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# Expression Patterns of Oxidative Stress-Related Genes of *Cucurbita pepo* and Relation to Cellular $H_2O_2$ under Short-Term Heavy Metal Stress

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**ABSTRACT:** Oxidative stress caused by biotic and abiotic stress factors is the most important cause of cellular damage. Due to their sessile structures, plants have evolved regulatory mechanisms to respond to various environmental stresses The increased cellular concentration of reactive oxygen species is one of the major consequences of oxidative stress. including $H_2O_2$ production. Also, $H_2O_2$ is produced as a by-product of respiratory and photosynthetic metabolisms in plants. $H_2O_2$ acts as a multifaceted molecule because of its dual role in cells. It has been found to act as a secondary messenger in signal transmission networks. In this study, the changes in expression levels of stress-related genes and their relationship with $H_2O_2$ in pumpkin (*Cucurbita pepo*) plant exposed to Cd heavy metal at different durations and concentrations were investigated. As a result of this study, we concluded that the expression of stress-related genes may be related to the oxidative status of the cell and the concentration of H2O2 in the signaling mechanism, the expression of stress-related genes may be up-regulated to a certain degree of concentration, while a higher concentration of $H_2O_2$ may down-regulate the expression of the genes.

**Keywords:** Stress-related genes, Expression level, Oxidative stress, Cadmium, Reactive Oxigen Species, *Cucurbita pepo*

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## INTRODUCTION

Reactive oxygen species (ROS) are generated as by-products of different metabolic processes such as photosynthesis and respiration. Because all the aerobic species use the oxygen molecule as the last electrons receiver during electron transport system, irregularities occur in the exchange of electrons in some cases, resulting in the formation of many reactive and therefore toxic intermediates such as single oxygen ($^1O_2$), radical superoxide ($O^{-2}$) and hydrogen peroxide ($H_2O_2$). varieties can cause serious damage to cell membranes and cellular components in their environment. Also, ROS can cause binding with high interest and lead to rather destructive results against DNA, carbohydrates, proteins, and lipids (Zimmermann et al., 2006). Due to these properties, cellular ROS concentration is tried to be kept in a stable balance with enzymatic and non-enzymatic detoxification mechanisms (Ahmad, 2014). The deterioration of this balance due to negative environmental factors leads to the phenomenon defined as the rapid increase of intracellular ROS levels called "oxidative burst." To reduce the possible effects of this stress, removal of ROS in the plant system becomes vital (Azarabadi et al., 2017).

However, the oxidative balance in question in the plant can be spoiled by many different abiotic environmental factors such as temperature, heavy metal ions, cold, salinity, drought, and light (Boyer, 1982).

Plants try to balance cellular ROS concentration using both enzymatic such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and non-enzymatic such as α-tocopherol ascorbate and flavonoids pathways.. (Khan et al., 2017).

Numerous studies have shown that, under different stress conditions, plants respond in a very complex manner involving numerous physiological and cellular changes. Plants use several hormone-driven signaling pathways to tackle environmental stress. Previous research has shown however that plants use ROS as signaling molecules to control growth and specific physiological responses. In recent years, scientists have concentrated on ROS generation and its incorporation in the control of plant growth and stress tolerance with various hormonal signal pathways. (Vanderauwera et al., 2009).

The precursor studies investigating the relationship between $H_2O_2$ and the role of signalization reported that it played the role of the signal cascade in events that are essential for plant development and growth like the strengthening of the plant cell wall, xylem differentiation and the relaxation of the cell wall, where $H_2O_2$ is an important part of oxidative metabolism. (Dempsey et al., 1994)

While the versatile molecule $H_2O_2$ serves as a significant signal at normal levels, the increase in its concentration in abiotic and biotic stress conditions causes oxidative stress. The fact that it has a small molecular structure, can easily be diffused between membranes, has a relatively longer half-life compared to other ROS, has unmatched stability, has less reactivity and acts as a central player in signal transduction pathways, puts $H_2O_2$ one step forward among other ROS molecules (Cuypers et al., 2016).

