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Gene expression dynamics in alfalfa under lead-induced stress

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Abstract
Gene expression in response to heavy metal stress plays a crucial role in plant adaptation and survival. High concentrations of metals like cadmium (Cd) and lead (Pb) can be toxic to cells, disrupting metabolic and physiological functions. To mitigate this stress, plants activate complex genetic regulatory networks, including genes involved in heavy metal homeostasis, detoxification, chelation, oxidative stress response, and transcriptional regulation. Phytochelatins, chelating proteins that bind toxic metals and reduce their harmful effects, are not directly produced by the expression of a heavy metal tolerance gene. Instead, they result from a metabolic pathway that utilizes glutathione as a substrate, involving the enzymes γ-glutamylcysteine synthetase, glutathione synthetase, serine acetyltransferase, and cysteine synthetase. This study aimed to evaluate the expression of genes encoding these enzymes in alfalfa plants exposed to varying lead concentration. Gene expression varied based on cultivar type (tolerant/sensitive), treatment duration, and Pb concentration in the substrate. Individual variability was also observed within the same cultivar. The most significant effect of Pb treatment occurred after one day, with increased expression of γ-glutamylcysteine synthetase and glutathione synthetase genes, particularly in the sensitive cultivar, which consistently exhibited higher gene expression levels.

Keywords: Medicago sativa, biotic stress, heavy metals, RT-qPCR

Introduction

Heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg), arsenic (As) and copper (Cu) are chemical elements that can become harmful to plants when found in high concentrations. Although some metals, like copper and zinc, are essential micronutrients in small quantities, their excessive accumulation can severely impact plant health and disrupt environmental balance.

In natural ecosystems, these metals are introduced into the soil mainly through human activities, such as mining operations, industrial emissions, synthetic fertilizers, and irrigation with contaminated wastewater. Once present in the soil, heavy metals can be absorbed by plant roots and interfere with key physiological processes, including photosynthesis, seed germination, plant development, and reproductive function [7].

Over time, the accumulation of heavy metals in plant tissues not only adversely affects agricultural productivity by inhibiting plant growth and reducing crop yields but also facilitates the transfer of these toxic elements into the food chain [13,20,21]. This bioaccumulation poses significant health risks to both animals and humans who consume contaminated plant matter, potentially leading to chronic toxic effects, including neurological, renal, and developmental disorders. Consequently, the presence of heavy metals in agricultural systems represents a critical concern for food safety and environmental sustainability [1,2,4].

Throughout evolution, plants have developed complex natural detoxification mechanisms to manage the accumulation of heavy metals from the environment, thereby maintaining homeostasis and physiological integrity. These mechanisms include the sequestration of metals within subcellular compartments (e.g., vacuoles), controlled exclusion at the rhizosphere level, and chelation through the formation of stable complexes with organic ligands such as phytochelatins and metallothioneins, which effectively reduce the bioavailability and toxicity of these elements [8].

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The process of metal hyperaccumulation is tightly regulated at four interdependent functional levels: (1) the uptake of metal ions at the root system, mediated by specialized transporters located in the plasma membrane; (2) radial transport through root tissues toward the vascular system, involving both apoplastic and symplastic pathways; (3) systemic translocation via the xylem to aerial organs, facilitating the redistribution of metals to the above-ground parts; and (4) final storage and compartmentalization in leaves in non-toxic forms, either by vacuolar sequestration or through binding to chelating molecules to prevent harmful oxidative reactions.

These adaptations enable certain hyperaccumulator species to tolerate high concentrations of heavy metals, granting them an essential role in phytoremediation strategies and environmental decontamination efforts.

In plant cells, in response to stress caused by heavy metals, phytochelatins ((γGlu-Cys)*n*-Gly), peptides with an essential role in detoxification mechanisms, are synthesized. Through complexation with metal ions, their toxicity is significantly reduced. The resulting complexes are transported and stored in vacuoles—cellular compartments that contribute to metal sequestration and the protection of essential cellular functions [6, 7].

