Effects of Lead (Pb) and Cadmium (Cd) Elements on Lipid Peroxidation, Catalase Enzyme Activity and Catalase Gene Expression Profile in Tomato Plants

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ABSTRACT

Heavy metals are significant abiotic stress factor, affecting various response mechanisms in plants. These responses include: changes in membrane composition, production of small molecules and free radicals, and alterations in the activities of antioxidant enzymes and their gene expressions. For this reason, lipid peroxidation levels (MDA), catalase enzyme activity, and gene expression profiles, quantified by real-time PCR, were analyzed in tomato plants exposed to various concentrations (0, 80, 160, 320, 640 and 1280 μM) of Cd^{2+} and Pb^{2+}. All concentration of Cd^{+2} or Pb^{+2} contamination led to increased lipid peroxidation and catalase enzyme activity, except for 320 and 640 μM Cd^{+2} contamination levels. As a result, gene expression patterns at the mRNA level and changes in MDA content under different concentrations of Pb^{+2} and Cd^{+2} contamination revealed a positive correlation, although no correlation was found between gene expression patterns at the mRNA level and catalase enzyme activity. These results might be explained by the regulation of genes at the transcriptional, posttranscriptional, and also translational or posttranslational levels.

Keywords: Tomato (*Solanum lycopersicum* L.); Lipid peroxidation; CAT enzyme activity; Gene expression; qRT-PCR

Domates Bitkisinde Kurşun (Pb) ve Kadminyumun (Cd) Lipid Peroksidasyonu, Katalaz (CAT) Enzim Aktivitesi ve Gen Ekpresyon Profiline Etkisi

ÖZET

Önemli bir abiyotik stres faktörü olan ağır metaller bitkilerde çok çeşitli yanıt mekanizmalarını uyarabilirler. Bu yanıt mekanizmaları; membran kompozisyonunda değişiklik, küçük molekül ve serbest radikallerin üretimi, antioksidant enzimlerin aktivitelerinin ya da gen ekspresyonlarının değişimini içerir. Bu sebeple, bu araştırmada çeşitli konsantrasyonlardaki (0, 80, 160, 320, 640 ve 1280 µM) Pb^{2+} ve Cd^{2+} kontaminasyonuna maruz kalan domates bitkilerinde lipid peroksidasyon seviyesi (MDA), katalaz enzim aktivitesi ve real-time PCR aracılığı ile katalaz gen ekpresyon seviyesi belirlenmiştir. 320 ve 640 µM Cd^{+2} kontaminasyonu hariç tüm Cd^{+2} ve Pb^{+2} kontaminasyonları lipid peroksidasyonuna ve katalaz enzim aktivitesinde artışa neden olmuştur. Sonuç olarak; çeşitli konsantrasyonlarda Pb^{2+} ve Cd^{2+} kontaminasyonuna maruz kalan domates bitkisinde, CAT gen ekpresyonu ve lipid peroksidayonu arasında pozitif korelasyon bulunurken, CAT gen ekpresyonu ve enzim aktivitesi arasında korelasyon tespit edilememiştir. Bu durum genlerin transkripsiyonel, posttranskripsiyonel ve aynı zamanda translasyonel veya posttranslasyonel seviyelerdeki regülasyonu ile açıklanabilir.

Anahtar Kelimeler: Domates (*Solanum lycopersicum* L.); Lipid peroksidasyonu; CAT enzim aktivitesi; Gen ekpresyonu; Real-time PCR

1. Introduction

Pollution of the environment by heavy metals is a serious problem throughout the world. The most common heavy metals in the environment are cadmium, chromium, copper, mercury, lead and zinc and some of their main sources are emissions from burning fossil fuels, industrial activities, automotive emissions, pesticides usage and domestic wastes (Kabata-Pendias & Pendias 1989).

Heavy metal contamination of economically important plants, such as fruits and vegetables, poses a threat to their quality and leads to alterations in the health statuses of humans and animals. Several researches have indicated that consumption of vegetables loaded with heavy metals such as Cd^{+2} and Pb^{+2} can generate carcinogenic effects (Trichopoulos 1997; Türkdoğan et al 2002). Climatic changes, nature of the soil, time of harvest, and concentrations of heavy metals in the soil are significant impacts on assumed threats of heavy metals in vegetables (Lake et al 1984; Scott et al 1996; Voutsa et al 1996). In addition, post-harvest vegetables could be influenced by air pollution during transportation and marketing, which can lead to elevated levels of heavy metals (Sinha et al 2005; Sharma et al 2006; 2007).