In plants, $H_2O_2$ works as a key factor in concentrations at the non-toxic level. As a signaling molecule, it merges with various pathways and tolerates biotic and abiotic stress (Baxter et al., 2014; Kar, 2018). $H_2O_2$ transmits the local effect of an abiotic stressor to systemic tissues and acts as a general preparation signal (Karpinski et al., 1999). The mechanism of responses activated by signals that are triggered by $H_2O_2$ as a response to abiotic stress is expressed as systematically acquired adaptation (SAA) and this adaptation is realized thanks to the $H_2O_2$ molecule (Suzuki et al., 2013).

In a study conducted in recent years, it was revealed that there was a relationship between the genes responsible for stress in chickpea plants exposed to heavy metal stress and $H_2O_2$ and that $H_2O_2$ increased the transcript accumulation of stress response genes up to a certain time and concentration, but caused a decrease after a certain concentration (Kar, 2018). Similarly, Tombuloğlu et al. (2012) reported a

reduction in gene expression due to prolongation of exposure in tomato plants exposed to boron stress (Tombuloglu et al., 2012).

Understanding the fine and precise mechanisms that plants use to regulate the cellular $H_2O_2$ amount and the related signaling pathways may be the key to improving agriculture in the future. Although it potentially damages plant cells, ROS oxidative signalization is considered to be useful predictors that provide systemic acquired resistance in cooperation with systemic acquired acclimation and hormones. Despite the scientific researches conducted in recent years, the cellular mechanisms of ROSs have not been fully elucidated (Hossain et al., 2015).

The aim of this study is mainly to reveal the effect of the genes of cellular $H_2O_2$ responsible for stress on the expression levels. In this context, the roots of pumpkin plants were exposed to cadmium (Cd) heavy metal, which is very toxic, and which causes irreversible damages in plants in different duration and concentrations. MDA accumulation and cellular $H_2O_2$ content were calculated. Also, the change in expression patterns of stress-related genes was calculated and its relationship with cellular $H_2O_2$ was tried to be explained.

## MATERIALS AND METHODS

### Plant growth conditions and Cd application

After surface sterilization, *Cucurbita pepo* seeds were planted in 40x25x5 cm plastic vials filled with sterile agricultural perlite. The seedling was incubated growth chamber (25 0C temperature 70% humidity) until it became seedlings for 15 days. The medium was the Hoagland solution. After this; the seedlings were exposed to 50, 100, and 200 micromolar CdCl solutions in the beaker for 12, 24, and 48 hours. All applications were repeated thrice.

### Lipid peroxidation

Lipid peroxidation in leaf tissues (250 mg) was measured in terms of malondialdehyde (MDA) determined by thiobarbituric acid (TBA) reactions as described by Heath and Packer (Heath et al., 1968).

### $H_2O_2$ determination

After modification, the method used by Junglee et al. was used to evaluate cellular $H_2O_2$ (Junglee et al., 2014). root sample (100 mg) was grinded in nitrogen and dissolved in 1 ml solution containing, 0.5 ml KI (1mM), 0.25 ml TCA (0.1% w /v) and 0.25 ml potassium phosphate for 10 minutes at + 4 ° C. After that the homogeneous centrifuged at + 4 degrees at 10,000 g for 15 minutes. Around 20-22 0C, the supernatant was incubated 20 minutes into the night. The content of $H_2O_2$ was measured at 280 nm, and the concentration of $H_2O_2$ in the cell was determined using the incremental concentration of $H_2O_2$. (Junglee et al., 2014). The formula obtained from a gradual concertation curve.

### RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis

RNA isolation from plant samples was carried out according to the Trizol protocol. The amount and quality of isolated RNA were determined by spectrophotometric measurements on the donovix nanodrop device. cDNA synthesis from RNA samples was performed using the first-strand cDNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit).

Real-time PCR applications were performed by Bioneer Exicycler Tm 96 FaST device using SYBR Green I Master dye. Primers used in the study are listed in Table 1. The actin gene was used as the control primer- Housekeeping gene-. Following quantitation using SYBR Green I dye, Melting Curve Analysis was performed to determine the effectiveness of PCR and to observe the presence of any dimer formation.

