the extent that its quantity cannot be quantified in the polluted environment (788.27 µg/ml vs not detectable). More details about the polyphenolic profile in the *T. hispida* plant are given in Figures 2 and 3.

Gene expression levels of *NAC* genes in *T. hispida*: The gene expression levels of *NAC* genes in *T. hispida* are depicted in Figure 4. The analysis of variance (ANOVA) manifests significant disparities in gene expression levels across the two examined locations for all genes under study ($P < 0.01$).

In the present study, a significant down-regulation of the mRNA levels of *NAC5* and *NAC6* genes was observed in *T. hispida* plants grown in RWP locations as compared to AKD ($P< 0.01$). The comparative analysis of transcript abundance between the RWP and AKD samples revealed a significant reduction of 48% and 52% in the expression levels of the *NAC5* and *NAC6* genes, respectively, in the RWP sample as compared to the AKD sample.

Additionally, the *NAC7*, *NAC9*, and *NAC17* genes displayed a significant upregulation in mRNA abundance in the contaminated RWP area compared to the non-contaminated AKD location ($P < 0.01$). Specifically, the *NAC7* gene exhibited a 17.1-fold increase in expression, while the *NAC9* gene showed an 18.5-fold increase and the *NAC17* gene displayed a 3-fold increase in expression.

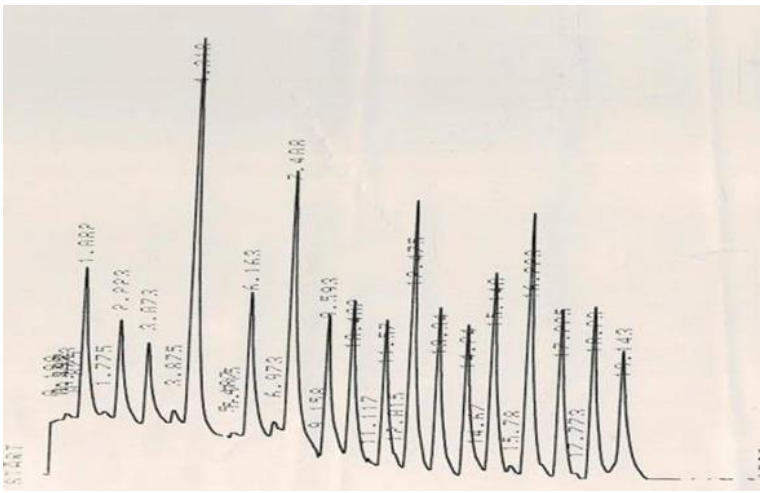
Correlation analysis between heavy metal pollution and gene expression levels: The correlation coefficients between pollution levels and gene expression levels are shown in Table 4. The results of Pearson's correlation analysis indicated that there were significant positive correlations between the pollution levels of Pb, Cr, and Hg in water and the transcript level of the expression of the NAC7, NAC9, and NAC17 genes in *T. hispida* ($P < 0.01$). However, significant negative correlations were detected between the heavy metals' levels of Pb, Cr, and Hg and the mRNA levels of NAC5 and NAC6 genes in *T. hispida* ($P < 0.01$).