According to the structure of phytochelatins, they are not directly produced by the expression of a heavy metal tolerance gene, but rather by a metabolic pathway that utilizes glutathione as the substrate. This process is catalyzed by phytochelatin synthase (PCS), an enzyme whose activity is triggered by the presence of heavy metal ions in the cell. Glutathione is synthesized through a two-step, ATP-dependent process. γ-Glutamylcysteine synthetase catalyzes the first step, combining glutamate, cysteine, and ATP to form γ-glutamylcysteine, while generating ADP and inorganic phosphate (Pi) as byproducts. In the second step, glutathione synthetase adds glycine to γ-glutamylcysteine, generating glutathione in another ATP-dependent reaction.

These reactions are regulated at both transcriptional and translational levels, as well as through a negative feedback mechanism, in which glutathione inhibits the activity of γ-glutamylcysteine synthetase. An additional important element influencing glutathione biosynthesis is the cellular level of cysteine. Cysteine is synthesized in the final step by cysteine synthase, which adds a sulfhydryl group (–SH) to O-acetyl-L-serine—a compound previously obtained by acetylating L-serine with the help of an acetyltransferase.

To verify these considerations, genetically modified plants with enhanced expression of genes involved in phytochelatin synthesis were obtained and analyzed. Thus, the gene encoding cysteine synthase was introduced via genetic modification into Arabidopsis thaliana plants, Columbia ecotype. Northern blot analyses revealed a sevenfold increase in transcription in the presence of Cd in the genetically modified plants compared to the control, accompanied by an increase in metal accumulation [5]. Additionally, Brassica juncea plants were obtained by introducing the gshI gene from *E. coli*, which encodes γ-glutamylcysteine synthetase (γ-ECS). These plants exhibited higher Cd tolerance and accumulated greater amounts of metal in the shoots, with increased levels of phytochelatins and γ-ECS compared to the control [19]. Genetically modified tobacco plants expressing the cysteine synthase gene were also studied. These plants showed increased tolerance to Cd and higher phytochelatin concentrations, although their capacity for cadmium accumulation was not enhanced [10]. In other experiments on transgenic tobacco expressing the cysteine synthase gene, the increased metal tolerance was accompanied by higher metal accumulation in plant tissues [14]. To date, studies on the expression of genes involved in metal tolerance and accumulation processes have been conducted mainly on model plants [9]. Genetically modified lines with high tolerance and accumulation capacity have been developed.

However, these species typically exhibit low growth rates, produce relatively low biomass, and often can only be cultivated in their natural habitats. Moreover, genetic modification is a laborious process requiring long testing periods and sometimes faces resistance from users. Therefore, the direct use of such plants in phytoremediation is challenging. In practical bioremediation, other species can be used that produce high biomass, have good heavy metal tolerance, and high metal accumulation capacity, such as alfalfa. In addition, alfalfa has a taproot from which secondary roots branch, with approximately 80% located in the 30–50 cm soil layer, making it suitable for bioremediation of the surface soil layer, which is usually the most contaminated. Being a leguminous plant, it can grow on nitrogen-poor soils without requiring additional treatments, while enriching the soil with significant amounts of nitrogen. To understand the genetic mechanisms involved in heavy metal tolerance and bioaccumulation in alfalfa, a first step is to study the expression of genes shown to be involved in similar processes in model plants. This will enable the identification and isolation of genes that play key roles in phytoremediation [15,16,18, 22].

The aim of this study was to evaluate the expression of genes encoding the enzymes γ-glutamylcysteine synthetase and glutathione synthetase, which are involved in the biosynthetic pathway of

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phytochelatins, in *Medicago sativa* plants exposed to different concentrations of lead (10, 20, 50, 100, and 500 ppm), in order to understand the molecular response of plants to heavy metal stress.

Materials and Methods

In this study, seeds from two alfalfa (*Medicago sativa*) cultivars, Sigma and Satelit, were used. The seeds were surface-sterilized by briefly rinsing in 70% ethanol (10 seconds), followed by immersion in 0.1% $HgCl_2$ for 3 minutes, and then washed five times with sterile distilled water.

Germination was carried out on moistened filter paper in Petri dishes, kept in the dark for 5 days and then transferred to light for an additional 2 days. Seven-day-old seedlings were transferred to pots containing perlite moistened with KNOP [12] nutrient solution (pH 5.4), with four plants per cup and a total of 12 pots per cultivar.

