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Original Paper

Lipoxygenase activity and antioxidant capacity in pepper (*Capsicum annum*) exposed to high concentration of copper sulphate

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Esra Koç*, Belgizar Karayiğit

Ankara University, Faculty of Science, Department of Biology, Ankara, Turkey

*Corresponding author, E-mail: ekoc@science.ankara.edu.tr, esrakoc.es@gmail.com

Abstract

Excess Cu causes negative effects on plant growth and development by affecting important physiological processes in plants. In this study, the effect of high concentration of copper sulphate ($CuSO_4$) on lipoxygenase (LOX) activity, lipid peroxidation and antioxidant activity in pepper was investigated. The highest amount of LOX activity increase and lipid peroxidation in pepper seedlings' leaves exposed to $CuSO_4$ was detected 24 h following the application. An early antioxidant response to $CuSO_4$ application occurred, and the highest 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was detected 3 h after the application and the highest reducing power after 12 h. The increase in malondialdehyde of leaf samples taken 1.5 h and 3 h after the application, along with the increase in LOX activity, reducing power and DPPH scavenging activity (%), showed that $CuSO_4$ stimulates the defence system in a short time. The increased lipid peroxidation, despite generally decreased DPPH fragmentation activity and reducing power in samples taken after 6 h showed that 0.5 M $CuSO_4$ caused high oxidative stress. This result revealed that high $CuSO_4$ concentrations may also disturb the non-enzymatic antioxidant capacity.

Key words: antioxidant activity, *Capsicum annuum*, Cu, lipid hydroperoxide, lipid peroxidation, lipoxygenase.
Abbreviations: $CuSO_4$, copper sulphate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; LOX, lipoxygenase; MDA, malondialdehyde; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid..

Introduction

Pepper (*Capsicum*), a member of the Solanaceae family, has at least 30 species and is included in the ten most produced vegetables in the world (FAOSTAT 2013; FAO 2016). Also, pepper ranks second in global fresh vegetable exports (FAO 2016). *Capsicum annuum*, both chilli and sweet, is the most widely grown pepper type in the world. Hot peppers are additives that are widely used in spicy or salty foods in many countries around the world. Hot pepper has a high economic value due to its colour, flavour and tang in addition to its economic importance, and pepper is also an agricultural product used in pharmacology and medicine due to its biological and functional compounds, such as antioxidants, vitamins and secondary metabolites (Barbero et al. 2016). *C. annuum* is exposed to various biotic stresses that limit its production and yield (*Phytophthora capsici*, *Xanthomonas vesicatoria, Alternaria* spp., *Fusarium* spp., *Pseudomonas syringae* etc.) (URL1 2011; Parisi et al. 2020) and abiotic stresses such as heavy metals (Antonious, Kochhar 2009; Vodyanitskii et al. 2016; Desoky et al. 2018), which cause major problems in agriculture and forestry. Sources of haevy metals are volcanic activity and exhaust gas of motor vehicles, fertilizer, pesticides, mining, urban waste and industrial activities (Vodyanitskii et al. 2016; Desoky et al. 2018).

Copper (Cu) is a essential micronutrient element for plant growth and development (Yu et al. 2018). It acts as a catalyst in photosynthesis and respiration and plays an important role in the formation of lignin in the cell wall. Copper sulfate ($CuSO_4$) is frequently used an active ingredient agricultural fungicide, algicide, herbicide, and as nutritional supplement, fertilizer (Zhu et al. 2012). Cu ions in $CuSO_4$ bind to functional proteins in pathogens and cause cell damage and leakage by causing protein denaturation (RED-Cu 2009). Nevertheless, at high concentrations copper can become extremely toxic to plants by affecting electron transport in photosynthesis (Yruela et al. 2005).