**Table 1**. List of genes and primer used for PCR amplification and the resulting product sizes

| Gene | Primer Pair | Sequence (5' – 3') | PRC product Size | Gene Bank Accession no |
|---|---|---|---|---|
| Metallothionein | MT F<br>MT R | GAGTGGGAAGGGGTAAGTGC<br>ACCAACCCAATACCACACCG | 105 | XM_023657362.1 |
| Superoxide dismutase Cu-ZnSOD | Cu-ZnSOD F<br>Cu-ZnSOD R | TCGCCATGCTGGTGTTT<br>ATGGAGATAGGTCCAGATAGAGG | 102 | MG014229.1 |
| Catalase | Cat F<br>Cat R | GTCACCCATGAGATCCGCA<br>CCAAGAGACCTATCCGCCTG | 161 | D55645 |
| Ascorbate peroxidase | APX F<br>APX1 R | TAGGCTCTTGGAGCCCATCA<br>AACCCTTGGTAGCATCAGGC | 179 | KF954415.1 |
| Actin-11 | Actin F<br>Actin R | CCTCTCAATCCCAAAGCTAACAG<br>CTGTTGGCTGTTCTGCTATCT | 91 | HM594170 |

## RESULTS AND DISCUSSION

When the accumulation of MDA was analyzed, statistically the highest MDA accumulation was observed to take place in 48 hours 200 µM application. While the lowest MDA accumulation occurred in the control group as expected, no statistically significant difference was found between the 50 µm application of 12 and 24 hours and the control group (Fig. 1A) ($p<0.05$)

When Cd concentrations are considered in terms of cellular $H_2O_2$ accumulation, the amount of $H_2O_2$ produced at different times within the same application concentration is statistically different. However, in terms of application times, no significant difference was determined between the concentrations. Statistically, the highest $H_2O_2$ accumulation was detected in 48 hours 50, 100, and 200 µm Cd application, and no statistically significant difference was found between these 3 application concentrations (Fig 1B)($p<0.05$).

MT expression, which is an antioxidant system element, was highest in the application of 48 hours, 50 µM and a tendency to decrease was observed in the expression level in the following applications. No statistically significant difference was found between 48 hours of 50 µM and 12 hours of 50µM. (Fig. 2A) ($p<0.05$)

When the Cu-Zn/SOD expressions were examined, the highest expression level was observed at a concentration of 50µM for 12 hours, and even though the expression level was higher than the control group in the following application concentrations and periods, nothing statistically significant was found within. (Fig 2B) ($p<0.05$).

When the changes in APX expressions were examined, the highest expression level was observed in the application of 12 hours of 200 µM. There was then a decrease in expression level due to increased exposure time. However, the reduction concerned did not take place as evidently as that of MT and Cu/Zn SOD expression levels (Fig. 2C) $P<0.05$).

The highest expression level in the CAT enzyme was found in the 50µM 12 hours application. However, a significantly higher expression level was observed in other application periods and concentrations than in the control. CAT expression was maintained at all concentrations (Fig. 2D) ($p<0.05$), although there was a tendency to decrease in the expression level.