Plants were watered every two days with KNOP solution and maintained in a growth chamber at a constant temperature of 24°C with a photoperiod of 16 hours light/8 hours dark. Lead treatment was applied when plants reached four weeks and developed 2–4 trifoliate leaves, using five concentrations of Pb (10, 20, 50, 100, and 500 ppm), each accompanied by an equimolar amount of EDTA. This single-dose treatment ensured the desired concentration within the substrate volume. Previous studies have shown that EDTA, as a chelating agent, forms a complex with heavy metals, enhancing their mobility and increasing Pb translocation from roots to leaves by up to 300%. For each treatment variant, 8 plants were used. Plant growth was monitored weekly after the treatment, by measuring the stem length of each plant.

Sampling for gene expression studies was performed at three time points: 1 day, 1 week, and 1 month after treatment. From each plant, two trifoliate leaves were harvested, placed in Eppendorf tubes, and kept at –80°C until RNA isolation.

RNA extraction from plant tissue

Total RNA extraction was performed using the kit RNAgents Total RNA Isolation System (Promega, USA) following the manufacturer's recommendations.

Reverse-transcriptase PCR

For reverse transcription PCR (RT-PCR) analysis, a *one-step* kit (**AccessQuick RT-PCR System)** from Promega was used, allowing both complementary DNA (cDNA) synthesis and subsequent amplification with specific primers to occur in a single reaction.

The primers for the genes encoding enzymes γ-glutamylcysteine synthetase and glutathione synthetase were used. The gene encoding tubulin was used as a reference gene for normalization of gene expression data. Its stable expression under the experimental conditions provided a reliable baseline for comparing the relative expression levels of the target genes.

The primers sequences were as follows:
γ-glutamylcysteine synthetase (GCS) F-5'CTTAGTGGAGCCCCTCTGGAA3' and R-5'CTGGAAACCAATCCCCAAAAA3', glutathione synthetase (GS) F-5'CAATCTTCTGCTGTCAAATGCCCTTCAA3' and R-5'TGCTTTTCTAACAATATCCGAGTCATCCA3', MtEF1α (EF1) F-5'ATTCCAAAGGCGGCTGCATA3' and R-5'CTTTGCTTGGTGCTGTTTAGATGG3'.

Results and Discussion

Assessment of the morphological effects of lead exposure

Throughout the four weeks of treatment, the Sigma cultivar showed a slight increase in growth at lower lead concentrations (20 and 50 ppm), although this positive trend diminished as the concentration of Pb increased. In contrast, the Satelit cultivar proved to be the most sensitive to lead exposure, with plants exhibiting a marked reduction in growth rate and visible symptoms of leaf chlorosis. At the highest concentration tested (500 ppm Pb), the effects were particularly severe, resulting in complete growth inhibition, pronounced leaf yellowing and desiccation—symptoms that ultimately led to the death of most plants.

The results of the measurements conducted for all experimental variants, in both the tolerant and sensitive cultivars, are summarized in the table and figure below (Tab. 1 and Fig. 1). These data provide a comparative overview of the growth responses and highlight the differences in lead tolerance between the two *Medicago sativa* cultivars under various treatment conditions.

Both *Medicago sativa* cultivars showed a gradual reduction in growth as the concentration of Pb in the growth medium increased. The Sigma cultivar demonstrated better tolerance to lower lead concentrations (10–50 ppm), maintaining relatively stable growth compared to the control, while the Satelit cultivar responded more sensitively, with a more pronounced growth reduction starting at 50 ppm.