In chemical control with pathogens that cause various diseases, the wrong, unnecessary, long-term and excessive use of especially high copper-containing fungicides and drugs cause morphological, physiological and biochemical changes in pepper and reduce the product quality and yield (Sonmez et al. 2006). In addition, these anthropogenic activities accelerate Cu accumulation in soil (Fernandes,

Henriques 1991). Prolonged exposure to Cu causes major deterioration in root morphology, reduced root and shoot growth and leaf chlorosis (Kolbert et al. 2012; Verma, Bhatia 2014). Excessive accumulation of this element damages biomolecules, including protein, DNA, enzymes and membrane lipids, causing the formation of reactive species (ROS) that cause oxidative stress (Burzynski, Klobus 2004; Köksal 2009). The increase in food contamination caused by these toxic metal ions, which increase due to many sources, is also a global problem.

Different lipoxygenase (LOX) isozyme forms are involved in plant growth and development (Siedow 1991). LOX are also responsible for membrane degradation as they catalyze the dioxygenation of polyunsaturated fatty acids that produce hydroperoxy fatty acids that are toxic to the cell (Ali et al. 2005; Shi et al. 2020). These lipid hydroperoxides are then converted into secondary compounds (jasmonic acid etc.) that act in defense (Feusser, Wastenack 2002). ROS in plant system leads to membrane damage and many other changes. Both enzymatic and nonenzymatic antioxidant defense mechanisms of the plant system coordinate and alleviate oxidative damage in plant (Sahitya et al. 2018). Antioxidants prevent the formation of ROS and provide scavenging of the reactive metabolites or their conversion to stable molecules or subtances of lower reactivity (Santos-Sánchez et al. 2019).

Plant tolerance for stress factors differ. The type of the plant, stress factor, duration and severity of exposure to stress, and the structure of the tissue or organ exposed to stress influence the tolerance. In literature reviews, no study on LOX activity in peppers exposed to copper sulphate has been found. Therefore, this study aimed to determine activity of the LOX enzyme in pepper plants at high concentration of $CuSO_4$, and also to determine the amount of lipid peroxidation and its effects on antioxidant capacity.

Materials and methods

Plant material

KM-121 (*Capsicum annuum* cv. Kahramanmaras-Acı.) cultivar was used in this study as plant material. The KM-121 pepper cultivar was supplied from Kahramanmaras Agricultural Research Institute (Turkey). After germination, pepper seedlings were sown in plastic pots containing a soil/fertilizer/sand mix (1:1:1, v/v/v). The plants were grown in a growth chamber under controlled environmental conditions (25 ± 2 °C and 16-h light, 8-h dark periods; Digitech GLO-PG42). At the end of two months, once they reached the six-seven leaf stage, plants were used as material for experiments.

Plants were collected, then the roots were washed with tap water and kept in 0.75% sodium hypochlorite for 1 to 2 min for disinfection. Then the roots were washed with sterile distilled water containing 1 to 2 drops of Tween 20 in 1 L water. Each application was repeated three times; three bottles were used for each repetition. There were 15 seedlings in each bottle. Fifteen seedlings were put into a sterile glass bottle containing 400 mL Hoagland solution, and then incubated for 3 days at 22 ± 3°C, 60% humidity, and 14-h light period for acclimation. The application of solution containing 0.5 M $CuSO_4$ and 0.05% Tween 20 was performed by superficial spraying on plant foliage. $CuSO_4$ was sprayed on leaves of each repetition; $CuSO_4$ spraying was performed once. Control plants were sprayed with distilled water containing 0.05% Tween 20. Random samples were collected from these seedlings at 0, 0.5, 1.5, 3, 6, 12 and 24 h later. The leaves were separated and tissues were frozen in liquid nitrogen and ground in a pre-chilled mortar. Afterwards, they were put into nylon bags, labeled and stored at –70 °C until analysis.

Lipoxygenase activity

Lipoxygenase (LOX, EC 1.13.11.12) enzyme assay was performed on leaf extract (Lanna et al. 1996). Leaf samples were ground in liquid nitrogen and extracted with 50 mM sodium phosphate buffer with pH 6.5, 1% polyvinylpolypyrrolidone and 1 mM phenylmethylsulfonyl and then centrifuged at 20 000 g for 25 min at 4 °C. The supernatant obtained was used to determine the LOX enzyme activity.