Abiotic stress factors, such as soil salinity, drought, high temperatures, and heavy metals are known to cause oxidative stress in plants by the production of reactive oxygen species (ROS), such as O_2^-, H_2O_2, $^1O_2$, HO_2^-, OH, ROOH, ROO, and RO (Smirnoff 1993). The most important intracellular generators of ROS are chloroplasts, mitochondria, and peroxisomes. As a result of stomatal closure and limited CO_2 availability, chloroplasts generate O_2^- and H_2O_2 by the electron acceptor of photosystem I, while singlet oxygen is produced by the transfer of an electron from an activated chlorophyll molecule to molecular oxygen (Asada & Takahashi 1987; Hernandez et al 1995). It has been estimated that, under stress conditions, 1-2% of O_2 consumption leads to the formation of ROS, which causes lipid peroxidation, membrane defects, and instability of enzymes in higher plants (Mittler 2002; El-Beltagi et al 2010). On the other hand, it is important to emphasize that ROS can act not only as damaging factors, but also as protective or signaling factors, which depend on the equilibrium between ROS production and scavenging mechanisms (Gratao et al 2005).

Plants are able to develop antioxidant defense systems to protect themselves against ROS and cope with different stress factors (Rao et al 2006). The antioxidant system restricts and removes ROS damage and maintains ROS homeostasis in plant cells. The components of this system are enzymatic, such as monodehydro ascorbate reductase

(MDHAR), dehydro ascorbate reductase (DHAR), glutathione reductase (GR), ascorbate peroxidase (APX), superoxide reductase (SOD), catalase (CAT) and non-enzymatic antioxidants, such as glutathione (GSH), proline, carotenoids, and tocopherol (Mittler et al 2004).

The various antioxidants have partially overlapping functions and can functionally compensate for each other. In this regard, SOD dismutases O_2^- into H_2O_2, which, in turn, is detoxified by CAT, APX, or PRX. CAT is one of the enzymatic antioxidants with the highest turnover rates among all enzymes, and it is located mainly in the peroxisomes. One molecule of CAT can convert approximately 6 million molecules of H_2O_2 to O_2 and H_2O per minute (Lee & An 2005). In this manner, it prevents longer H_2O_2 action, which could lead to cell disturbances and DNA damage (Shim et al 2003). There have been many reports regarding catalase enzyme activity, lipid peroxidation (MDA), and gene expression levels in several different plant species under stress conditions, such as salt stress (Mittova et al 2003), boron (B) toxicity (Cervilla et al 2007), Cu toxicity (Cui et al 2010), and Pb toxicity in tomatoes (Wang et al 2008), low temperature stress in wheat (*Triticum aestivum* L.) (Matsumura et al 2002), Mn toxicity in spruce trees (*Picea abies* L.) (Polle et al 1992), pecan trees [*Caryaillinoinensis* (Wangenh.) C. Koch, cv. Kiowa] (Henriques 2003), and Cd and Cu toxicity in mouse-ear cress (*Arabidopsis thaliana*) (Skorzynska-Polit et al 2010).

The control of gene expression in all eukaryotic cells is a complex process that involves molecules such as RNA polymerases, numerous transcription factors, the DNA template, RNA produced by transcription, and protein produced by translation, with its attendant processing. The examination of gene expression often involves quantifying the abundance of a particular transcript. Contemporary methods, such as real-time PCR, that examine gene expression reveal the dynamic nature of this biochemical process. Real-time PCR allows precise measurements of mRNA steady-state levels and provides advantages, such as very high sensitivity and precise quantification of expression levels

under different conditions or treatments. This method only measures immediate levels or final accumulation of RNA in the cell. Also other modern methods, such as microarray hybridization, or more conventional methods, like Northern hybridization, do not provide information about the transcriptional activity of genes. These methods fail to quantify the stability of the RNA or the ratio of transcription at the specific loci under investigation. In order to detail understanding the nature of gene expression modulation, it is essential to evaluate alteration of transcript and corresponding protein levels which both are associated with a phenotypic change (Farrel 2007). As such, the experiments in the current study were conducted in three phases. In the first phase, after Cd^{+2} and Pb^{+2} stress treatments, MDA levels were determined in order to obtain evidence that the plants were in stress as a result of the treatments. In the second phase of the study, the steady-state level of CAT mRNA was determined by quantitative real-time PCR. Finally, in the third phase, CAT enzyme activity was determined in order to obtain an idea about the final stage of CAT gene expression in tomato samples exposed to different concentrations of Cd^{+2} and Pb^{+2} stress.

