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Exogenous cysteine alleviates mercury stress by promoting antioxidant defence in maize (*Zea mays* L.) seedlings

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Abstract: Mercury (Hg) is one of the most important environmental pollutants that negatively affects plant growth and development. Cysteine (Cys) plays an important role in plant response to various environmental stress factors. In the present study, the alleviation of Hg stress through exogenous Cys treatment to maize seedlings was evaluated. For this purpose, a hydroponic experiment was set up to investigate the effect of $HgCl_2$ (100 µM) and in combination with Cys (200 µM) on plant growth, total chlorophyll content, reactive oxygen species, antioxidant enzyme activities, and mRNA expression levels of some antioxidant genes in maize seedlings. The results showed that $HgCl_2$ treatment significantly decreased both root and shoot growth and total chlorophyll content, also increased the malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and superoxide levels ($O_2^{\cdot-}$) in maize seedlings. After treatment with 200 µM exogenous Cys combined with 100 µM $HgCl_2$, root and shoot growth and total chlorophyll content increased and the concentration of MDA, H_2O_2, $O_2^{\cdot-}$ in seedlings notably decreased and catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), and peroxidase (POD) activities in seedlings increased significantly. In addition, $HgCl_2$ treatment alone or combination with Cys markedly increased the root and leaf Hg content. The higher amounts of Hg accumulated in the roots of the seedlings. Furthermore, qRT-PCR results showed that the mRNA levels of *CAT*, *GR*, and *SOD* genes were up-regulated at $HgCl_2$ + Cys treatment groups compared to the $HgCl_2$ treatment alone. The results of the study indicated that exogenous Cys improved resistance to Hg-stress in maize seedlings by activating antioxidant defence system.

Key words: Antioxidant, cysteine, gene expression, glutathione, mercury

1. Introduction

Higher plants develop several adjustments to almost all different environments. Due to their immobile structure, plants are constantly subjected to abiotic and biotic stress factors that negatively affect plant growth, development, and reproduction (Rejeb et al., 2014). The impact of unfavourable conditions on the ecosystem is increasing at an alarming rate due to the rapid increase in soil and water pollution and destruction of vegetation through the excessive release of wastes produced by human activities such as industrialization and urbanization (Nagajyoti et al., 2010; Mantri et al., 2012).

Heavy metals are important environmental pollutants and their toxicity is of increasing importance due to ecological, evolutionary, nutritional, and environmental reasons. They have the greatest availability in soil and water ecosystems and present a relatively small proportion in the atmosphere as particulates or vapours. The extent of heavy metal toxicity in plants differs according to the plant species, metal specificity, concentration, chemical form, exposure type, and duration (Nagajyoti et al., 2010; Yadav, 2010).

Mercury is known to be one of the most important toxic heavy metals in nature (Tchounwou et al., 2012). The source of natural mercury is from "hot spots" through natural processes such as hot springs or volcanic explosions. Although mercury is present in the earth's crust in low concentrations, human activities such as mining, burning of fossil fuels, release of industrial wastes into aquatic environments cause an increase in the mercury level in the atmosphere, soil, and water (Boening, 2000; Patra and Sharma, 2000; Lopes et al., 2013; Mahbub et al., 2017). Besides, the widespread use of mercury in chlorine-alkali processes, dental amalgams, thermometers, and fungicides has contributed significant quantities of Hg into the aquatic and terrestrial ecosystems (Jarup, 2003). When mercury is released into the air, it can travel long distances and then be deposited into the water and ground.

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Mercury is considered a key global pollutant because of its persistent bioaccumulation and toxicity in the environment (Hylander and Goodsite, 2006). Therefore, in the European Union (EU), the mercury exportation was forbidden since 2011, about 6×10^3 tons of mercury is released to the environment each year as direct results of human activities (Ronchetti et al., 2006).

The toxicity of mercury is linked to the form in which it is found in the environment. Mercury can be found in most ecosystems in 3 different oxidation forms: metallic, monovalent or divalent and the last form being the most abundant in the soil (Stein et al., 1996; Goldman et al., 2001). The plants pick up the mercury from the contaminated soil by means of their roots and accumulate it in their bodies. Mercury accumulation in plants causes inhibition of germination, slowing of growth rate, reduction of membrane permeability and mitochondrial activity, deterioration of photosynthesis, and oxidative stress by activating reactive oxygen derivatives (Patra and Sharma, 2000; Clemens, 2006; Meng et al., 2014).

Therefore, there is a need to develop certain effective methods to mitigate the effects of mercury toxicity in plants. In order to reduce the damage caused by mercury toxicity in plants, hydrogen sulphide, nitric oxide, salicylic acid, and carbon monoxide are exogenously applied to plants. The exogenous administration of such kind of substances at lower doses has been reported to be effective in alleviating the adverse effects of heavy metal accumulation (Hsu and Kao, 2004; Zhang et al., 2007; Meng et al., 2011; Chen and Yang, 2012). It has been reported that the exogenous application of Cys to plants can also be used to diminish the severity of the phytotoxicity of heavy metals such as cadmium and lead (Klaassen et al., 1999; Benavides et al., 2005; Sharma and Dubey, 2005). As known, Cys is one of the 20 amino acids that make up proteins and carry the sulphur group in the side chains. Cys has both D and L isomers like many amino acids. Unlike many other amino acids, Cys carries a thiol group. The side chain of this group is nucleophilic and is easily oxidized (Moller et al., 2007; Dickinson and Chang, 2011). Cys is also involved in GSH (glutathione-cysteine-containing tripeptide) synthesis in plants and can also form mixed disulphides primarily with glutathione. GSH can also be used to remove H_2O_2 and acts as an antioxidant in alleviating redox imbalance caused by toxic metal accumulation such as mercury. As an antioxidant molecule, Cys has been reported to inactivate free radicals in plant cells and protect cells from oxidative damage (Moller et al., 2007; Gill and Tuteja, 2010; Dickinson and Chang, 2011).