Discussion

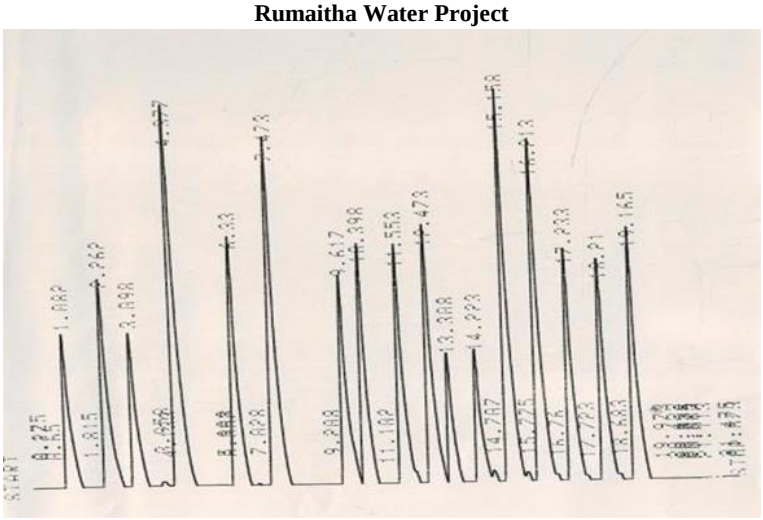
The presence of heavy metal contamination can adversely impact the alteration of polyphenols. Heavy metals are perilous and resistant to decomposition, presenting a threat to plants, animals, and the overall ecosystem (Arif *et al*., 2016). Polyphenols have diverse functions in the green fabrication of polyphenol nanomaterials and find utility in medical and environmental domains (Cheng and Wen, 2022). Heavy metals can induce genotoxicity in plants, leading to DNA damage and genomic instability (Dutta *et al*., 2018). Environmental heavy metal contamination, especially from industrial wastewater, can result in detrimental impacts on organisms, including humans, and give rise to a range of diseases (Koduru *et al*., 2017). Heavy metals and metalloids in soil can accumulate in living organisms and pose a threat to human health, plants, and animals (Pourakbar *et al*., 2007b; Abbasi *et al*., 2023). It is imperative to monitor the levels of heavy metals in the environment and implement strategies for their removal. Heavy metals exert detrimental effects on all living organisms,

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Rumaitha Water Project



Al-Khadir District

Figure 2. HPCL diagram of polyphenolic compounds of *Tamarix hispida* collected from Rumaitha Water Project (RWP) and Al-Khadir District (AKD).

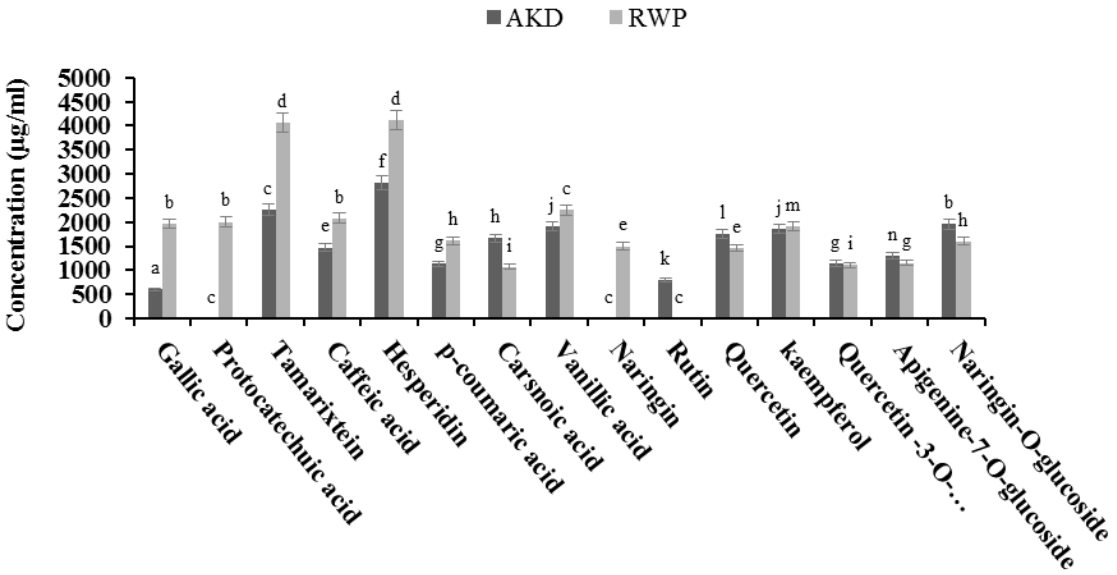


Figure 3. The amount of polyphenols of *T. hispida* plant in the control site compared to the site contaminated with heavy metals. Al-Khadir District (AKD) indicates the control site, and Rumaitha Water Project (RWP) indicates the heavy metal-contaminated site. The unit of measurement of polyphenols is micrograms per milliliter.

