



The Evulation of Stress Related Gene Expression Level and Relationship to Cellular H₂O₂ in Chickpea (*Cicer arietinum* L.) Under Copper Stress^A

Musa KAR^{1*} Nuriye ÖZTÜRK²

Abstract: The aim of this study is to determine the changes in expression levels of stress genes in chickpea (*Cicer arietinum*) plant leaves exposed to copper (Cu) at different times and concentrations. Also; the relationship between the changes in gene expression and cellular H₂O₂ was investigated. In this context, the amount of malondialdehyde (MDA) and reactive oxygen species (ROS) hydrogen peroxide (H₂O₂) levels were determined. Furthermore, the changes in gene expressions of Metallothionein (MT), Catalase (CAT) and superoxide dismutase (Cu / Zn-SOD) enzymes were determined based on the actin expression level that selected as a housekeeping gene. It was determined that MDA content increased significantly due to time and concentration, In all duration and concentrations, the expression of stress-related genes significantly differed from the control group. Hleowever, a decrease has been determined by all gene expressions after the highest expression. This phenomenon is associated with cellular H₂O₂, which shows a steady increase in stress. At the end of the study, it was concluded that the elevating duration and concentration of Cu induced oxidative stress and caused the expression of stress-related genes. Furthermore, cellular H₂O₂ might be acting as a signal molecule that, up-regulate gene expressions until a certain concentration and down-regulate until a certain concentration. Thanks to the results of this study; Plants in agricultural areas can be exposed to a certain concentration of H₂O₂ to provide an earlier response to oxidative stress against biotic or abiotic stresses. In this way, the use of chemical pesticides can be minimized due to obtaining more durable products.

Keywords: *Cicer arietinum*, Cu, H₂O₂, Oxidative signaling, Stress-related genes.

^A Bu çalışma yüksek lisans tezinden üretilmiştir.

* **Sorumlu yazar/Corresponding Author:** ¹ Musa KAR Nevşehir Hacı Bektaş Veli Üniversitesi Fen-Edebiyat Fakültesi, Moleküler Biyoloji ve Genetik Bölümü, Nevşehir, Türkiye, musa.kar@nevsehir.edu.tr, [OrcID 0000-0001-7983-4814](https://orcid.org/0000-0001-7983-4814) ² Nuriye ÖZTÜRK Nevşehir Hacı Bektaş Veli Üniversitesi, Fen Bilimleri Enstitüsü, Biyoloji Bölümü, Nevşehir, Türkiye, biolognuriye@hotmail.com, [OrcID 0000-0003-4857-013](https://orcid.org/0000-0003-4857-013)

Bakır Stresi altında Nohut Bitkisinde (*Cicer arietinum* L.) Stresle İlişkili Gen Ekspresyon Seviyelerinde Meydana Gelen Değişimlerin Belirlenmesi ve Hücrel H₂O₂ ile İlişkisi

Öz: Bu çalışmanın amacı, nohut (*Cicer arietinum*) bitkisinde farklı zaman ve konsantrasyonlarda bakıra maruz kalmış bitki yapraklarındaki stres genlerinin ekspresyon seviyelerindeki değişimlerin belirlenmesidir. Ayrıca; gen ekspresyonlarındaki değişim ile hücrel H₂O₂ arasındaki ilişki tespit edilmeye çalışılmıştır. Bu bağlamda hücrede oksidatif stresin göstergesi olan melondialdehit (MDA) miktarı ve reaktif oksijen türlerinden (ROS) H₂O₂ miktarları tespit edilmiştir. Ayrıca antioksidan savunma elemanlarından Metallothionein (MT), Catalase (CAT) ve superoksit dismutaz (Cu/Zn-SOD) enzimlerinin gen ekspresyonlarındaki değişim house-keeping gen olarak seçilmiş aktinin ekspresyon düzeyi baz alınarak tespit edilmiştir. Çalışma sonucunda MDA içeriğinin süre ve konsantrasyona bağlı olarak belirgin bir şekilde arttığı, stres alakalı gen ekspresyonlarının bütün konsantrasyonlarda kontrolden yüksek düzeyde eksprese edildiği ancak en yüksek ekspresyon gerçekleştikten sonra süre ve konsantrasyondan arttıkça ekspresyon düzeylerinde bir azalma olduğu belirlenmiştir. Bu durum stres anındaki kararlı bir artış gösteren hücrel H₂O₂ ile ilişkilendirilmiştir. Çalışma sonucunda Cu maruziyetinin oksidatif strese neden olarak stres alakalı genlerin ekspresyonlarını indüklediği tespit edilmiştir. Ayrıca hücrel H₂O₂'nin belirli konsantrasyona kadar gen ekspresyonunu up-regule ederken belirli konsantrasyondan sonra down regule etmiş olduğu düşünülmektedir. bu çalışmanın sonuçları sayesinde; tarım alanlarındaki bitkiler belirli konsantrasyonda H₂O₂ ye maruz bırakılarak biyotik ve ya abiyotik streslere karşı oksidatif stres cevabını daha erken evrede vermesi sağlanabilir. Bu sayede, daha dayanıklı ürünler elde edilmesine bağlı olarak kimyasal zirai ilaç kullanımı asgari düzeye indirilebilir.