LOX activity was determined according to Axelrod et al. (1981). The reaction mixture contained 1 mL 50 mM sodium phosphate buffer with p.H 6.0, 20 µL substrate (10 mM sodium linoleate, pH 9.0) and 10 µL supernatant. LOX activity was calculated using the molar extinction coefficient of linoleic acid (25 mM cm^{-1}) at 234 nm for hydroperoxide.

Measuring lipid peroxidation (malondialdehyde concentration)

Malondialdehyde (MDA) concentration in pepper leaves was measured as and indicator of lipid peroxidation using the method of thiobarbituric acid (TBA) reaction (Heath, Packer 1968). After the fresh sample was homogenized with 1% trichloroacetic acid (TCA), a solution containing 20% TCA and 0.5% TBA was added to the extract obtained, and then the solution was incubated in a water bath for 30 min at 95 °C, and absorbance was measured in a spectrophotometer at 532 and 600 nm. MDA content (155 mM cm^{-1}) was calculated using the extinction coefficient.

1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

The ability of methanol extracts of pepper plants to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals was determined using the method described by Blois (1958). A fresh leaf sample (100 mg) was incubated overnight at room temperature in 1.5 mL methanol. A solution of 0.1 mM DPPH (1.45 mL) was mixed with 150 µL of extract. Methanol (150 µL) was used as a control. The samples

were incubated in a water bath for 30 min at 24 °C, and the decrease in absorbance at 517 nm was measured. The lower absorbance of the reaction mixture showed a higher DPPH radical scavenging activity. Percent inhibition was calculated using the formula:

$$\text{DPPH scavenging activity (\% inhibition)} = [(Ac - As)/ Ac] \times 100,$$

where "*Ac*" was the absorbance of the control reaction and "*As*" was the absorbance of the extracts.

Determination of ferric reducing antioxidant power

The Fe^{3+} reducing power of extracts was determined by the method of Vijayalakshmi and Kandamsy (2016). To determine reducing power, a sample of 100 mg fresh leaves was incubated with 1.5 mL of methanol. The reaction mixture contained 250 µL extract, 1.25 mL 0.2 M pH phosphate buffer with p.H 6.6 and 1.25 mL potassium hexacyanoferrate ($K_3Fe(CN)_6$) (1%) and incubated at 50 °C for 20 min. The reaction was stopped by 1.25 mL of 10% TCA and centrifuged at 3000 g for 10 min. The upper layer (1.25 mL) was mixed with 1.25 mL distilled water and 250 µL ferric chloride ($FeCl_3$) (0.1%) at 24 °C for 10 min and the absorbance was measured at 700 nm. A higher value absorbance of the reaction mixture indicated greater reducing power.

Statistical analysis

Differences in LOX, MDA, DPPH and reducing power were analyzed by repeated measures analysis of variance (one way ANOVA). Variance analysis was conducted using the SPSS 24 software package. Significant differences were determined as $p < 0.05$). Statistical significance is indicated by appropriate letters in the tables. Different lowercase letters indicate significant differences between treatments. Data presented are mean values ± SD measures for three replicates ($n = 3$).

Results

$CuSO_4$ applied to pepper leaves affected the LOX enzyme activity 1.5 h after the application and caused significant increase in LOX activity, especially 3, 6, 12 and 24 h after the application (Table 1). Enzyme activity in leaf samples taken at 3, 6, 12 and 24 h after the 0.5 M $CuSO_4$ application was higher by 51.7, 53.2, 42.1 and 78.1%, respectively, than in the control plants. The highest enzyme activity occurred in leaf samples collected after 24 h (Table 1).

At 1.5 h after high $CuSO_4$ application in pepper seedlings, an increase in MDA concentration was also detected. The highest MDA concentration occurred at 12 and 24 h after the application, resulting in an 153 and 226% increase in the leaf samples, respectively, compared to the control plants (Table 2).