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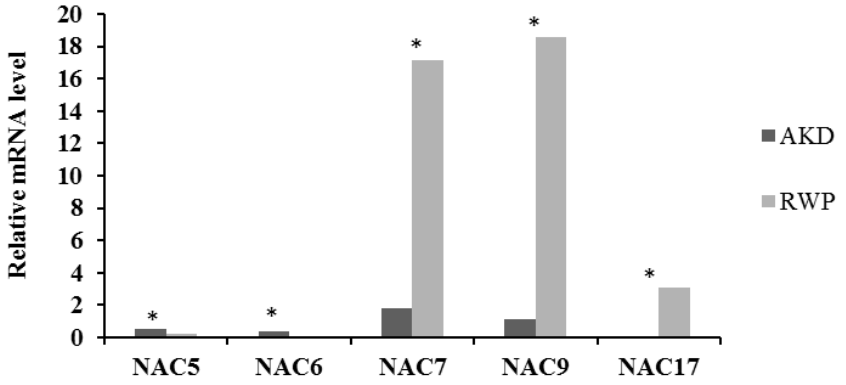


Figure 4. Gene expression levels of *NAC* genes in *T. Hispidae* relative to the control site Al-Khadir District (AKD) (mean ± SD, n = 3). Asterisks indicate significant differences among the sites for each gene (P < 0.05).

Table 4. Correlation coefficients between the pollution status of Pb, Cr, and Hg and the mRNA abundance of *NAC* genes in *T. hispida.*

Gene	Pb	Cr	Hg
NAC5	-0.76	-0.74	-0.72
NAC6	-0.79	-0.77	-0.75
NAC7	0.91	0.89	0.88
NAC9	0.92	0.90	0.89
NAC17	0.81	0.79	0.78

causing tissue damage, impairing organ functionality, and disrupting reproductive processes (Mohammadi *et al*., 2021). Accumulation of heavy metals in soils can result in diminished plant growth, modified metabolism, and DNA harm (Dutta *et al*., 2018).

The effects and functions of gallic acid, protocatechuic acid, tamarixtein, caffeic acid, hesperidin, p-coumaric acid, and naringin in heavy metal resistance in plants have been investigated. Multiple studies have demonstrated that these substances, specifically organic acids and phenolic compounds, possess the ability to function as chelators and mitigate the harm caused by heavy metal toxicity. They have been discovered to enhance plants' ability to tolerate heavy metals by affecting their physiological and molecular responses. In addition, plants have developed diverse defense mechanisms, such as root exudates, subcellular structures, chelation, osmoregulation, and antioxidant systems, to withstand the effects of heavy metal stress. Phenolic compounds, such as caffeic acid and ferulic acid, contribute to the plant's ability to adapt to heavy metal exposure. In general, these compounds help the plant withstand the harmful effects of heavy metals and improve its ability to tolerate heavy metals (Mousavi *et al*., 2021; Mohammadi *et al*., 2021; Wang *et al*., 2019).

Our research indicates that heavy metal contamination can induce various alterations in the polyphenolic composition of plants. In our study, we observed an increase in the levels of gallic acid, protocatechuic acid, tamarixtein, caffeic acid, hesperidin, p-coumaric acid, and naringin in the *T. hispida* plant when exposed to heavy metal stress conditions. Additionally, quercetin, carsnoic acid, and rutin levels experienced a substantial decline in the plant when it was subjected to an area contaminated with heavy metals.

The results of our study reveal a significant increase in the expression of the *NAC7*, *NAC9*, and *NAC17* genes in *T. hispida* at the site affected by pollution, in comparison to the unaffected region. The aforementioned findings indicate that these specific genes potentially possess a significant role in the intricate mechanism of heavy metal detoxification. The findings of this study also reveal a notable contrast in the expression of *NAC5* and *NAC6* genes in *T. hispida* at a polluted location. The down-regulation of these genes suggests a negative regulation in response to the presence of heavy metal stress.