Anahtar Kelimeler: *Cicer arietinum*, Cu, H₂O₂, Oksidatif Sinyalizasyon, Stres alakalı genler.

Introduction

The rapid increase in the world population, industrial development, and global warming have caused a dramatic decrease in the usable agricultural lands in recent years. It is estimated that abiotic stress conditions are one of the most important causes of future arable land loss because 19% of world soil is hyper arid and 56% is at risk of desertification (FAO, 2004)

For these reasons, it is necessary to increase the production of high-quality foods while decrease costs and losses are reduced. In this regard; the physiological responses of the plants to the biotic and abiotic stress is the most common research topic among the scientist. Due to their sessile life plants are exposed to various abiotic stresses such as drought, temperature, salinity, and heavy metal exposure (Sewelam et al., 2016). Heavy metal exposure is one of the most common stress factors, especially in farmlands. Heavy metal exposure can cause quite different cellular effects in plants. In general, the deterioration of ion balance in plants, cell membrane permeability and DNA damage because of very destructive effects. (Leblebici and Aksoy, 2011).

Cu is an essential element that plays an important role in many metabolic events required for growth and development in all plants. It has a vital role in many physiological processes such as photosynthesis, respiration, reactions to oxidant stress, cell wall metabolism and hormone perception (Hernández-Hernández et al., 2018). On the other hand, excess Cu concentrations have several side effects. These are an imbalance in photosynthesis and pigment synthesis causes oxidative stress and other metabolic disorders. (Yruela, 2005).

Abiotic conditions lead to excessive production of reactive oxygen species that are highly toxic to plants and cause cellular damage. Cellular ROS reaches excessive concentrations, it can also cause significant damage by reacting with lipids, proteins, DNA, and several cellular components. This may result in a structural change and/or inhibition of the function. Because of these toxic effects of ROS, it is very important to keep its production and toxins under strict control. Plants have a very complex antioxidant system to avoid potential damages caused by ROS to cellular compartments. The main enzymatic components of the defense system are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) (Ahmad, 2014; Sahin et al., 2017).

Plants can react to biotic and abiotic difficulties with rapid changes in stress-related gene expression and protein synthesis. The determination of roles of functional genes or proteins against metal toxicity is very important in explaining the molecular mechanisms of metal tolerance. In recent years, scientists have suggested that H₂O₂ acts as a protective or signaling factor, except oxidative damage (Mittler et al., 2004). Despite our knowledge of the mechanisms of detoxification of antioxidant enzymes in metal exposure, we have inadequate information about signal transduction for the regulation and activation of these enzymes. Because H₂O₂ production is an immediate response to increased Cu-exposure, it has been emphasized by many researchers that it can be a key molecule that can trigger signal transduction events in plants after exposure to the metal (Farnese et al., 2016; Gupta et al., 2016). Vanderauwera et al. emphasized that reactive oxygen species are not as dangerous as they are considered and that H₂O₂ signaling effect is required in certain concentrations in order to activate the antioxidant defense system (Vanderauwera et al., 2009)

In this study, MDA and H₂O₂ concentrations were determined in leaf cells of chickpea plants exposed to Cu heavy metal at different times and concentrations. In addition, changes in mRNA transcription levels of stress-related genes were investigated and their relations with reactive oxygen species, H₂O₂, were explained.

