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Research Article

RESPONSE OF TOMATO (*Lycopersicon esculentum* Mill.) SEEDLINGS TO CADMIUM STRESS WITH PHYSIOLOGICAL AND OXIDATIVE PARAMETERS

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ABSTRACT. Cadmium (Cd) is a kind of heavy metal and one of the hazardous abiotic stress factors for whole agricultural areas. In this study, it was aimed to study the effect of cadmium treatments on the growth and development of tomato seedlings *(Lycopersicon esculentum*, Mill. cv Astona). For this purpose, the seedlings were grown for 15 days in nutrient solution was studied with five Cd levels (0, 5, 25, 50 and 100 µM) under controlled environmental conditions. The following parameters were determined: dry matter, pigment concentration, antioxidative enzymes, lipid peroxidation and soluble protein. The results indicate that the shoot and root growth on all Cd concentrations was reduced. Among the Cd treatments, 25 µM Cd-treated plants showed higher values for many parameters and 5 µM Cd-treated plants were less affected by toxicity. The results indicate that high Cd levels cause oxidative stress, as expressed by the increase of lipid peroxidation products, ascorbic acid (AsA), sulphydryl (SH) groups and protein amount compared to controls.

Keywords: *tomato, cadmium, antioxidant enzymes, growth, pigment*

INTRODUCTION

Heavy metal pollution is a significant worldwide problem that threatens to the human life. In most places in the world, particularly close to industrial areas, are extremely polluted by heavy metals resulted from anthropogenic activity [1]. It is causing concern that agricultural soils are polluted with toxic metals due to industrial activities, fertilization, and waste disposal [2].The use of wastewater in agricultural areas and therefore the contamination of heavy metals to plants is an increasing danger for consumers that consume these plants. Plants tend to take metals naturally, which are essential nutrients despite some others cadmium (Cd), mercury (Hg), nickel (Ni) and lead (Pb), are toxic to plants [3]. It has been reported that Cd is a heavy metal which is rare in the ecosphere [4]; in areas where human activities are poor, it's source in that mineralization of rocks [5]. Primary sources of soil contamination by Cd that adversely affects plant growth and development are using of mineral fertilizers, some pesticides, wastewater and other soil amendments [6]. Because of soils contaminated with Cd in most agricultural area, plants uptake of Cd and translocated to various plant parts, in this manner food chain is a major pathway of its entry to human diet [7].

Some plant species can grow and survive in soil containing high-concentration metals that are toxic to other plants, while others cannot grow [8]. Recent studies have shown that heavy metals decrease some metabolic activity (photosynthesis, transpiration, chlorophyll synthesis), whereas increase oxidative stress which begun by

reactive oxygen species (ROS) in organisms and cellular systems [9]. Increasing by the amount of ROS cause the most severe effects of heavy metals on plants, especially chloroplast pigments, membranes and nucleic acids [10, 8]. As a result of these, some changes occur in the tissues such as chlorosis, necrosis, limited root growth, plant growth problems, reduction of water potential and activity of various key enzymes [11]. In plants exposed to Cd, some processes including photosynthesis, respiration and transpiration are inhibited in addition to these exhibit various symptoms such as leaf epinasty, chlorosis, dwarf growth [12]. Cadmium affects some events such as membrane damage at the cellular level, the presence of electron transport, changes in enzyme metabolism and the dealing with nucleic acids. High concentrations of Cd seriously damage such as membrane injury, growth inhibition and impairment of photosynthesis through the activation of oxidative stress [13]. As against Cd toxicity, cells have antioxidants such as α-tocopherol, β-carotene and some antioxidant enzymes (Superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR)) useful for removing ROS [14, 15].

Most of the plants contain compounds with antioxidant activity for example carotenoids and polyphenols, and an increased consumption of them in the diet could decrease the risk of cancer and other diseases. [16,17]. Tomato is the most essential food both for its general utilization and for its profitable use in antioxidant properties [18,19]. Consequently, tomatoes have acquired the status of a functional food.

The purpose of present study was to investigate the effect of Cd stress on growth parameters, pigment concentration, antioxidative enzymes, lipid peroxidation and soluble protein of tomato plants at some physiological levels.