The *NAC* genes play a crucial role in encoding transcription factors that fall into the NAM, ATAF, and CUC (*NAC*) families (Hu *et al*., 2006). Transcription factors are known to have a significant impact on the regulation of various stress responses in plants (Tran *et al*., 2010). The *NAC* genes have demonstrated significant involvement in plant defense mechanisms against both pathogenic organisms and non-living stressors, including drought, salt, cold, and heat (Manna *et al*., 2021; Barua *et al*., 2022; Anwar and Kim, 2020; Abdelraheem *et al*., 2019). This study presents a novel investigation into the distinct patterns of *NAC* gene expression in *T. hispida* in response to heavy metal-induced stress. It is the first documentation of such patterns, providing valuable insights into the molecular mechanisms underlying the plant's response to environmental stressors. The results obtained from our comprehensive study provide compelling evidence suggesting that the *NAC7*, *NAC9*, and *NAC17* genes

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possess the potential to act as facilitators of heavy metal resistance. These genes have been observed to play a crucial role in stimulating the transcription of downstream genes that are closely associated with detoxification or tolerance processes (Zhang *et al*., 2019). In contrast, it is plausible to consider the *NAC5* and *NAC6* genes as potential inhibitory modulators of heavy metal resistance. These genes may exert their influence by suppressing the transcription of downstream genes that are involved in stress adaptation or protective mechanisms.

NAC genes are recognized for their role in controlling the expression of stress-related genes through transcriptional regulation, contributing to the regulation of plant stress resistance (Mathew and Agarwal, 2018). NAC transcription factors modulate plant growth and development via influencing plant hormone signaling pathways, perhaps including the control of genes associated with phenolic chemical production (Zhang *et al*., 2019). NAC genes play a role in controlling the production of secondary metabolites, such as phenolic compounds, in plants. This indicates that NAC genes may have an impact on the accumulation of phenolic compounds in plants (Ma *et al*., 2016). Research has demonstrated that genetically engineering plants to increase the expression of NAC genes is feasible to improve the plant's biomass and ability to withstand stress. This, in turn, can have an indirect impact on the production of phenolic chemicals that play a role in the plant's defense mechanisms (Wang and Dane, 2013).

Conclusion

The concentrations of lead (Pb), chromium (Cr), and mercury (Hg) in the root tissues and shoot tissues of *T. hispida* provide insights into their respective capacities for accumulating or translocating heavy metals in response to stress. The findings of this study indicate that the *NAC7*, *NAC9*, and *NAC17* genes in *T. hispida* potentially play a role in augmenting the accumulation or translocation of heavy metals in plant. This effect is likely achieved through the enhancement of chelation or transport capacity within the plant. In contrast, the *NAC5* and *NAC6* genes identified in *T. hispida* have been found to potentially mitigate the accumulation or translocation of heavy metals within plants. This is achieved through a reduction in the plants' uptake or transport capacity for these metals.

The present study provides significant findings regarding the molecular mechanisms underlying plant resistance to heavy metals, while also identifying promising prospects for genetic engineering to improve phytoremediation approaches. Further research is necessary to elucidate the regulatory networks and precise functional roles of these genes. The investigation of the regulatory mechanisms governing these genes is of significant importance, particularly with regard to the upstream signaling pathways or transcription factors that possess the ability to detect and transmit heavy metal signals. Furthermore, it is highly beneficial to conduct a thorough examination of the influence exerted by these genes on subsequent physiological or biochemical processes that play a role in the establishment of resistance or tolerance towards heavy metals. In addition, it is crucial to evaluate the effectiveness of these genes in transgenic plants under actual field conditions and when subjected to different types of heavy metals.

In this study, we have made a significant breakthrough by identifying five genes (*NAC5*, *NAC6*, *NAC7*, *NAC9*, and *NAC17* for *T. hispida*) that demonstrate distinct patterns of gene expression in the presence of heavy metal stress. These findings were obtained through the application of Real-time PCR, a widely used technique in molecular biology. Evidence has been presented to establish a correlation between specific genes and the presence of Pb, Cr, and Hg contamination. This finding suggests that *NAC5*, *NAC6*, *NAC7*, *NAC9*, and *NAC17* genes may play a crucial role in enabling plants to develop resistance against heavy metal exposure. The findings of our research provide significant contributions to the understanding of the molecular mechanisms involved in plant adaptation to heavy metal stress. These insights offer promising opportunities for improving plant productivity and optimizing the effectiveness of phytoremediation strategies.

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