Material and Method

Plant Material and Growing Conditions

Cicer arrinetium seeds were purchased from Nevşehir Directorate of Provincial Agriculture and Forestry one commercial genotype (Kocbası) used in this study. The seeds were subjected to germination in 25⁰C of moist perlite until the roots and the first leaves sprouted. The plants were taken to beakers with Hoagland solution to elongate their roots. After the root length was 10-15 cm (14 days), CuSO₄ was added to the beakers to be 50, 100 and 200 µM, respectively. They were exposed to Cu for 1, 3 and 5 days. The light / dark photoperiod is set to 16: 8 in the growth chamber. All applications are made as three replicates.

MDA Determination

The 500 mg leaf sample was homogenized with 3 mL of a solution containing 20% TCA and 0.5% TBA ((w/v). For lipid peroxidation, the homogenate was allowed to stand for 30 minutes at 95 ° C, then 10 minutes ice was used to stop the reaction. The samples were centrifuged at 12,000 g for 15 minutes. and the absorbance of the supernatant was recorded at 532 and 600 nm. The amount of malondialdehyde (MDA) (extinction coefficient of 155 mM⁻¹ cm⁻¹) was calculated by subtracting the non-specific absorbance at 600 nm from the absorbance at 532 nm.

H₂O₂ determination

Root tissues (500 mg) were homogenized at + 4 ° C with 5 ml trichloroacetic acid (TCA) in 0.1% (w / v) and centrifuged at 12,000 rpm for 15 minutes. Afterward, 0.5 ml of the supernatant, 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide (1 ml) was added to a separate tube. The solution incubated at room temperature. Absorbance was measured 280 nm and H₂O₂ concentration in the cell was determined according to the standard curve calculated using gradual H₂O₂ concentrations (Junglee et al., 2014).

RNA isolation and cDNA synthesis

Thermo Gene JET Plant RNA Purification Kit used for total RNA isolation. Yielded RNA quality was determined by measuring the absorbance values in 260/280 and 230/260 nanometers by using Donovix micro-volume spectrophotometer and used in cDNA synthesis. For the first-strand cDNA synthesis, Thermo RevertAid First Strand cDNA Synthesis Kit was used.

The quantitative real-time PCR analysis

For quantitative real-time analysis accuPower®, DualStar™ qPCR PreMix was used. For the preparation of this premix, 1 µM forward primer, 1 µM reverse primer, 5 µM cDNA and 13 µM DEPC-water were mixed and the final volume was adjusted to 20 µM. Actin gene was selected as a house-keeping gene. The primer sequences are given in Table 1.

Table 1. Primer sequences for PCR amplification and product sizes

Gene	Primer Pair	Sequence (5' – 3')	PRC product Size	Accession no
<i>Metallothionein-like gene</i>	MT2 F MT2 R	ATGTCTTGCTGTGGTGGTAAC TCATTTGCAGGTGCAAGGGTTG	240	GQ900702
<i>Superoxide dismutase Cu-ZnSOD</i>	Cu-ZnSOD F Cu-ZnSOD R	TAACCTCAGTCAGGAGGGAG GGAGTTTGGTCCAGTGAGA	276	AJ012739.1
<i>Catalase</i>	Cat F Cat R	TGCCCGCAGATGGATAGA GGTTGGCGAGGACCTTAACT	161	AJ131046.1
<i>Actin</i>	Actin F Actin R	TGTCTTGAGTGGTGGTTCTAC CTTGCCGCCAGATATTGTA	202	XM_004493535.1

Statistical Analysis

$2^{\Delta\text{ct}}$ calculation method by Livak and Schmittgen (2001) was used to determining changes in stress-related gene expression levels.