Reducing power increased due to $CuSO_4$ stress at 1.5, 3 and 12 h, compared to control plants (Table 3). Although the highest activity of reducing power occurred at 12 h; the highest reducing power increase (by 41.76%) compared to the control group was found at 3 h after the application (Table 3). Although there was a decrease in reducing power in leaf samples taken at 6 and 24 h compared to the control group, the difference was statistically significant only for 6 h (Table 3).

DPPH radical scavenging activity did significantly differ betaeen the treatments and control groups at all times (Table 4). The highest DPPH radical scavenging activity increase compared to the control group was by 5.63% at 1.5 h after the application and by 14.83% at 3 h. DPPH radical scavenging activity decreased at later times after the application and the lowest relative DPPH radical scavenging activity was observed at 24 h (Table 4).

Discussion

It has been reported that LOX has functional roles in response to abiotic as well as biotic stresses (Hwang, Hwang 2010; Padilla et al. 2012). The increase in LOX enzyme activity due to $CuSO_4$ application in this study is in line with previous studies (Somashekaraich et al. 1992; Gallego et al. 1996; Bhattaxhajee 1997; Skorzynska-Polit, Krupa 2003; Djebali et al. 2005; Rucins, Gwozdz 2005; Mahmud et al. 2017) that reported an increase in th LOX enzyme activity in plants exposed to heavy metals. In addition in

Table 1. Effect of $CuSO_4$ on LOX activity in pepper at different times after treatment. Data presented are mean values ± SD for three replicates

Time (h)	LOX activity (unit g^{-1} FM)	
	Control	$CuSO_4$-treated
0	0.364 ± 0.020 e	0.361 ± 0.020 e
0.5	0.375 ± 0.038 e	0.378 ± 0.018 e
1.5	0.397 ± 0.004 e	0.565 ± 0.032 c
3	0.379 ± 0.022 e	0.575 ± 0.041 c
6	0.449 ± 0.012 d	0.688 ± 0.030 b
12	0.460 ± 0.018 d	0.654 ± 0.009 b
24	0.463 ± 0.046 d	0.825 ± 0.026 a

Table 2. Effect of $CuSO_4$ on MDA concentration in pepper at different times after treatment. Data presented are mean values ± SD for three replicates

Time (h)	MDA (nmol g^{-1} FM)	
	Control	$CuSO_4$-treated
0	0.923 ± 0.145 e	0.992 ± 0.034 e
0.5	0.964 ± 0.081 e	0.888 ± 0.108 e
1.5	1.014 ± 0.071 e	1.863 ± 0.005 c
3	0.991 ± 0.079 e	1.292 ± 0.084 d
6	1.022 ± 0.090 e	1.754 ± 0.026 c
12	0.954 ± 0.049 e	2.410 ± 0.060 b
24	0.994 ± 0.323 e	3.244 ± 0.150 a

Table 3. Effect of $CuSO_4$ on reducing power in pepper at different times after treatment. Data presented are mean values ± SD for three replicates

Time (h)	Reducing power (A_{700})	
	Control	$CuSO_4$-treated
0	0.646 ± 0.061 c	0.634 ± 0.039 c
0.5	0.604 ± 0.004 c	0.622 ± 0.022 c
1.5	0.661 ± 0.020 c	0.854 ± 0.033 b
3	0.692 ± 0.024 c	0.981 ± 0.142 a
6	0.804 ± 0.132 b	0.576 ± 0.038 c
12	0.825 ± 0.075 b	1.017 ± 0.022 a
24	0.691 ± 0.032 c	0.666 ± 0.019 c

Table 4. Effect of $CuSO_4$ on DPPH radical scavenging activity in pepper at different times after treatment. Data presented are mean values ± SD for three replicates