MATERIALS AND METHODS

Plant Growth

Seedlings of tomato (*Lycopersicon esculentum* Mill. cv. Astona; thirty days old) were obtained from Güney Seedling Company, Mersin, Turkey. Roots of seedlings were washed with distilled water series and CaSO4 solution, after this plants transferred to rectangle plastic pots containing continuously aerated Hoagland's nutrient solution (700µM K_2SO_4, 100 µM KCl, 2000 µM $Ca(NO_3)_2$, 750 µM $MgSO_4$, 200 µM KH_2PO_4, 100 µM FeEDTA, 10 µM H_3BO_3, 1 µM $MnSO_4$, 0,2 µM CuSO4, 0,01 µM $(NH_4)_6Mo_7O_24.4H_2O$, 1 µM $ZnSO_4$). Plants were grown under controlled conditions (light/dark regimes of 16/8 h light photoperiod, photosynthetic photon flux of 320 µmol $m^{-2}s^{-1}$ at canopy height provided by fluorescent lamps, 22/18 °C light/dark temperature and relative humidity 60-70%) [20]. After one week of hydroponic culture, different Cd concentrations (0-5-25-50 and 100 µM) applied to tomato seedlings as $CdCl_2$. Each application had ten seedlings totally. Nutrient solution renewed every four days and plants were harvested ten days after Cd application.

Analysis

Plants root and shoot parts were separately harvested for the determination of root and shoot dry weight and the concentrations of Cd. At harvest, the roots were washed twice in deionized water and then in 0.5 mM $CaSO_4$ solution to remove Cd. After drying at 70°C and measuring the shoot and root dry weights, 0.2 g dry shoot and root samples were powdered and ashed at 500 °C for five hours. The ash was dissolved in 2

ml 37% HCl and evaporated. Later 2 ml 1/3 HCl put on to samples and diluted until 20 ml volume with distilled water and filtered with Whatman filter paper. Atomic absorption spectrometer (AAS) was used for determining Cd content of samples.

Pigment analysis was done according to [21,22]. 200 mg fresh leaf sample was homogenized with 80% acetone. The homogenate filtered and read at 470, 646 and 663 nm spectrophotometrically. Chlorophyll a, chlorophyll b and carotenoid content were measured by using the following formulas.

Chlorophyll a = 12.21*A663-2.81*A646

Chlorophyll b = 20. 13*A646-5.03*A663

Carotenoid = (1000*A470-3.27* Chlorophyll a-81.4* Chlorophyll b) / 229

Membrane lipid peroxidation was estimated by measuring malondialdehyde (MDA) amount according to modified [23]'s protocol. 500 mg fresh leaf material was extracted with 80% ethyl alcohol and centrifuged for 10 minutes at 3000 g. After this, treatment was conducted as two stage. For first stage 2 ml (-) TBA solution which contains 20% TCA and 0,01% BHT in it; samples incubated in 95°C water bath 25 minutes; after ice bath centrifuged again 15 minutes at 3000 g. Distinctively from the first stage, (+) TBA solution was used instead of (-) TBA solution, which had also 0.65% in it, used in the second stage. First samples read at 532 and 600 nm; and others read at 440, 532 and 600 nm spectrophotometrically. Absorbance values put into formulas below and MDA content calculated.

1. $[(ABS_{532}+TBA)-(ABS_{600}+TBA)-(ABS_{532}-TBA)-(ABS_{600}-TBA)]=A$
2. $[(ABS_{440}+TBA-ABS_{600}+TBA)*0.0571)]=B$
3. nmol MDA / ml =(A-B)/157 000) $*10^6$

Ascorbic acid (AsA) and sulphydryl (SH) compounds were determined by using [24,25]'s methods. One g fresh leaf tissue was homogenized with 10 ml 5% meta-phosphoric acid and centrifugated at 5000 g 15 minutes. For determination of total ascorbic acid, 200 µL supernatant put into a glass tube. 500 µL 150 mm phosphate buffer (pH 7.4) with 5 mm EDTA was added on the sample. Later 100 µL DTT (1,4 dithiothreitol) put and waited 10 minutes at room temperature and 100 µL N-ethyl maleimide was added. Reaction mixture was adjusted by using 400 µL 10% TCA, 400 µL 44% ortophosphoric acid, 400 µL 4% 2,2'bipyridyl in 70% ethanol and 200µL 3% FeCl3. Samples were in 40°C for 40 min and pink color was read at 525 nm spectrophotometrically. L(+) ascorbic acid was helped for standard curve. For SH compound analysis 500 µL homogenate mixed with 2.5 ml of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and 500 µL 6 mmol/L DTNB (5-5-dithiobis-(2-nitrobenzoic acid). Samples incubated at room temperature for 20 minutes. Later samples read at 412 nm spectrophotometrically and glutathione was used for standard curve.