Analysis of variance (ANOVA) was performed to confirm significant differences among treatments. Post-hoc Duncan's test used to determine differences among groups ($p<0.05$). All statistical analyses were performed with the SPSS 22.0 software package.

Result and Discussion

The amount of MDA was calculated to determine the damage caused by the cell membrane and to determine the oxidative stress status of the cell.

MDA concentration increased as Cu duration and concentration increased. Statistically the highest MDA concentration on day 5, 200- μM concentration. (Fig 1 A) ($P<0.05$).

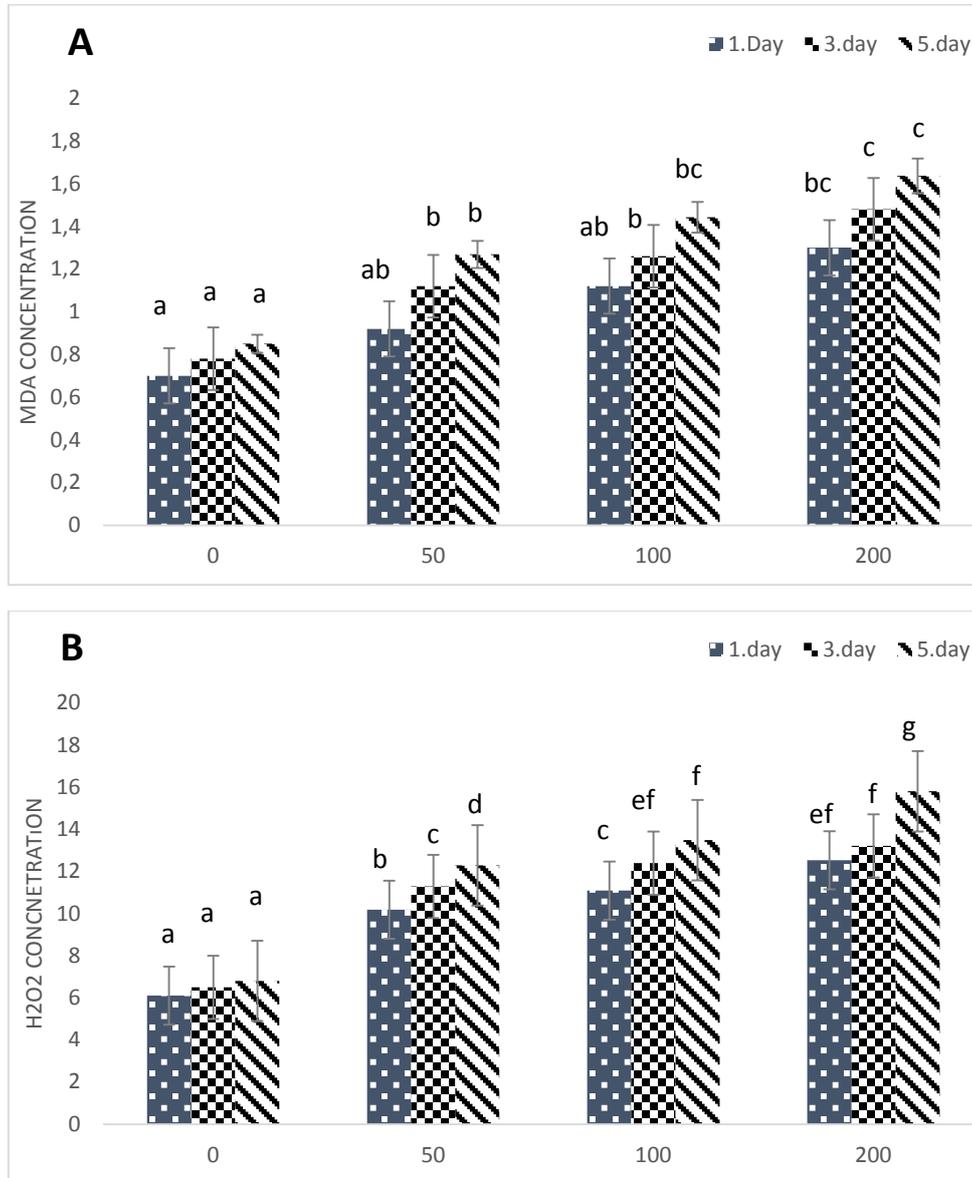


Fig.1. The differential of MDA content (A) and cellular H₂O₂ concentration (B) in Cu exposed Chickpea leaves. Different letters show the significance between groups ($p < 0.05$). The bar shows standard errors. (x axis: Different Cu concentrations)