2. Material and Methods

2.1. Plant material, growth conditions, and stress treatment

Plant growth conditions and stress treatments were performed as previosly reported by Soydam Aydın et al (2013). Tomato (*Solanum lycopersicum* L. 'Falcon') seeds were germinated and grown hydroponically in pots containing 0.2 L of modified 1/10 Hoagland's solution. After germination, seedlings were transferred to pearlite and watered with 1/10 Hoagland's media in regular time interval (daily). Twenty-five-day-old six tomato plants grown in 1/10 Hoagland's media were used for the stress treatments. For the heavy metal application, Pb^{+2} and Cd^{+2} were added to the hydroponic solution for 24 h at concentrations of 0 (control), 80, 160, 320, 640 and 1280 μM. Harvested tomato leaves were ground in liquid nitrogen and used for estimation

of lipid peroxidation, CAT enzyme activity assay, RNA extraction, and gene expression analysis.

2.2. Estimation of lipid peroxidation

Malondialdehyde (MDA) content is a marker of oxidative lipid injury was performed as described and previously reported by Hodges et al (1999) and Soydam Aydın et al (2013). ELISA microtiter reader (SpectraMax M2) was used to read absorbance at 440 nm, 532 nm, and 600 nm. MDA equivalents were calculated as described by Hodges et al (1999) using Equation 1, 2 and 3.

$$[(Abs532_{+TBA}) - (Abs600_{+TBA}) - (Abs532_{-TBA} - Abs600_{-TBA})] = A \quad (1)$$

$$[(Abs440_{+TBA} - Abs600_{+TBA}) \times 0.0571] = B \quad (2)$$

$$\text{MDA equivalents (nmol mL}^{-1}) = (A - B/157000) \times 10^{6} \quad (3)$$

2.3. RNA extraction and cDNA preparation

RNA extraction was performed using a TRIzol protocol followed by RNeasy mini cleanup kit (Qiagen, Cat no: 74104). RNA quantity and quality were measured with a NanoDropND-1000 spectrophotometer. Quality of RNA was also confirmed by gel electrophoresis, containing 1.5% agarose and formaldehyde. The cDNA synthesis based on reverse transcription reactions were performed with 2 μg of RNA and a high fidelity cDNA synthesis kit (Roche) containing 2.5 μM anchored oligo(dT)18, 1X transcriptor high-fidelity reverse transcriptase reaction buffer, 20 U protector Rnase inhibitor, 1 mM deoxynucleotide mix, 5 mM DTT, and 10 U transcriptor high-fidelity reverse transcriptase at final concentration. And, the program applied was 65 °C, 10 min; 55 °C, 30 min; 85 °C, 5 min.

2.4. Real-time RT-PCR and quantification of mRNA levels

Quantitative real-time PCR was performed with a LightCycler® 480 System (Roche) thermal cycler. The sequences of primers and probes (Table 1) of the target gene catalase (CAT) and actin (ACT), which is used for normalization, were designed based on the sequences of tomato genes available in the databank (NCBI 2013a; NCBI 2013b). Amplifications of

PCR product were monitored via intercalation of hybridization probes (HyProbe, FRET probes) that allow exact and specific identification of the target gene. After pre-denaturation at 95 °C for 10 min, 45 cycles of 95 °C 10 s, 60 °C 30 s, and 72 °C 15 s were applied. Data collection for quantification was accomplished during the annealing period. Copy numbers of the genes (CAT, ACT) under stress treatment were determined by using standard curves. PCR efficiency for CAT standard curve was 1.937, while ACT was found to be 1.954.