As mentioned above, although there are several studies on the role of Cys in relieving phytotoxicity caused by some heavy metals, the role of exogenous Cys on mercury toxicity has not been investigated yet. In the present study, hydroponic experiments were performed to examine the effects of exogenous Cys on maize seedlings under mercury stress. For this purpose, the expression of 5 genes (*CAT, SOD, POD, GR*, and *GPX*) related to oxidative stress defence was determined by using qRT-PCR. In addition, root and shoot elongation, total chlorophyll, protein and proline contents, ROS accumulation and antioxidant enzyme activities were analysed.

2. Materials and methods

2.1. Plant material and plant growth conditions

Throughout the study, maize (*Zea mays* L. cv. Arifiye-2) seeds obtained from Sakarya Agricultural Research Centre were used. For experiments, the seeds were short-term washed with ethanol (96%) prior to sowing and subjected to surface sterilization for 5 min in 5% sodium hypochlorite. Then, the seeds were rinsed 5 times with sterile distilled water and immersed in distilled water for 6 h. Seeds were kept in a growth cabinet between a double layer of filter paper wetted with 10 mL of ¼ Hoagland solution at 25 °C for 72 h until germination. Germinated seeds were transferred into a hydroponic medium containing ¼ diluted Hoagland solution. In the hydroponics environment, hydrotone (expanded clay) is used as a support medium. The seedling development was carried out in a plant growth cabinet with adjustable light intensity, temperature (24 ± 2 °C; day/night and 16/8 h photoperiod) and humidity (65%) for 9 days.

2.2. $HgCl_2$ and Cys treatments

In this study, control (1st group), $HgCl_2$ application alone (2nd group), Cys application alone (3rd group), and $HgCl_2$ + Cys application groups (4th group) were created to perform all experiments. After germination, when the seedlings reached the 2-leaf stage (day 9), 200 µM Cys was sprayed on the leaves of the 3rd and 4th group for 3 h before the application of mercury. 100 µM $HgCl_2$ was both added into the Hoagland solution of $HgCl_2$ alone (2nd group) and $HgCl_2$ + Cys (4th group) application groups (Figure 1). The toxic dose of $HgCl_2$ and the regenerative dose of Cys were determined according to our preliminary studies (Supplementary Table 1). Three days after the application of $HgCl_2$ and Cys (3-leaf stage-12th day), the root and shoots of the seedlings were harvested separately and used as experimental material in the study. Fresh samples were harvested and immediately used for some experiments, while the remaining samples were stored at –80 °C after freezing in liquid nitrogen.

2.3. Determination of chlorophyll content

In order to determine the total chlorophyll content in the leaves of the maize plants, the experimental procedure recommended by Witham et al. (1971) was applied to grounded leaf samples. Fresh leaf samples (0.2 g) were

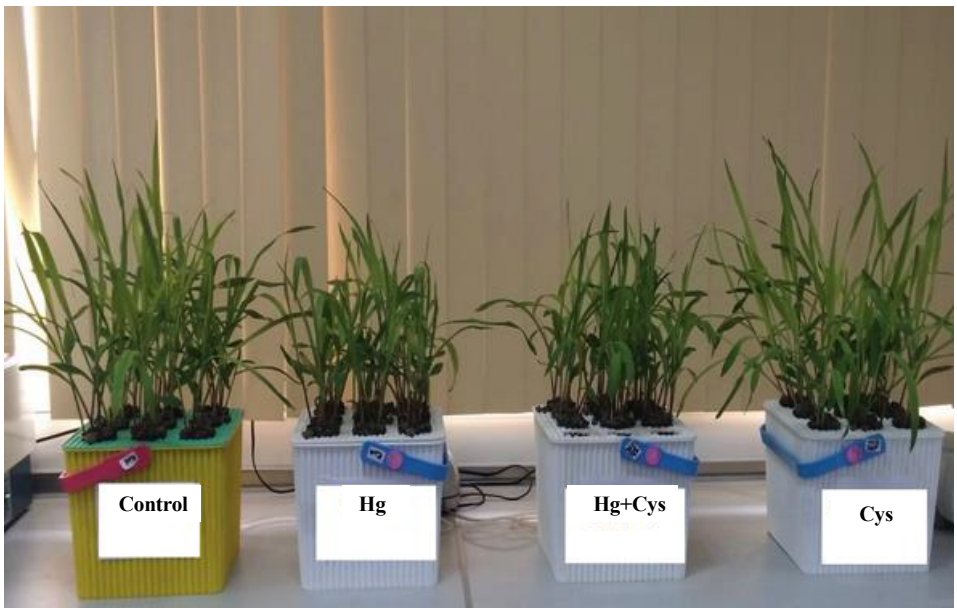

Figure 1. Application groups in hydroponic environment.