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Table 1. Plant stem length (cm) in Sigma and Satelit cultivars under various Pb treatment concentrations

Variants	initial	1 week	2 weeks	3 weeks	Difference (3 weeks-initial)
Sigma					
control	8.81±1.79	12.94±2.57	16.94±4.26	19.31±5.47	10.5±5.76
10ppm	7.31±1.44	10.31±2.39	13.63±3.43	16.69±5.21	9.38±5.41
20ppm	6.75±1.49	9.13±2.22	12.13±2.52	14.44±3.41	7.69±3.72
50ppm	5.56±2.29	8.00±2.30	10.63±1.83	13.25±2.14	7.69±3.13
100ppm	8.06±1.92	10.50±2.51	12.69±2.72	14.75±2.93	6.69±3.50
500ppm	6.63±1.66	7.38±2.07	7.94±2.15	7.50±3.63	0.87±3.99
Satelit					
control	6.69±2.20	9.69±2.43	14.81±3.46	19.19±4.29	12.5±4..82
10ppm	6.94±2.90	10.50±2.99	15.13±3.73	18.50±3.75	11.56±4.74
20ppm	6.50±2.04	8.56±2.15	13.06±2.16	16.88±2.03	10.38±2.88
50ppm	5.25±1.77	6.75±2.71	9.75±3.45	12.37±3.85	7.12±4.24
100ppm	5.88±2.40	7.63±3.13	9.50±3.88	12.13±3.72	6.25±4.43
500ppm	4.81±2.22	5.13±2.03	4.81±3.50	4.13±3.70	-0.68±4.31

At low concentrations (10–20 ppm), both cultivars exhibited moderate reductions in stem length, but the differences compared to the control became significant at higher concentrations (100–500ppm), highlighting clear toxic effects of lead on plant development.

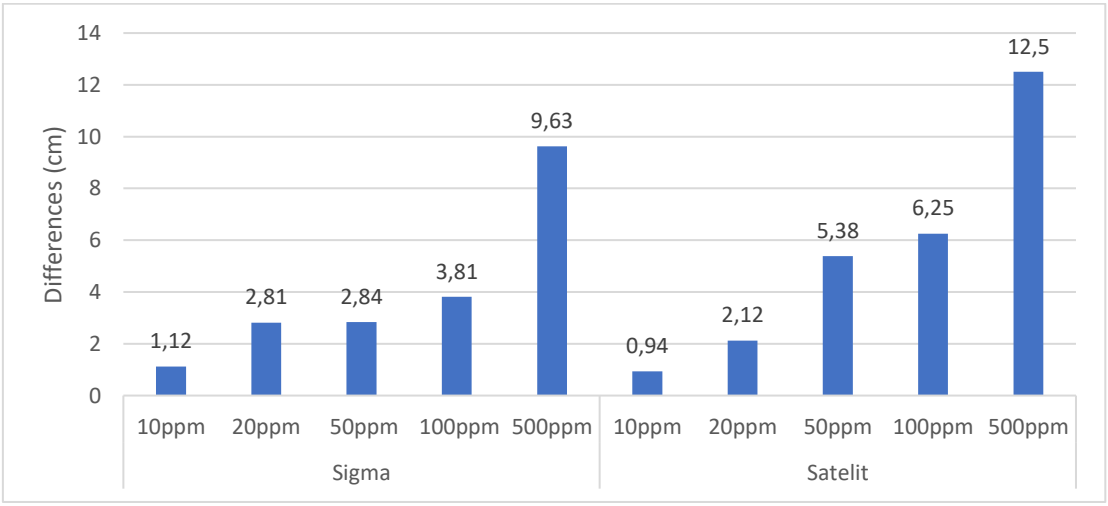

Figure 1. Growth differences relative to the control in Sigma and Satelit cultivars under treatments with varying concentrations of Pb.

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At the highest concentration (500 ppm), growth was severely inhibited: Sigma showed nearly stagnant growth (a difference of 0.87 cm from the initial value), while Satelit even exhibited a decrease in average stem length (–0.68 cm), suggesting acute toxicity.

Overall, the differences in growth relative to the control increased in proportion to the Pb dose applied, indicating a clear negative dose-response relationship. Comparatively, the Sigma cultivar displayed a higher apparent tolerance to lead stress, whereas Satelit was more adversely affected, particularly at high concentrations, highlighting significant genotypic differences in the ability to cope with heavy metal pollution.

Molecular analysis

DNA was first extracted from alfalfa plants to enable the optimization of amplification reactions. To determine the optimal annealing temperature for each gene, gradient PCR (45–65°C) was used, which also allowed the evaluation of the length of the amplified fragments (Fig. 2).