Table 1- Primer and probe sequences of CAT and Actin

Çizelge 1- CAT ve Actin için primer ve prob sekansları

Primer and probe name	Sequences
CAT2 F	*CTTTCCTCTTCGACGATATTGGTA*
CAT2 S	*TATTCCCCAAGATTACAGGCAT*
CAT2 A	*CCGACTCGGATTGCCTT*
CAT2 R	*GTGATTTGCTCCTCCGACTC*
CAT2 FL	*CAACAGGGCTGGAAAATCAACTTATGT-FL*
CAT2 LC 640	*AAGTTCCACTGGAAGCCCACATGT p*
Actin S Fw	*TCTGTTTCCCGGTTTTGCTATTAT*
Actin R Rev	*TGCATCAGGCACCTCTCAAG*
Actin FL	*ATTCATAGCCCCCACCACCAAAC-FL*
Actin LC 640	*TCTCCATCCCATCAAAAAAACAAATTGACTp*

2.5. Catalase enzyme activity assay

Catalase enzyme activities were performed as previosly reported by Aebi (1984) and Soydam Aydın et al (2013). Powdered tomato tissues were suspended in extraction buffer and homogenates were centrifuged at 15000 *g* for 20 min, and the supernatant fraction was used for the enzyme activity assays (Jovanovic 2006). All steps were carried out at 4 °C. The catalase activity assay was performed according to the method reported by Aebi (1984), based on 240 nm absorbance.

2.6. Statistical methods

The abundance of target gene transcripts was normalized to ACT and set relative to the control plants (no stress exposure), according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). Changes in

relative expression levels (REL) of the CAT gene were checked for statistical significance according to one-way ANOVA. The results were considered statistically significant if the P value was <0.05 in Dunnett's test.

3. Results and Discussion

In the current study, the effects of heavy metal stress (Pb^{+2} and Cd^{+2}) on cell membrane, gene expression and CAT enzyme activity profiles in tomato plants (*Solanum lycopersicum* L.) were analyzed. It is known that heavy metal contamination can lead to an increase in different forms of ROS.

Malondialdehyde (MDA) analysis was used as a marker of oxidative lipid injury, which might have changed in response to Cd^{+2} and Pb^{+2} heavy metals that led to stress in tomato seedlings. Changes in MDA content were observed in the tomato seedlings treated with different concentrations of Pb^{+2}, indicating that the samples were under stress. It has been demonstrated that increased lipid peroxidation is a characteristic feature of oxidative stress caused by unbalanced equilibrium between ROS production and scavenging or defense mechanisms under heavy metal stress (Smirnoff 1993; Mittler 2002; Lima et al 2006).

Small hydrocarbon fragments, such as ketones and MDAs, which are considered the first evidence of stress in plants, have been used as indicators of lipid peroxidation or membrane damage, (Lyons 1973). In recent studies, increased lipid peroxidation has been observed in plants under heavy metal stress, such as tomato (Krupa & Baszynski 1985; 1989; Quariti et al 1997; Ben Ammar et al 2005), wheat (Malik et al 1992), barley (Vassilev 2004), and mustard (Gaur & Grupa 1994; Nouairi et al 2006). Results of the current study indicate that MDA content substantially increased with all concentrations of Pb^{+2} contamination. The maximum level of MDA content was determined in the samples exposed to 320 μM concentrations of Pb^{+2} contamination while a significant decrease was measured at 1280 μM among treated samples (Figure 1). The data also show that ROS-induced lipid peroxidation began in a short period of time and that the level of injury was dependent on the exposure concentrations

of Pb^{+2} contamination. However, MDA contents, which were obtained in the samples exposed to Cd^{+2} contamination, showed an increase only with the 80 μM and 1280 μM Cd^{+2} concentrations. The highest level of inhibition in MDA content was observed in the treatment with 1280 μM Cd^{+2}. In addition, the maximum decrease in MDA content was observed in the tomato seedlings exposed to the 320 μM concentration of Cd^{+2}. Surprisingly, lower MDA levels were observed at 160, 320, and 640 μM of Cd^{+2} treatment compared to the control samples (Figure1).
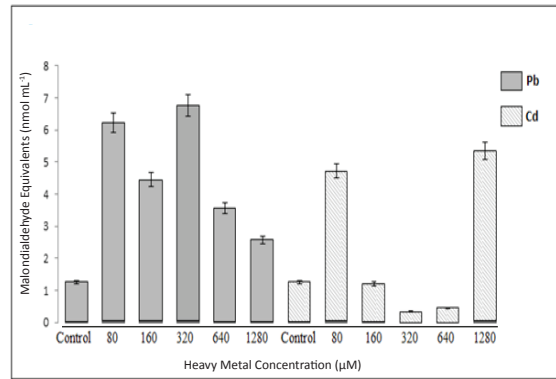

Figure 1- Lipid peroxidation (malondialdehyde, MDA content) in the total tissues of tomato plants exposed to different concentrations of Pb^{2+} and Cd^{2+}