Time (h)	DPPH radical scavenging activity (%)	
	Control	$CuSO_4$-treated
0	136.162 ± 4.921 c	135.733 ± 4.892 c
0.5	133.615 ± 5.217 cd	136.784 ± 4.530 bc
1.5	134.847 ± 4.233 cd	142.414 ± 1.002 b
3	133.911 ± 3.237 cd	153.748 ± 0.783 a
6	132.887 ± 1.265 cd	131.463 ± 4.004 cd
12	137.817 ± 5.259 bc	128.714 ± 0.064 d
24	134.977 ± 3.036 cd	117.589 ± 1.556 e

recent studies on different plants, it has been reported that metals such as Cd, Pb, As, Cr, Al induce lipid peroxidation and accumulation of ROS, indicating oxidative stress, as well as cause a high increase in LOX enzyme activity (Nahar et al. 2017; Hasanuzzaman et al. 2018; Kabala et al. 2019; Yadu et al. 2019; Ahanger et al. 2020). In a study with basil plants exposed to Cu stress, it was shown that high Cu concentration caused cellular damage by significantly increasing the MDA and H_2O_2 concentration, while Cu applied at low concentration did not cause a significant increase in the concentration of MDA (Georgiadou et al. 2018).

Polyunsaturated fatty acids, which are the main components of membrane lipids, are the main target of the oxidative effect of heavy metals (Morsy et al. 2012). MDA is the end product of lipid peroxidation process (Gawel et al. 2004). Therefore, an increase in the concentration of MDA is an indicator and parameter of the intensity of oxidative stress. Therefore, the increasing amount of MDA after a high concentration of $CuSO_4$ application, whichwas observed in this study, shows that $CuSO_4$ stimulates oxidative stress in peppers, probably due to an increase of ROS. In our study, the highest increase in MDA amount was observed 24 h after treatment with Cu. Lipid hydroperoxides, which are formed as a result of fatty acid peroxidation in plant cells, occur in both enzymatic and non-enzymatic pathways. In non-enzymatic lipid peroxidation, a lipid radical is formed as a result of hydrogen removal from the unsaturated fatty acid and a radical chain reaction occurs with the addition of molecular oxygen (Kappus 1985).

Also, it has been reported that transition metal ions are involved in the initiation of lipid peroxidation (Kappus 1985). One of the most important targets of Cu ions is proteins in the cell membrane. Cu causes damage to the cell membrane by attacking the sulfhydryl groups of these proteins (De Vos, Schat 1989). Transition metals such as Cu^{2+} catalyze the formation of hydroxyl radicals from the non-enzymatic chemical reaction between superoxide and H_2O_2 (Halliwell, Gutteridge 1984). These results support the view that transition metals such as Cu participate in the formation of ROS in excessive doses and stimulate oxidative stress and consequently increase lipid peroxidation (Lu et al. 2010, Gou 2010; Liu et al. 2018).

Transition metals such as Fe and Cu stimulate LOX activity more strongly (Gallego et al. 1996; Skorzynska-Polit, Krupa 2003). In addition, these metals contribute to LOX-mediated ROS formation (Mitler 2017; Choudhary et al. 2020). Enzymatic lipid peroxidation is catalyzed by lipoxygenase enzyme. The lipoxygenase enzyme catalyzes the dioxygenization of free fatty acids containing linoleic and linolenic acid and lipid hydroperoxides are formed. These lipid hydroperoxides are then converted into secondary compounds (Feusser, Wastenack 2002). The increase in the amount of lipid peroxide in parallel with the increase in LOX enzyme activity in the current study shows that the increased activity of LOX enzyme under $CuSO_4$ application may have contributed to the lipid peroxidation of the plant tissue. These results also support the findings that lipid peroxidation can start parallel with LOX activation and this may result in high levels of peroxides (Skorzynska-Polit 2007). A study conducted with *Phaseolus vulgaris* treated with Cd showed that the LOX enzyme activity on the sixth day was higher than on the fourth day, but it was not correlated with the lipid peroxide content (Somashekaraiah et al. 1992). The amount of non-enzymatic lipid peroxidation was higher in the plants treated with Cd compared to the control group, but there was no change in LOX enzyme activity (Skorzynska-Polit, Krupa (2006).