The change in the amount of cellular H₂O₂ gives an idea about the oxidative state of the cell. Cellular H₂O₂ concentration determined in chickpea leaves exposed to Cu is showing a significant increase depending on duration and concentration. Statistically, the highest H₂O₂ concentration was found in the 5th day at a concentration of 200 μM (Fig. 2-B) According to the post hoc analysis, it was determined that all groups were different from each other ($p < 0.05$).

Relative changes of stress-related antioxidant genes expression levels were calculated based on changes of actin gene expression which selected as a house-keeping gene.

When the CAT expression was examined, the highest exposure level was observed on day 5 at a concentration of 50 μM . In prolonged duration and concentrations, CAT enzyme expression was statistically different from the control group. However, a statistically significant decrease was found with respect to the concentration at which the highest expression was observed. (Fig 3-A) ($p < 0.05$).

In the 50 μM concentration 5th day application, MT gene expression was found to be approximately 100-fold higher than actin gene expression. Afterward, it was found that there were a decrease in expression levels at 100 and 200 μM concentrations according to the concentration level of 50 μM . In addition, no statistically significant difference was found between Day 3 and Day 5 200 μM concentrations. (Fig 3-B) ($p < 0.05$).

When Cu/Zn- SOD gene expression is examined; similar to the other stress-related gene expressions, the highest expression level was found at 5th day 50 μM concentration. On the other hand; the expression levels are reduced moreover, there was no statistically significant difference between the expression level at 1st day 200 μM and the control group (Fig. 2-C) ($p < 0.05$).

In this study, the product of lipid peroxidation and an indicator of oxidative stress MDA concentration and a by-product of oxidative stress ROS/ H_2O_2 concentrations investigated. In addition, changes in expression levels of MT, Cu-Zn SOD and CAT antioxidant stress-related genes were determined based on housekeeping gene actin expression. The linkage between cellular H_2O_2 concentration and changes in the levels of expression was explained.

Free radicals that cause oxidative stress can react with all the cellular structures, but the most sensitive component to these interactions are lipids. Malondialdehyde (MDA) is a highly reactive metabolic product that occurs as a result of a series of reactions during lipid peroxidation, which is caused by the effects of free oxygen radicals on tissues (Gaweł et al., 2004). In this study, a steady increase in all concentrations was observed depending on the Cu exposure. In previous similar studies, they emphasized that the primary indicator of stress caused by heavy metal exposure is lipid peroxidation and MDA content (Duman et al., 2010).

MTs are low molecular mass proteins that are thought to play a key role in response to metal toxicity and oxidative stress through Cys thiol groups. (Gu et al., 2014; Guo et al., 2008; Mir et al., 2004). Kumar et al. reported that; MT gene expression is elevated as a result of the different concentrations of Cu exposure to *Neurospora crassada* (Kumar et al., 2005). 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). In this study we found that; MT gene expression increased all durations and concentrations according to the control group. Liu et al. conducted a study with rice exposed to Cu at a different time and concentration they put out that; there was a decrease in MT expressions as the prolonged duration and concentration. As a result, they stated that; the *OsMT* expression increase is not related to Cu concentration it is directly regulated by H_2O_2 (Liu et al., 2015). Our study is supporting these findings.