Şekil 1- Farklı konsantrasyonlarda Pb^{2+} ve Cd^{2+}'ye maruz kalan domates bitkisinin tüm dokularındaki lipid peroksidasyonu (malondialdehit, MDA içeriği)

When evaluated SOD enzyme activity and gene expression results of tomato samples exposed to same concentration of Cd^{+2} contamination in another study conducted in our laboratory (unpublished data), it was difficult to suggest that prevention of lipid peroxidation in the cells of the tomato samples exposed to 160 μM, 320 μM, and 640 μM Cd^{+2} concentrations in the current study might result from increased activation of antioxidant mechanisms. Further analysis is necessary to explain this complex connection between lipid peroxidation and antioxidant mechanisms. Results also revealed that lipid peroxidation induced by ROS was observed after contamination of low (80 μM) and high

(1280 μM) Cd^{+2} concentrations, which generated an imbalance between production of ROS and antioxidant enzyme activity or gene expression as a component of scavenging and defense mechanisms of ROS.

ROS have a dual action, not only acting as protective or signaling factors, but also as oxidative damaging factors at the cellular level due to an imbalance between the production and removal of ROS under stress conditions (Mittler 2002). To combat the negative effects of this imbalance, an enzymatic antioxidant defense system is activated to protect plants from cellular injury with removing excessively produced H_2O_2 with the enzymes, such as peroxidase (POD), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT) (Qilin et al 2009). In recent years, genes involved in response to abiotic stress have become the main field for biotechnological studies, as it constitutes the basis for improving the stress tolerance of plants. Many genes play a crucial role in responding to environmental stresses at the post-transcriptional level, and CAT is one of these genes that encode the catalase enzyme. Studies about the protective activity of CAT in response to different abiotic stresses have been demonstrated by previous research studies conducted with *Coffea* sp., oilseed rape (*Brassica napus* L.), pea (*Pisum sativum* L.), and tomato (*Lycopersicum esculentum* L.) plants (Goupil et al 2009; Qilin et al 2009; Fortunato et al 2010).

In the current study, exposure of tomato seedlings to Pb^{+2} and Cd^{+2} led to significant changes in the abundance of CAT gene transcripts, suggesting the involvement of the gene in heavy metal tolerance (Figure 2). To evaluate the stability of our results, CAT transcript levels of all samples were measured three times for each stress condition, which indicated steady-state mRNA levels in the cells for both CAT and the reference gene ACT. With regard to the control and to each other, different expression levels were recorded in all stress conditions applied. The highest level of transcript was recorded at 320 μM of Pb^{+2} and 160 μM of Cd^{+2} concentrations, and these results were found to be statistically significant. The lowest levels of mRNA were recorded at the 640 μM of Pb^{+2} and 1280 μM of Cd^{+2} concentrations.

Contamination with different concentrations of Pb^{2+}, 80 and 320 μM Pb^{+2} led to a statistically significant increase in mRNA level in the tomato seedlings compared to the control, while the 640 μM concentration led to a reduction in the expression of the gene; these results were statistically significant (P_1= 0.001; P_2= 0.001; P_3= 0.000). Changes in the rate of expression level in the catalase gene compared to the control were statistically insignificant upon exposure to 160 and 1280 μM of Pb^{+2} concentrations. CAT expression levels decreased significantly in response to 640 μM of Pb^{+2}, which indicates that CAT was strongly downregulated by this concentration (Figure 2). CAT mRNA levels in the tomato seedlings increased in response to 80 μM and 160 μM of Cd^{+2} treatments; these results were statistically significant (P_1= 0.000; P_2= 0.000) when checked for significance according to one-way ANOVA. The 320, 640, and 1280 μM of Cd^{+2} treatments led to a decrease in catalase gene expression level compared to the control, but only the 640 μM and 1280 μM of Cd^{+2} treatment results were statistically significant (P_1= 0.005; P_2= 0.000) (Figure 2). When we analyzed this complex profile of gene expression patterns at the mRNA level and changes in MDA content under different concentrations of Pb^{+2} and Cd^{+2} contamination, the results revealed positive correlations between each other (Figure 1 and 2).
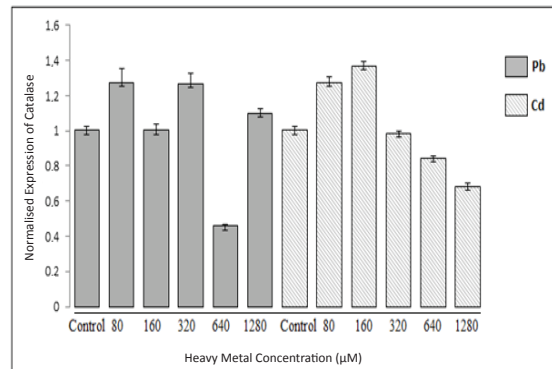