Table 1. Primer pairs used for the amplification of target sequences.

GENE	Forward	Reverse	Access. No
β-actin	AGCAACTGGGATGACATGGA	GGGTTGAGAGGTGCCTCAGT	NC_000071.6
POX1	CTGCTGAGTGACCCTGTCTTC	GGATAGGGTCTATTTAAGCATCAG	NC_024459.2
CAT1	TGTTCTAACAGGCTGTCGTGAG	TGTCAGTGCGTCAACCCATC	NC_024463.2
SOD-1A	GTTGTTGGGAGAGCATTTGTG	GAGCAACCTACAATGGAGTCAG	NC_024459.2
GPX	CGCTATGCTCCAACCACTTC	GCTCTCAGAGCAATGTTCATACAG	NC_024468.2
GR	CACCAAAGCAGACTTCGACA	AAGTTCGTCTTTGGCTTGGA	NC_024463.2

homogenized in 80% cold acetone with a final volume of 10 mL. Then, the extract obtained by filtering the homogenate through the filter paper was centrifuged at 3000 × g for 5 min. Supernatants were taken from the tubes, the absorbance values were recorded at 450, 645, and 663 nm for each tube and the chlorophyll amounts were calculated.

2.4. Determination of soluble protein and proline contents

The shoots (0.5 g) were thoroughly extracted in liquid nitrogen and the powdered extract was ground in a 4-fold volume of 0.1 M phosphate buffer (pH: 6.75) to complete the homogenization. The homogenate was then centrifuged for 15 min at 15,000 × g. The clear supernatant was used for protein determination. Protein content was determined spectrophotometrically at 562 nm (Smith et al., 1985). Colorimetric detection of proline was determined (Bates et al., 1973) based on proline's reaction with ninhydrin. The amount of proline was calculated by a constructed standard curve.

2.5. Determination of intracellular mercury

The roots and leaves of harvested maize seedlings were dried at 65 °C for 48 h. The samples were thoroughly ground into a fine powder in liquid nitrogen and passed through a 1 mm sieve. Dried and ground samples (0.2 g) were taken and dissolved in 10 mL of HNO_3 + 4 mL of H_2O_2 solution at approximately 200 °C in a microwave oven under high pressure. Mercury amounts in the roots and leaves were measured by using ICP/OES (ICP/OES; Perkin-Elmer, Optima 2100 DV, ICP/OES, Shelton, CT 06484-4794, USA).

2.6. Determination of lipid peroxidation, hydrogen peroxide content, and superoxide anion production

Lipid peroxidation was determined by calculating the MDA content (Ohkawa et al., 1979) in 0.2 g fresh weight (FW) of leaves. Tissues were homogenized in liquid

nitrogen and suspended in 0.1 % trichloroacetic acid (TCA) solution. The homogenate was then centrifuged and after centrifugation, supernatant from each sample was taken and freshly prepared TCA-TBA (thiobarbituric acid)-HCl (1: 1: 1) solution was added into the tubes. Samples were incubated at 95 °C for 1 h. After incubation, tubes were immediately placed into ice until reaching room temperature. The concentration of MDA was calculated using 532 nm absorbance. Unspecific turbidity correction was done by subtracting the 600 nm absorbance value.

H_2O_2 content was measured according to a revised protocol prepared by Velikova et al. (2000). Plant tissue (0.4 g) was homogenized in cold 0.1% TCA and the homogenate was centrifuged at 10,000 × g. 10 mM KH_2PO_4 (pH: 7.0) buffer and KI solution was then added onto the resulting supernatant immediately. Absorbance values were measured and recorded at 390 nm by using a UV spectrophotometer. H_2O_2 concentration was calculated by a constructed standard curve and defined as µmol/g FW.

$O_2^{\cdot-}$ production rate was determined by a modified version of the protocol used by Elstner and Heupel (1976). The homogenate was prepared from fresh plant tissues in 50 mM potassium phosphate buffer (pH: 7.8) and then centrifuged. After homogenization, the supernatant was taken carefully. 50 mM potassium phosphate buffer (pH: 7.8) and 1 mM hydroxylammonium chloride solution were added for the preparation of the incubation mixture. Samples were incubated at 25 °C for 20 min. 17 mM sulphanilic acid and 27 mM a-naphthylamine were also put into the mixture. The absorbance was measured at 530 nm after 15 min incubation at room temperature and the superoxide radical amount was calculated via a standard curve.