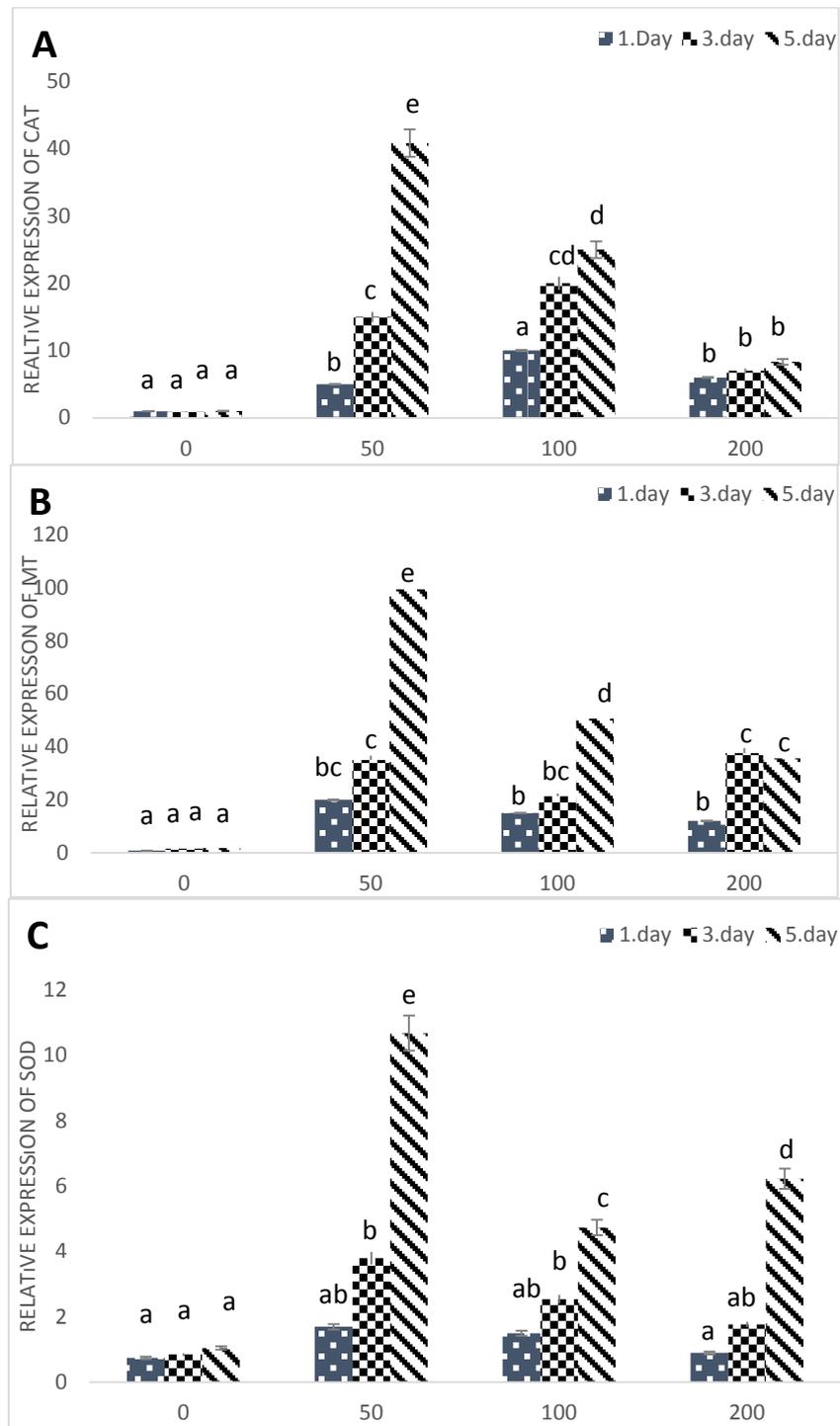


Fig. 2. Relative expressions of stress-related genes; Catalase (A) Metallothionein (B) and Cu/Zn-SOD (C) in Cu exposed *Cicer arietinum* leaves. Different letters show the significance between groups ($p < 0.05$). The bar shows standard errors. (x axis: Different Cu concentrations)

The most important function of the catalase is to detoxify H_2O_2 as H_2O and O_2 . Rosatto et al. reported that CAT expression levels are increased under stress factors (Rossatto et al., 2017). Kim et al. stated that; as a result of salt stress in rice CAT gene expression induced positively (Kim et al., 2007). In our study in parallel with these studies, there was a significant increase in CAT expression levels as a result of stress. It is notable that; a decrease of CAT gene expression, after the highest expressions observed, is not distinct as other studied gene expressions. Rossatto et al. emphasized that; the signaling molecule effect of H_2O_2 on CAT expressions is lower than that of other antioxidant genes. In their studies, similar results reported from Kar and Sougir et al. (Kar, 2018; Sougir et al., 2013). Our findings are in parallel with the literature.