The effect of metal on LOX activity usually depends on type as well as concentration of the metal. For example, high amounts of lipid peroxides and jasmonic acid were observed in plants under excess Cu stress, suggesting that linolenic acid as the main substrate for LOX was involved in formation of jasmonic acid in response to heavy metal stress, with jasmonic acid being involved in the transduction of signal as a response to abiotic stress effects for the systemic response formation (Farooq et al. 2018; Bali et al. 2019). The increases in enzyme activity found in our study indicate that copper stimulates the LOX enzyme in pepper plants. The highest increases in LOX activity were observed at 6, 12 and 24 h after application. Therefore,

this indicates that these increases in LOX activity may be associated with synthesis of jasmonic acid, which acts as a defense signaling molecule against stress, and that jasmonic acid may have alleviated oxidative stress through improved ROS scavenging mechanisms in pepper plants facing copper stress.

Another known way of preventing ROS excess caused by various stresses is the non-enzymatic mechanism of some metabolites synthesized from secondary metabolism, especially by the phenylpropanoid pathway (Noctor, Fayer 1998). Some of these metabolites have the potential to detoxify free radicals. DPPH free radical scavenging activity and reducing power indicate the induction of non-enzymatic antioxidant mechanism (phenolic, flavonoids etc.) in plants exposed to stress (Contreras et al. 2018). DPPH is a free radical that easily damages the cell membrane. The high radical scavenging activity of an organism is directly proportional to its protective effect against oxidative damage (Subramanian et al. 2013). This view is also supported by a significant increase in DPPH and reducing power in *Colobanthus quitensis* exposed to high copper (Contreras et al. 2018).

In our study, the decrease in the MDA amount in the leaf samples taken at 3 h, compared with that in leaf samples taken 1.5 h after the application, may be the result of weakening of the cytotoxicity induced by $CuSO_4$ by decreasing the cellular ROS level that increases over time with an increase in DPPH scavenging activity and reducing power of the nonenzymatic antioxidant.

The high level of radical scavenging activity of an organism is directly proportional to its protective effect against oxidative damage (Subramanian et al. 2013). A higher absorbance indicates a higher reducing power (Bursal, Köksal 2011). In our study, an early antioxidant response to $CuSO_4$ application has occurred. The increase in DPPH scavenging activity and reducing power together with the increase in MDA content in leaf samples taken at 1.5 and 3 h after the application shows that $CuSO_4$ also activates the defence system. These findings are in line with studies showing that Cd and Cu stress can increase LOX activity, but at the same time, increased activity of nonenzymatic antioxidants (phenolics, flavonoid etc.) can reduce lipid peroxidation levels (Somashekaraiah et al. 1992; Skorzynska-Polit, Krupa 2006; Skorzynska-Polit et al. 2006; Smeets et al. 2008). Therefore, increased non-enzymatic antioxidant activity is a defensive response that reduces stress damage caused by $CuSO_4$ and improves stress tolerance. However, the increase in the amount of MDA indicates that this antioxidant power is not enough. However, despite the decrease in DPPH radical scavenging activity and reduction power in leaf samples taken at 24 h following the application, the increase in the MDA amount shows that the antioxidant capacity can no longer cope with $CuSO_4$ toxicity. This result revealed that high $CuSO_4$ may also disturb the non-enzymatic antioxidant capacity. Studies indicate that the scavenging power of nonenzymatic antioxidants may decrease during stress conditions, including that induced by toxic heavy metals (Ahmad et al., 2011; Siddiqui et al. 2014). This study showed that exposure to excessive $CuSO_4$ stimulated MDA accumulation and caused oxidative stress in pepper leaves, but also increased nonenzymatic antioxidant capacity and LOX activity shortly after the application.

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