2.7. Determination of antioxidant enzyme activities

The leaf samples (0.5 g) were first extracted thoroughly in liquid nitrogen and the extract was homogenized in 5 mL of 0.1 M KH_2PO_4 (pH: 6.75), 1% PVP, 1 mM EDTA buffer. The homogenate was then centrifuged for 15 min at 15.000 × g. The supernatant was taken carefully and used as the enzyme source in the studies. All experiments were carried out at +4 °C. The method proposed by Gong et al. (2001) was performed for the determination of CAT activity. This method is based on the monitoring of the absorbance change in the CAT activity during the conversion of H_2O_2 to oxygen and water at 240 nm. POX activity was determined by measuring the increase in the absorbance generated by a coloured compound, which is the product of guaiacol and H_2O_2 at 470 nm (Ye et al., 2003). SOD activity is assessed by spectrophotometric determination according to the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) with superoxide radicals to blue formazan by SOD enzyme (Agarwal and Pandey, 2004; Yordanova et al., 2004). GR activity was measured spectrophotometrically via using a revised protocol of Foyer and Halliwell (1976) by monitoring the decrease in the absorbance at 340 nm due to NADPH oxidation. APX function was detected by the decrease in the absorbance at 290 nm according to the reduction and oxidation reaction of ascorbic acid (extinction coefficient of 2.8 mM^{-1} cm^{-1}) (Nakano and Asada, 1981).

2.8. Determination of nonenzymatic antioxidants

Shoot tissue (0.2 g) taken from the powder obtained from liquid nitrogen grinding was homogenized in 2 mL of 5 % TCA. The extract was centrifuged at 12,000 × g for 10 min at 4 °C. The obtained supernatant was used for the determination of glutathione and ascorbic acid contents. Total (GSH + GSSG), oxidized (GSSG), and reduced glutathione (GSH) amounts were determined according to a modified protocol of Wu et al. (2009) that is generated by Hodges et al. (1996). The levels of reduced ascorbate (AsA), oxidized ascorbate (dehydroascorbate, DHA), and total ascorbate (ASA + DHA) were measured by the change in the absorbance at 525 nm (Wu et al., 2009).

2.9. Gene expression studies

The procedure described in our previous work was followed in the gene expression studies (Aksakal et al., 2017). The RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used for RNA isolation from the maize leaves. The RNA quality and quantity were determined by using the absorption of light at 260 nm and 280 nm (A260/A280) on a spectrophotometer and samples were stored at –80 °C. RevertAid First Strand cDNA Synthesis Kit (Fermentas, catalog no: K1622) was used to synthesize cDNAs from the RNA samples. After cDNA synthesis, qRT-PCR reactions were performed using the Quantitect SYBR Green kit (Qiagen, cat. no: 204143) according to the manufacturer's instructions. All the qRT-PCR reactions were run on Rotor-Gene 6000 (Corbett, Qiagen) cycler. Specific primers (Table 1) were designed for the selected genes according to the Primer 3 software (version 0.4.0). Expression levels (fold change) of the detected genes were normalized against the reference gene *β-actin*. Reaction efficiency incorporated ΔΔCt formula was used to perform the relative quantification. Three independent biological replicates with 3 technical replicas per experiment were used for each qRT-PCR. The Student's t-test provided by GenGlobe data analysis software (Qiagen- https://www.qiagen.com/en/geneglobe) was used to evaluate the statistical significance of fold change of the expressed genes studied in the control group and experimental groups. The statistical significance is marked with * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$).

2.10. Statistical analysis

SPSS 20.0 package program (IBM Corp., Armonk, NY, USA) was used to evaluate the results and one-way analysis of variance (ANOVA) was performed all data

except gene expression analysis. The statistical significance was determined by using Duncan's multiple comparison test for comparison of the mean differences at $P \leq 0.05$ significance level.

3. Results

3.1. Early growth parameters of maize seedlings (root, shoot length, and dry weight)

At the end of the 12th day of plant growth, root, and shoot lengths of maize seedlings were measured and the results are given in Table 2. It was determined that 100 µM $HgCl_2$ application decreased the root and shoot length compared to control and also 200 µM Cys application alone significantly increased ($P \leq 0.05$) the root and shoot length compared to control. It was also found that combined application of $HgCl_2$ and Cys to maize seedlings increased root and shoot growth compared to $HgCl_2$ application alone. On the other hand, it was noted that the $HgCl_2$ application notably reduced the dry weight of samples when compared to the control, and the application of Cys alone and $HgCl_2$ + Cys increased the dry weight.

3.2. Effect of $HgCl_2$ and Cys on chlorophyll, soluble protein, proline, root, and shoot Hg content

The $HgCl_2$ concentration (100 µM) used in the study significantly reduced the chlorophyll content in maize seedlings (Table 2). $HgCl_2$ + Cys applied seedlings showed a significant increase in the chlorophyll content compared to the $HgCl_2$ application alone group. In addition, when compared to control one, the Cys application alone significantly increased the chlorophyll content ($P \leq 0.05$). $HgCl_2$ application significantly increased both the total protein and proline content compared to control (Table 2). Besides, $HgCl_2$ and Cys application together led to a significant increase in both total protein and proline amounts ($P \leq 0.05$). Intracellular mercury content was not detected in control and Cys groups. However, the amount of intracellular Hg was measured in $HgCl_2$ and $HgCl_2$ + Cys application groups. The intracellular Hg content of Cys applied group was decreased significantly by 30.8% in the shoot and 18.4% in the root as compared to the $HgCl_2$ treatment group (Table 2).

3.3. The amount of lipid peroxidation and the levels of reactive oxygen species

The amount of MDA, H_2O_2, and $O_2^{\cdot-}$ radicals increased with the application of 100 µM $HgCl_2$. On the other hand, Cys application alone significantly reduced the MDA, H_2O_2, and $O_2^{\cdot-}$ levels compared to $HgCl_2$ alone. In addition, $HgCl_2$ + Cys administration caused a significant reduction in MDA, H_2O_2, and $O_2^{\cdot-}$ levels compared to $HgCl_2$ alone (Table 3).