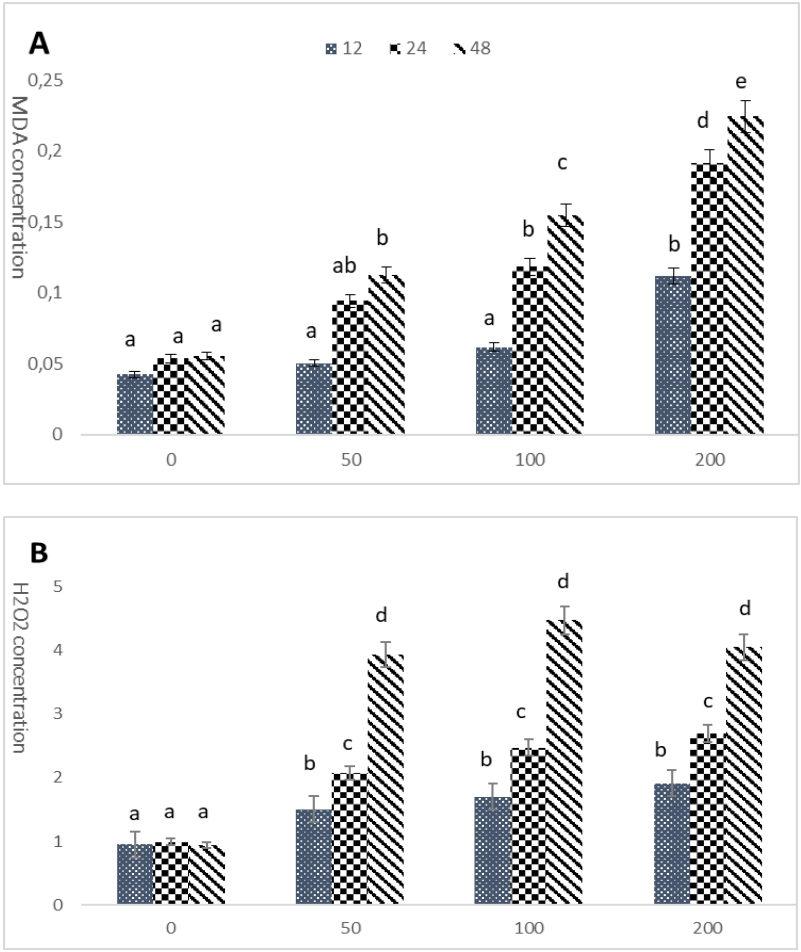


**Figure 1**. Cellular MDA (A) and H2O2 (B) concentrations. Different letters indicate differences between Cd concentrations and durations $p<0.05$ (ANOVA) the bars show standard deviations (SD)

Metallothionines (MTs) are the main transition metal ion binding proteins in cells (Hassinen et al., 2011). Also, it was suggested that MTs function in both metals chaperoning and ROS scavenger tasks (Wang et al., 2010). In the study of Tamas et al., where they investigated the effect of Cd on barley plant roots, it was found that as Cd concentration increases in plant roots *MT* expression level also increased (Tamás et al., 2008). Jain et al., in the study they conducted with sugar cane plants exposed to selenium (Se), found that the *MT* gene was expressed at a higher level than all concentrations. However, they found that *MT* expression level increased up to a certain Se concentration and decreased after a certain Se concentration (Jain et al., 2015). In the study conducted by Tombuloğlu et al. on the effect of boron element on the tomato plant, they found that *MT* gene expression level increased up to a certain concentration in root and shoot parts of the plant *and* but decreases after a certain concentration(Tombuloglu et al., 2012). Souguir et al. studied the mRNA accumulation of some genes related to stress as a result of Cd exposure at different duration and concentrations in broad bean (*Vicia faba*) plant. After all, after 12 hours, they found an increase in *MT* gene expression but a decrease when the period was prolonged (Souguir et al., 2013).

In this study, *MT* expression was found to be higher in all application periods and concentrations compared to the control group. However, *MT2* expression level increased up until 50 µM Cd application and then showed a tendency to decrease. The results of this study show parallelism with the results of the studies conducted with the *MT* gene.