In addition, 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 Sougir 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 (Sougir 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 our study, CAT expression did not depend on the elevation of H_2O_2 concentration and continued to express in chickpea roots.

SOD plays a decisive role in protecting against the toxic effects of oxidative stress by scavenging superoxide radicals and ensuring their conversion to O_2 and H_2O_2 (Verma and Dubey, 2003). Many studies have reported that ROS associated with oxidative stress under drought and salt stress in plants induces SOD enzyme activity (Baloğlu et al., 2012; Ishibashi et al., 2011). Soydam et al. determined that; tomato plants subjected to salt and drought stress and there was an increase of SOD enzyme expressions. (Aydin et al., 2014). Similarly, in this study, an increase in SOD enzyme expression is observed in response to oxidative stress triggered by Cu exposure.

Also; Rossatto et al. found that SOD expression increased in rice plants up to 15-days exposure but decreased after 20-day salt stress exposure (Rossatto et al., 2017). In the current study, also found a significant decrease in SOD expression due to increased time and concentration. 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 our 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.

In recent years; scientists have been approached a different view of cellular H₂O₂ function and emphasized that; reactive oxygen species are less harmful than formerly known. (Farnese et al., 2016; Gupta et al., 2016; Del Río, 2015). Sharma et al. reported that; H₂O₂ and other ROS species can serve as a signal molecule because it is useful in low concentrations and toxic when excess a certain concentration (Sharma et al., 2012). Rosatta et al. emphasized that; after salt stress in rice plant H₂O₂ concentration was low and do not cause any damage to cells, on the contrary, they reported that low H₂O₂ expression of stress-related genes. (Rossatto et al., 2017). Gill and Tuteja have stated that because of the long life of H₂O₂ and the permeability of the membranes, ROS is considered as a secondary messenger and acts as a key regulator in a wide range of physiological processes. (Gill and Tuteja, 2010). 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 O₂^{•-} and H₂O₂ 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 and Hirt, 2004). In the study they conducted, Gill and Tuteja maintained that since H₂O₂ 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 and Tuteja, 2010). Laloi et al. emphasized that the increases in H₂O₂ concentration were usually based on different mechanisms and that H₂O₂ served as a signal (Laloi et al., 2004). Kar, exposed chickpea the plant roots to Cd and reported that; antioxidant gene expression is associated with H₂O₂ concentration. In a certain level of H₂O₂ concentration stress-related gene expressions up-regulated and a significant decrease observed in gene expressions after a certain H₂O₂ concentration. (Kar, 2018). In addition, Del-rio emphasized the cellular importance of H₂O₂ to secondary messenger tasks and emphasized that it is appropriate to use the term “oxidative signaling” for H₂O₂ activities in the cell. (Del Río, 2015). Our findings support these findings.

Conclusions

As a result of the study, Cu exposure to chickpea at different duration and concentrations increases cellular MDA content and H₂O₂ accumulation. In order to prevent oxidative damage of Cu exposure, stress-related genes were highly expressed than the control group at all application concentrations. Thanks to the results of this study; Plants in agricultural areas can be exposed to a certain concentration of H₂O₂ to provide an earlier response to oxidative stress against biotic or abiotic stresses. In this way, the use of chemical pesticides can be minimized due to obtaining more durable products.

Additionally; expressions of stress-related genes were highest when H₂O₂ concentration was at a certain level but decreased expression levels due to increased H₂O₂ concentration. The findings may contribute further oxidative signaling studies.

Acknowledgment

All authors participated in the conception of the topic. M.K and N.Ö conducted %50-%50 laboratory studies. M.K and N.Ö have written %75-%25 of the manuscript. All authors read and approved the final manuscript after critically revising it for important content. The authors declare that there is no conflict of interest regarding the publication of this article.

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