Table 2. The effect of mercury, cysteine, and the combination of the 2 on the root and shoot length, dry weight, root and shoot Hg content, chlorophyll, proline, and protein levels in maize seedlings.

Treatments	Root length (cm)	Shoot length (cm)	Dry weight (g plant^{-1})	Root Hg content (ppm)	Shoot Hg content (ppm)	Chlorophyll content (mg g^{-1} FW)	Proline (µg.g^{-1})	Soluble Protein (mg.g^{-1})
Control	12.58 ± 0.4b	27.57 ± 0.5c	0.39 ± 0.003c	ND	ND	3.51 ± 0.05a	0.29 ± 0.04d	15.3 ± 0.26d
Hg	9.51 ± 0.3c	23.19 ± 0.5d	0.29 ± 0.003d	8.423 ± 0.15a	0.0396 ± 0.006a	2.68 ± 0.04c	0.38 ± 0.03b	16.7 ± 0.11c
Cys	16.02 ± 0.4a	28.94 ± 0.4b	0.45 ± 0.004a	ND	ND	3.56 ± 0.05a	0.34 ± 0.02c	17.4 ± 0.14b
Hg+Cys	12.66 ± 0.7b	29.76 ± 0.6a	0.40 ± 0.001b	6.876 ± 0.17b	0.0274 ± 0.003b	3.30 ± 0.04b	0.41 ± 0.05a	19.0 ± 0.17a

Different letters on the same column indicate that the difference is significant at the level of $P \leq 0.05$, ND: Not detected.

Table 3. The effect of mercury, cysteine, and the combination of both on the MDA amount and the levels of reactive oxygen derivatives.

Treatments	Superoxide anion content (µg g^{-1} FW)	Hydrogen peroxide content (µmol g^{-1} FW)	Malondialdehyde content (nmol ml^{-1} FW)
Control	4.18 ± 0.13b	1.05 ± 0.06c	2.75 ± 0.09c
Hg	4.77 ± 0.11a	1.67 ± 0.05a	3.90 ± 0.07a
Cys	3.92 ± 0.14c	0.98 ± 0.04c	2.27 ± 0.11d
Hg+Cys	4.14 ± 0.12b	1.27 ± 0.07b	3.09 ± 0.10b

Different letters on the same column indicate that the difference is significant at the level of $P \leq 0.05$.

3.4. Antioxidant enzyme activities

It was determined that SOD and POX enzyme activities were affected differently from $HgCl_2$ and Cys applications. $HgCl_2$ treatment alone inhibited the activity of these enzymes; whereas it was identified that Cys alone increased the activity of these enzymes compared to control (Table 4). It was also noted that when compared to $HgCl_2$ alone, $HgCl_2$ + Cys application increased the activity of these enzymes. On the other hand, $HgCl_2$ alone increased the activity of GR, CAT, and APX enzymes compared to the control (Table 4). The treatment of Cys alone increased the activity of GR and CAT compared to the control group and $HgCl_2$ alone group, however, it decreased the activity of APX ($P \leq 0.05$). In addition, when compared with control, $HgCl_2$ + Cys application increased the activity of these 3 enzymes (Table 4).

3.5. Total GSH and total ASA content

The total amount of GSH and ASA in maize seedlings increased with all 3 applications. In spite of the other applications, the combination of $HgCl_2$ + Cys application significantly increased the amount of both GSH and ASA compared to other application groups (Table 4) ($P \leq 0.05$).

3.6. Antioxidant gene expression studies

According to the qRT-PCR results, it was determined that $HgCl_2$ + Cys application significantly increased the expression levels of *CAT, GR*, and *SOD* genes compared to $HgCl_2$ alone (Figure 2). On the other hand, it was found that $HgCl_2$ application alone did not significantly change the mRNA levels of *SOD* and *POX* genes compared to control. However, *CAT*, *GR*, and *GPX* mRNA levels significantly increased after the application of $HgCl_2$. In comparison to the control group, it has been observed that Cys treatment alone increased the expression of all studied genes except *GPX* (Figure 2).

In our study, a heat map of the gene expression change of selected 5 genes in all groups is presented according to the control group (Figure 3). As a result, it was determined that the 5 genes studied in the root and leaf of the seedlings were differentially expressed from each other in different application groups.

4. Discussion

Mercury is one of the most dangerous heavy metals for plants with phytotoxic properties that cause redox imbalance, change in photosynthetic activity, impair growth rate, and a decrease in production (Cui et al., 2015; Gontia-Mishra et al., 2016; Zhang et al., 2017). The high amount of mercury accumulation in plants leads to the death of cells and tissues (Sahu et al., 2012). In this study, it was determined that the $HgCl_2$ applied maize seedlings showed root and shoot growth inhibition and significantly reduced dry weight (Table 2). Root and shoot growths are the most important indicators for understanding the response of plants against stress conditions. Since the root is the first organ that comes into contact with water and absorbs water, toxic substances such as heavy metals are mostly taken by the roots (Peralta et al., 2001). Mercury strongly impaired organ growth and accumulated mainly in roots. The effect of mercury applied to maize seedlings on root and shoot growth may be due to the fact that mercury changes the water uptake of the plant or disrupts the activity of enzymes in various metabolic pathways. In previous studies on mercury toxicity in plants, it has been reported that mercury inhibited the root and shoot growth in various plants similar to our study (Cho and Park, 2000; Cargnelutti et al., 2006). On the other hand, according to our findings, growth inhibition by $HgCl_2$ in maize seedlings was determined to be reversed by Cys application to seedlings from outside. Exogenous Cys application to the seedlings promoted the root and shoot growth by reducing the toxic effects of $HgCl_2$ (Table 2). In addition to its role in proteins as an amino acid, Cys functions as a precursor for a large number of basic biomolecules such as vitamins and cofactors (Teixeira et al., 2017). Thus, the exogenous application of Cys is a promising application due to its important role in biomolecule synthesis.