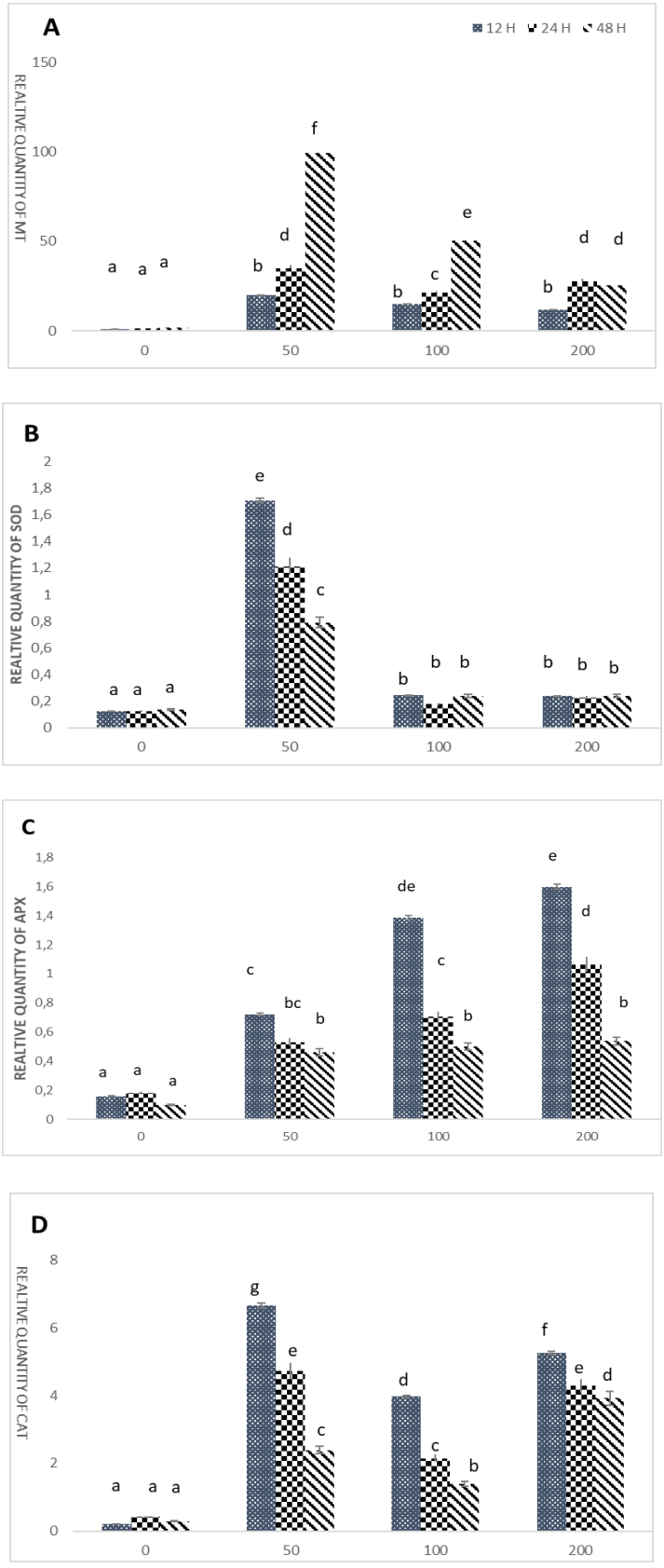


**Figure 2.** Relative expressions of MT(A), Cu/Zn SOD (A), APX (C), and CAT(D) house-keeping gene actin. Different letters indicate differences between Cd concentrations and durations $p<0.05$ (ANOVA) the bars show standard deviations (SD)

The enzyme *Cu-Zn/SOD* is present in the nucleus and cytoplasm of the cell and is involved in the first step of defense in protecting the cell from the harmful effects of ROS. SOD expression level increases under abiotic stress conditions as stated in previous studies. Increased SOD expression level, is considered to be proof that $O_2^-$ radical accumulates in the cell and that the $H_2O_2$ amount also increases. This situation shows that the SOD enzyme removes the excess amount of $O_2^-$ radicals and increases the resistance of the plant under stress (Liochev et al., 2007). In a study where Dixit et al. applied Cd on green pea plant, they found that the SOD expression level in plant leaves increased up to a certain period in all Cd applications and decreased after a certain period (Dixit et al., 2001). Dai et al. applied different concentrations of salt stress to the canola plant. As a result of the study, they found that the *SOD* expression level increased up to 200 mmol $L^{-1}$ concentration and that it decreased in 250 and 300 mmol $L^{-1}$ concentrations (Dai et al., 2009). Rossatto et al., who worked with rice plants under salt stress found that SOD expression increased compared to control up until 15 days of exposure but decreased after 20 days of exposure (Rossatto et al., 2017). In this study, which is similar to the studies in the literature, SOD expression level increased up to 50 µM Cd application and reached the highest level in 12 hours 50 µM Cd application, but as the concentration amount increased, SOD expression level decreased in all application periods. Although the expression levels detected as a result of prolonged exposure were higher than the control levels, there was no statistical difference between the expression levels. This shows that there is a gradual increase in expression with the introduction of the defense mechanism in the early times when the plant encounters stress and a decrease in expression when the plant is unable to tolerate stress.