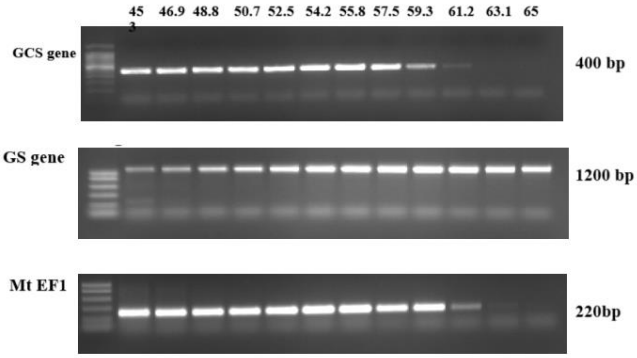

Figure 2. Electrophoretic analysis on a 1.5% agarose gel of the amplification products generated from temperature gradient PCR

For the γ-glutamylcysteine synthetase (GCS) gene, the optimal primer annealing temperature was observed to be between 45°C and 57.5°C, with the amplification product having the expected size of 388 bp.

For the primers specific to the glutathione synthetase (GS) gene, the optimal annealing temperature was determined to be between 55.8°C and 65°C. The resulting amplification product had the expected length of 1200 bp.

In the case of the reference gene (Mt EF1α) the desired amplification product of 220 bp was obtained, with an optimal primer annealing temperature between 45°C and 55.8°C. For all three genes analyzed, the specificity of the primers was confirmed by the synthesis of a single DNA fragment, which was visualized as a single distinct band on the gel

The analyses continued with the evaluation of gene expression. Following RNA extraction from plants treated with different concentrations of Pb, belonging to the Sigma (tolerant) and Satelit (sensitive) varieties, high-quality RNA free of DNA contamination was obtained. The resulting concentration ranged from 108 to 1126 µg/ml. Before amplification, the RNA from each sample was adjusted to a concentration of 0.2 µg/µl

The expression of selected genes was compared across all treatment variants in both *Medicago sativa* cultivars—Sigma (tolerant) and Satelit (sensitive)—to assess genotypic differences in molecular response to lead stress Fig. 3). To investigate the temporal variation in gene expression, molecular analyses were conducted at three distinct time points: one day, one week, and one month after treatment. This approach allowed for a comprehensive evaluation of how gene expression patterns change over time in response to prolonged exposure to heavy metal stress.

Gene expression was assessed by comparing the intensity of amplification bands obtained through RT-PCR with those of the untreated control and normalized against the reference gene (tubulin). The band intensity serves as an indicator of gene expression levels, allowing the evaluation of treatment-induced changes in both cultivars. This semi-quantitative approach provides insight into the upregulation or downregulation of specific genes involved in the plant's response to heavy metal stress.

After just one day of exposure to lead, the sensitive *Medicago sativa* cultivar- Satelit treated with 500 ppm Pb exhibited a marked upregulation of gene expression, particularly notable for genes involved in the biosynthesis of phytochelatins, suggesting an acute molecular response to heavy metal stress. This early

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activation indicates a rapid attempt by the plant to mitigate Pb toxicity through enhanced synthesis of protective compounds. By one week after treatment, both the tolerant and sensitive cultivars showed similarly elevated expression levels of the **γ-glutamylcysteine synthetase** and **glutathione synthetase** genes, especially at the 500ppm concentration, indicating a sustained activation of the phytochelatins biosynthesis pathway as a general defense mechanism regardless of cultivar sensitivity. However, by one-month post-treatment, gene expression had significantly declined and leveled out across all samples, possibly reflecting an exhaustion of the plants' stress response capacity or an adaptive rebalancing of gene activity following prolonged exposure.