Table 4. The effect of mercury, cysteine, and the combination of both on the amount of antioxidant enzyme activities, total glutathione, and total ascorbate levels in maize seedlings.

Treatments	SOD (U mg^{-1} protein)	POX (U mg^{-1} protein)	GR (U mg^{-1} protein)	CAT (U mg^{-1} protein)	APX (U mg^{-1} protein)	Total GSH content (ng g^{-1} FW)	Total ASA content (ng g^{-1} FW)
Control	4.64 ± 0.07a	524 ± 13.1b	3.50 ± 0.04d	2.84 ± 0.07d	766 ± 21.4c	488 ± 24.2d	304 ± 5.4d
Hg	3.51 ± 0.09d	472 ± 11.7c	4.03 ± 0.09b	3.53 ± 0.13c	1094 ± 42.6a	628 ± 31.3b	344 ± 3.1c
Cys	4.37 ± 0.11b	589 ± 10.6a	4.21 ± 0.10a	3.94 ± 0.09b	691 ± 24.5d	553 ± 12.7c	422 ± 6.4b
Hg+Cys	3.95 ± 0.07c	517 ± 11.1b	3.84 ± 0.06c	4.61 ± 0.10a	913 ± 32.1b	739 ± 28.9a	450 ± 8.3a

Different letters on the same column indicate that the difference is significant at the level of $P \leq 0.05$.