Rout and Sahoo applied different levels of Cu stress on *Withania somnifera plant*. As a result of the study, they determined that the CAT expression found in the leaf tissues of the plant increased up until the 50 µM Cu application but decreased in 100 and 200 µM Cu applications (Rout et al., 2013). In their studies, Cantarello et al. found that *CAT* expression level increased in 48-hour stress application and decreased in 56 and 72-hour periods (Cantarello et al., 2005). Kar applied Cd to chickpea plant at varying duration and concentrations. As a result of the study, the researcher found that the *CAT* gene was expressed at a high level after 24 hours of application, and after 48 hours of application, there was no significant decrease in expression level compared to the decrease in other antioxidant related gene expressions and it continued to be expressed (Kar, 2018). Besides, in the study where Rossatto et al. applied salt stress to rice plants, *CAT* expression was found to be higher at all application periods compared to control and it was stated that the expression level increased as the application period was prolonged (Rossatto et al., 2017). In the study of Souguir et al., in the rice seedlings to which Cd was applied, it was found that CAT expression level increased depending on the increasing concentration *and* the prolonged period (Souguir et al., 2013). Luna et al. concluded that CAT regulation serves to limit excessive $H_2O_2$ accumulation while allowing essential signaling functions to occur (Luna et al., 2005). Contrary to these findings, in this study CAT expression did not depend on the elevation of $H_2O_2$ concentration and continued to express in *C.pepo* roots.

Luo et al. exposed the perennial ryegrass plant to Cd at different times and concentrations and found that APX expression was regulated upwards in the first 24 hours of Cd application; however, they reported a decrease in expression levels due to prolonged concentrations (Luo et al., 2011). The induced expression of APX by other heavy metals has been reported in other plants. Cuypers et al. observed that Cu stress-induced APX2 of Arabidopsis thaliana (Tony et al., 2010). APX gene transcript increased in grass pea (*Lathyrus sativus L*) treated with Pb (Brunet et al., 2009). In this study, in parallel with the

literature, APX expression showed a significant decrease in the concentration of the highest $H_2O_2$ accumulation.

Baxter et al. reported that while early research on ROS metabolism focused on the potential toxicity of ROS and the different ROS cleaning mechanisms, more recent studies have been focusing on the role ROS plays as signaling molecules. In the studies conducted in recent years, it was emphasized that reactive oxygen species are less harmful than expected (Del Río, 2015; Farnese et al., 2016; Gupta et al., 2016). Choudhury et al. stated that ROS affects the expression of some genes and that ROS acts as a biological signal in the regulation of stresses. They emphasized that $O_2^{\bullet -}$ and $H_2O_2$ considered as the primary ROS in plants serves as secondary messengers regulating various functions in the growth and development of the plant (Choudhury et al., 2017). Apel and Hirt maintained that activate signal transmission could affect gene expression in 3 different ways by (1) activating the ROS sensors, (2) oxidizing the signal path components directly by ROS and (3) by changing the activity of transcription factors of ROS (Apel et al., 2004). In the study they conducted, Gill and Tuteja maintained that since $H_2O_2$ is long-lived and the permeability between membranes is high, regarding the signals produced through ROS, ROS was started to be accepted as a secondary precursor. They also stated that ROS acts as a key regulator in a wide range of physiological processes (Gill et al., 2010). Laloi et al. emphasized that the increases in $H_2O_2$ concentration were usually based on different mechanisms and that $H_2O_2$ served as a signal (Laloi et al., 2004). Kar exposed chickpea plant roots to Cd and stated that antioxidant gene expressions are associated with $H_2O_2$. He emphasized that at a certain level $H_2O_2$ amount provided the expression of genes at a high level while depending upon the increasing $H_2O_2$ concentration, there was a significant decrease in gene expression levels (Kar, 2018).

## CONCLUSION

In this study, we concluded that there is a close relationship between elevating cellular H2O2 concentrations and MT, Cu/Zn-SOD, and APX expressions. CAT was not affected by the elevated concentration.

In recent years, scientists have shown that H2O2 is the key molecule in many vital functions in plants. They emphasized that it is necessary for biotic and abiotic stress adaptation, signal transduction network in plants, and control of developmental processes. In this study, we tried to contribute to the literature about the signaling effect of H2O2. The findings of this study will shed light on the subsequent oxidative signaling and studies.

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