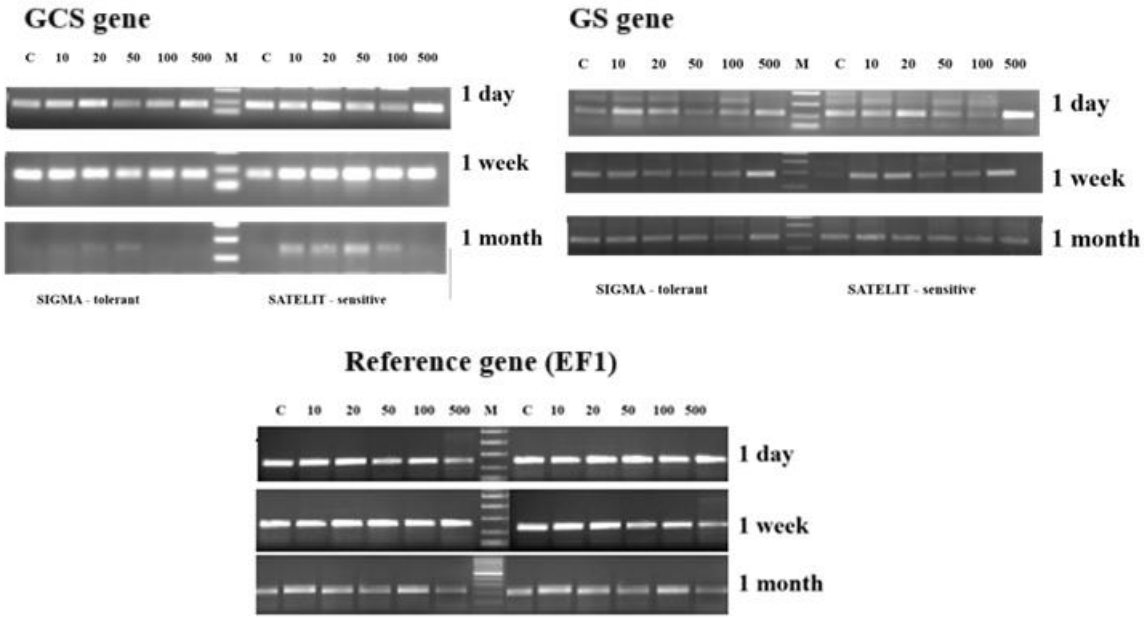

Fig. 3. Agarose gel electrophoresis (1.5%) of RT-PCR products from RNA extracted from *Medicago sativa* plants treated with different Pb concentrations, using GGS, GS, and EF1 primers, at 1 day, 1 week, and 1 month after treatment.

The results of the present study are consistent with findings reported by other authors, reinforcing the significance of specific molecular pathways in heavy metal stress response in *Medicago sativa*. Chen et al. (2018) demonstrated that the legume–rhizobium interaction plays a pivotal role in regulating the expression of genes linked to antioxidant defense, phytochelatin (PC) biosynthesis, and metallothionein biosynthesis in seedlings subjected to Cu stress [3]. Similarly, Helalui et al. (2020) observed a significant upregulation of genes involved in the phytochelatin biosynthesis pathway under varying nickel concentrations, highlighting their essential role in Ni detoxification in alfalfa plants [11]. Moreover, additional studies have emphasized the critical involvement of **glutathione reductase (GR)**, **phytochelatin synthase (PCS)**, and the **metal transporter NRAMP1** genes as key components of the molecular networks that mediate metal stress tolerance in *M. sativa* [17]. Collectively, these findings provide strong evidence for the complex genetic regulation underlying heavy metal stress adaptation in this species.

Conclusions

In conclusion, increasing lead concentrations negatively affected plant growth in both cultivars, with Sigma showing greater tolerance and Satelit exhibiting higher sensitivity, especially at elevated Pb levels.

Gene expression varied depending on the cultivar (tolerant vs. sensitive), the treatment duration, and the Pb concentration in the substrate. Individual variation was also observed among plants of the same cultivar.

The most pronounced effect of Pb treatment was recorded after one day, when a marked upregulation of γ-glutamylcysteine synthetase and glutathione synthetase genes was observed. The sensitive cultivar consistently exhibited higher expression levels. After prolonged exposure (1 month), gene expression stabilized, with no significant differences observed between cultivars, Pb concentrations, or among individual plants. Research on the expression of genes involved in phytochelatin biosynthesis will continue, with the present study serving as a foundation and starting point for future investigations.

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