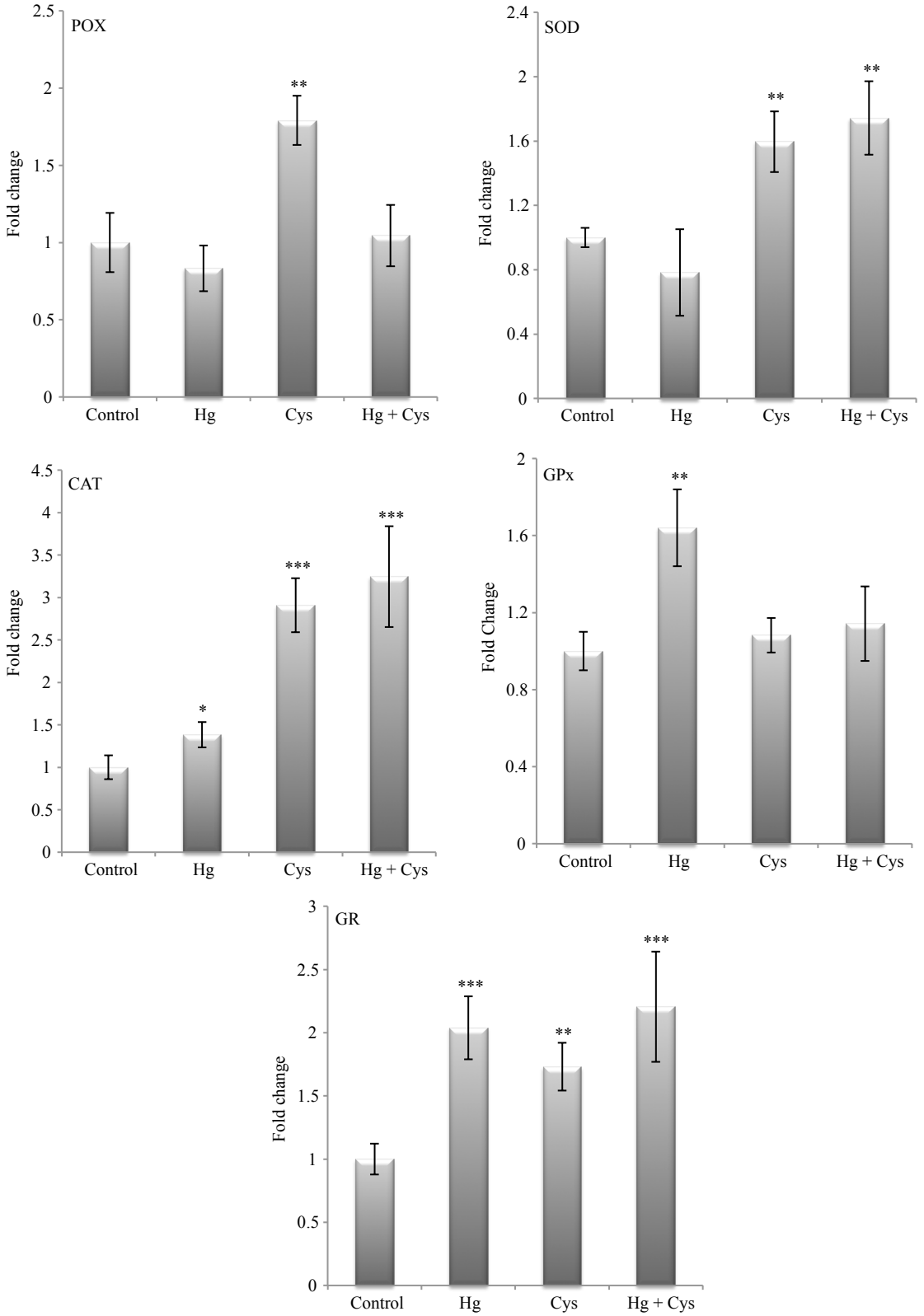

Figure 2. Antioxidant genes' expression levels of maize seedlings (mRNA expression levels of **A.** *POX*, **B.** *SOD*, **C.** *CAT*, **D.** *GPX*, and **E.** *GR*) (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

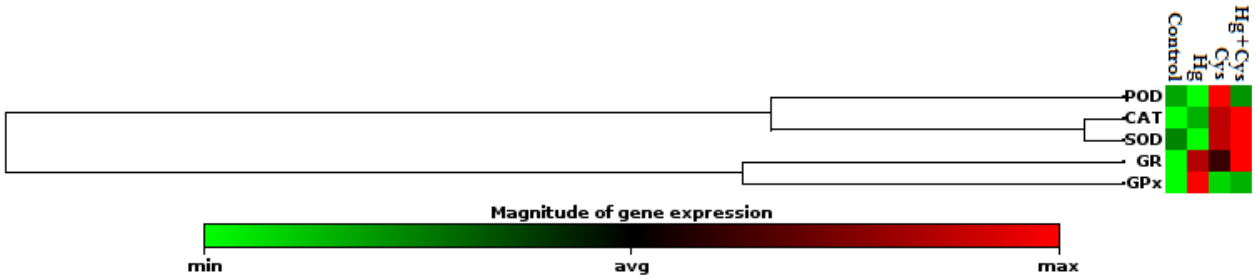

Figure 3. Hierarchical cluster diagram of antioxidant genes in mercury and cysteine treated maize seedlings.

Measurement of soluble protein content and proline in plants are considered as important indicators for the determination of the physiological status of cells under various stress conditions such as heavy metal, cold, and salt stress (John et al., 2008; Lamhamdi et al., 2013; Alayat et al., 2014). In addition, the content of soluble proteins is one of the most important indicators in determining the reversible and irreversible changes in plant metabolism. Morphological changes in plants are generally interpreted together with the changes in proteins (Sandalio et al., 2001). In our study, it was found that mercury stress significantly increased the amount of soluble proteins in maize seedlings compared to the control group. It is probable that most of these proteins have low molecular weight and they are related to stress pathways. It was also found that Cys alone and $HgCl_2$ + Cys applications increased the amount of soluble proteins compared to the control (Table 2). An increase in soluble proteins could be based on the activation of genes for synthesis of stress-specific proteins functioning in the protection of vital cellular activities, maintaining the membrane stability and retaining the integrity of plant cells (Alayat et al., 2014). Moreover, it has been reported that the amount of proline increases significantly in such cases causing changes in osmotic values in cells and increases the resistance in stress conditions by activating the cell defence system (Sharma and Dietz, 2006). In the present study, $HgCl_2$ application to maize seedlings significantly increased the proline amount compared to control (Table 2). The accumulation of proline in stress conditions serves as a mechanism of stress tolerance in plants (Szabados and Savoure, 2010). $HgCl_2$ and Cys co-administration to the maize seedlings led to a significant increase in the proline amount ($P \leq 0.05$). In this study, the higher proline accumulation was determined to correlate with the earlier stress response of maize plants against mercury stress (Table 2). Heavy metals limited metabolic activities by inhibiting the effect of enzymes, which may be the most important cause of inhibition. Chlorophylls are important components of pigment systems in chloroplasts and therefore, directly affect plant growth. Decreased chlorophyll content associated with heavy metal stress may be a result of enzyme inhibition responsible for chlorophyll biosynthesis (Zengin and Munzuroglu, 2005). Determination of the chlorophyll content is one of the most frequently used methods to understand the effects of biotic and abiotic stress factors on plants. Effects of various environmental pollutants (e.g., heavy metals) on plants have been examined by taking into account toxic properties such as chlorophyll degradation and deterioration of membrane structure (Mocquot et al., 1996; Zengin and Munzuroglu, 2005). It was found that heavy metals prevented chlorophyll synthesis by reducing the intake of Fe^{+2} and Mg^{+2} from the soil. The $HgCl_2$ concentration used in the study significantly reduced the chlorophyll content in maize seedlings. $HgCl_2$ + Cys co-administration to the seedlings significantly increased the chlorophyll content compared to $HgCl_2$ application alone. It has also been found that the exogenous application of Cys to the seedlings has led to a change in the amount of chlorophyll, helping to alleviate the damage caused by $HgCl_2$ (Table 2). The reduction of total chlorophyll content by $HgCl_2$ may be due to reduced uptake of essential elements (Fe^{+2} and Mg^{+2}) and replacement of metal ions by Hg in photosynthetic pigments (Safari et al., 2019). Both Cys application alone and its combination with $HgCl_2$ resulted in an increase in the total chlorophyll content of the maize seedlings (Table 2). This may have arisen from the accelerating activity of Cys to photosynthetic activity or helping to maintain the balance among metabolic pathways. In this case, it can be said that the Cys in the leaves increases the rate of photosynthetic pigments and helps to increase the resistance of the plant to mercury toxicity. Similar to our results, Erdal and Turk (2016) found that Cys application increased the chlorophyll content of 12-day-old maize seedlings.