Figure 2- Catalase mRNA levels in the total tissues of tomato plants exposed to different concentrations of Pb^{2+} and Cd^{2+}

Şekil 2- Farklı konsantrasyonlarda Pb^{2+} ve Cd^{2+}'ye maruz kalan domates bitkisinin tüm dokularındaki katalaz mRNA seviyesi

Also, catalase enzyme activity assay revealed different data from gene expression patterns at the mRNA level in tomato seedlings treated with different concentrations of Pb^{+2} and Cd^{+2}. In particular, 640 μM Pb^{+2} and 80 μM Cd^{+2} contamination levels led to a significant increase in catalase enzyme activity, while gene expression levels under these contamination levels were under the control level or slightly increased (Figure 2 and 3). Increased activity of CAT enzyme indicates the ability of the cell to scavenge increased concentrations of H_2O_2; this ability was not weakened by Pb^{+2} or Cd^{+2} treatment according to the results of the current study, in accordance with previous reports (Mead 1976). The differences recorded in the enzyme activities of the plants under Pb^{+2} and Cd^{+2} stress may be due to the balance of ROS generation and antioxidant activities that must be achieved to enhance the protection of the cell.
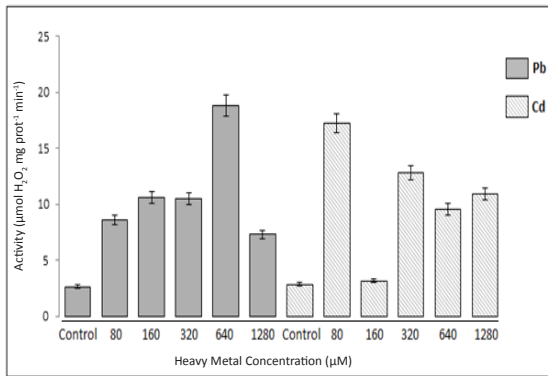

Figure 3- Catalase enzyme activity in the total tissues of tomato plants exposed to different concentrations of Pb²⁺ and Cd²⁺

Şekil 3- Farklı konsantrasyonlarda Pb²⁺ ve Cd²⁺'ye maruz kalan domates bitkisinin tüm dokularındaki katalaz enzim aktivitesi

The differential recovery in CAT enzyme activity at various concentrations of Pb^{+2} treatments, 640 Pb^{+2} and 1280 μM Pb^{+2}, might imply that the enzyme contributes to cellular defense with differing performances, depending on the concentrations of the heavy metals. In the current study, CAT gene

expression patterns at the mRNA level and changes in CAT enzyme activities under different concentrations of heavy metal treatment revealed no positive correlation or inverse proportion. For example, although the steady-state level of mRNA did not change after the 160 μM Pb^{+2} treatment (compared to control), a significant increase was observed in enzyme activity levels at the same concentration. In addition, while the mRNA level of CAT decreased in tomato plants exposed to the 640 μM Pb^{+2} treatment, enzyme activity significantly increased, and while the mRNA level of CAT increased in tomato plants exposed to the 1280 μM Pb^{+2} treatment, enzyme activity slightly increased compared to the control. The results suggest that a translationalpost-translational level of control for CAT gene expression or enzyme activity might be regulated by the reversible modulation of the functionality of its mRNA, depending on the supply of the methyl group donors from glycine and serine and that production of these amino acids is greatly enhanced by photo respiratory carbon flow. Therefore, catalase protein synthesis is associated with the photo respiratory and photosynthetic pathways (Schmidt et al 2002).

4. Conclusions

Results of the current research verified that oxidative stress alerts plant antioxidant defense systems in unsupportable concentrations of heavy metals, and that in most cases, the CAT gene is induced to encode the CAT enzyme to scavenge ROS generated under stress conditions (Lee & An 2005). The data on gene expression and enzyme activity of CAT obtained in the current study could provide a better understanding of the antioxidant mechanisms in tomato plants.

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