It is well known that mercury leads to oxidative damage by increasing lipid peroxidation and the formation of various reactive oxygen derivatives in plants (Cho and Park, 2000; Zhang et al., 2007). Lipid peroxidation is the oxidative degradation of unsaturated fatty acids in the membranes of plant cells, resulting in an increase in the amount of MDA as a final biproduct by the degradation of these fatty acids. MDA is a good indicator of deterioration of the structural integrity of membranes in cells and an increase in the

amount of MDA is extremely dangerous for cell survival (Draper and Hadley, 1990). In our study, it was found that $HgCl_2$ application to maize seedlings significantly increased the amount of MDA and Cys application together with $HgCl_2$ to the seedlings significantly decreased the amount of MDA (Table 3). The reduction of the MDA amount by Cys can be attributed to the dismutation of the superoxide radical, in particular by the effect of SOD activity (Table 4). In this study, it was determined that exposure to mercury enhanced H_2O_2 level and O_2^- content and co-administration of Cys with mercury decreased these parameters (Table 3). These results suggested the role of Cys in reducing the amount of ROS formation. Previous studies also indicated that low molecular weight antioxidants like cysteine help to scavenge various types of ROS (Singh et al., 2006). Excessive ROS production in plants damages to macromolecules such as lipids, proteins, and nucleic acids (Gill and Tuteja, 2010). In order to eliminate ROS, plants evolve enzymatic (CAT, POX, SOD, GR, and GPX) and nonenzymatic (ASA and GSH) antioxidants. Hence, we investigated the effects of Cys on enzymatic and nonenzymatic antioxidants (Table 4) in order to explain how Cys plays a role in ROS detoxification. In this study, exposure to $HgCl_2$ reduced the activity of SOD and POX and their related genes. However, the Cys application together with $HgCl_2$ increased the amounts of these enzymes and the expression of the encoded genes (Table 4, Figure 2). In this context, the increment of SOD and POX activities by exogenous Cys application in $HgCl_2$-treated plants in association with a decrease of ROS indicates the important role played by this amino acid in the alleviation of mercury toxicity.

In addition to enzymatic antioxidants, nonenzymatic antioxidant GSH plays an essential role in ROS scavenging. It has several activities as a metabolic regulator and antioxidant. These activities are attributed to its oxidation to glutathione disulphide (GSSG). The reduction of GSSG to GSH occurs in the presence of NADPH by GR, an important antioxidant enzyme (Moller et al., 2007; Dickinson and Chang, 2011). Herein, $HgCl_2$ administration increased the GSH amount; GR and APX enzyme levels and increased the expression of *GR* and *GPX* genes compared to the control (Table 4, Figure 2). In addition, $HgCl_2$ + Cys administration increased the amount of GSH and also increased the activity and gene expression levels of these 2 enzymes. Previous studies have been indicated that there is an important link between Cys application and elevated GSH biosynthesis (Moller et al., 2007; Erdal and Turk, 2016). According to the data obtained from our study and the literature, it can be said that the plant increases the tolerance against heavy metal stress by accelerating the biosynthesis of GSH (Zhou et al. (2009). ASA, like GSH, is a strong and water-soluble antioxidant that prevents damage to cells due to excessive accumulation of ROS (Zhou et al., 2008). It acts as an electron donor in many enzymatic and nonenzymatic reactions (Asada, 2006; Gill and Tuteja, 2010). APX and the ASA-GSH cycle can also contribute to the scavenging of hydroxyl radicals (Miller et al., 2010). In this study, the amount of ASA in maize seedlings increased with $HgCl_2$ application ($P \leq 0.05$). $HgCl_2$ + Cys application also increased the amount of ASA significantly (Table 4). Inconsistent with our results, the Cys application to maize plants prior to mercury stress treatment increased the antioxidative capacity of the plant by supporting the enzymatic antioxidant system via increasing both GSH and ASA levels from the nonenzymatic system parameters. Also, Cys has been reported to increase the ASA and GSH in plants against toxicity of heavy metals (Hsu and Kao, 2004; Laspina et al., 2005). Heavy metal (cadmium) treated plants had higher ASA and GSH content (Hossain et al., 2012) and the increase in ASA has been associated with the increase in tolerance to heavy metal stress (Tsuji et al., 2002).

In conclusion, it was determined that the Cys application to the maize seedlings before exposure to mercury stress contributes to the plant's stress tolerance by stimulating the response mechanisms at the morphological, biochemical, and molecular levels. This contribution is achieved by induced antioxidant enzyme activities and decreased MDA and ROS level increased chlorophyll and soluble protein content, elevated total GSH and ASA amount, and altered gene expression levels of related antioxidants.

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