Soluble protein amount determined as described in [27] with some modifications. Reaction reagent had 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid and 10% glycerin. 0.5 g fresh leaf material homogenized with 5 ml K-P buffer of which pH 7.6 and contains 0.1 mM Titriplex in it then homogenate centrifuged at 15000 rpm 20 minutes. 5 ml reaction reagent put in glass tubes and 100 µL supernatant was added on it. Later samples read at 595 nm spectrophotometrically and Bovine serum albumin was used for standard curve.

Enzyme activities were determined according to [27,25] with some differences. 1 g fresh leaf material was homogenized with 10 ml pH 7.6 K-P buffer which had 0,1 mM Titriplex. Plant extract centrifuged at 15000g for 20 min and the whole processes were

conducted at 3-5°C. Superoxide dismutase (SOD) activity was determined in little glass bottles. The reaction medium (totally 5 ml) 50 mmol/L phosphate buffer (pH 7.6), 0.1 mmol/L Na-EDTA, enzyme aliquots (50-150 µL), 50 mmol/L Na2CO3 (pH 10.2), 12 mmol/L L-methionine, 75 mmol/L NBT and 2 mmol/L riboflavin, in order. Reactions were conducted at room temperature and under 400 mmolm2/s light intensity 10 min. One unit of SOD activity was described as the amount of enzyme required to cause 50% blockage of the rate of NBT reduction read at 560 nm spectrophotometrically. Glutathione reductase (GR) activity was determined by following the reduction sin absorbance at 340 nm due to NADPH oxidation. The reaction medium (totally 1 ml) pH 7.6 K-P buffer, 0.5 mM GSSG, 0.12 mm NADPH, and the enzyme aliquot. Corrections were made for NADPH oxidation lack of GSSG.

The experiments were organized in a randomized design. Differences amongst Cd applications were checked using the IBM SPSS 20 software. Statistical variance analysis with four replicates, except for growth parameters and antioxidative enzyme analyses, was operated using ANOVA and equated with least significant differences (LSD) at the 5% level.

RESULTS AND DISCUSSION

Growth Parameters and Cd Concentration

No noticeable evidences of toxicity were detected in plants with all concentrations except for 100 µM Cd. For supporting this, 100 µM Cd-treated plants' shoots appeared shorter and the root system is longer and more dense as seen in Fig. 1. The detrimental effects of Cd on plant growth and development; becomes visible with reduced biomass in Cd-applied tomato plants. Growth inhibition is a reaction to heavy metal injury in plants [28].



Fig. 1. *Effects of several rates of Cd application to tomato plants*

Root length of tomato seedlings didn't have a significant change against Cd applications. However, shoot length decreased by high Cd concentrations (50 and 100 µM Cd) significantly. In addition to these high Cd levels also decreased root fresh weight, while there is no serious effect on root dry biomass. Whole Cd doses had a significant negative effect on shoot fresh and dry biomass, but the lowest Cd dose (5 µM Cd) didn't affect shoot dry weight (as shown in Table 1).

Table 1. *Effect of increasing Cd on growth parameters*

Cd treatment (µM)	Total length (cm)	Total fresh weight (g)	Total dry weight (g)
Control	75.7±3.8	50.2±3.8	3.3±0.2
5	72.3±1.2	42.3±3.9	3.0±0.5
25	70.7±2.5	34.5±1.9	2.3±0.2
50	64.7±3.8	33.7±2.7	2.3±0.2
100	63.3±3.8	23.6±1.8	1.5±0.1
$LSD_{0,05}$	7.2	6.7	0.6

In our study, shoots and roots' weights constantly decreased with increased Cd concentrations in the growth medium, similar results were observed in [29,30]. Cadmium toxicity causes degradation of plant water status [31] and reduced the synthesis of chlorophyll enzymes, as a consequence, inhibited the photosynthesis and the plant's growth [32,33]. Similarly; [34] have been proposed that Cd inhibits the chlorophyll biosynthesis and thus decreases chlorophyll content and consequently declining in shoot length and biomass

The plants subjected to the various treatments of Cd as 0, 5, 25, 50 and 100 µM, showed different accumulation levels. Most of the Cd accumulated in the roots as expected, then in the shoots, respectively (As shown in Table 2).

Table 2. *Effect of increasing Cd on root and shoot Cd concentrations*

Cd treatment (µM)	Cd Concentration (ppm)	
	Shoot	Root
Control	0.04±0.01	2.92±0.38
5	4.55±0.17	68.02±9.37
25	5.42±0.06	153.79±10.22
50	7.02±0.12	301.54±5.20
100	12.15±1.22	632.58±125.25
$LSD_{0.05}$	1.254	128.05

Most studies about Cd showed that accumulate in the root system in a lot of plant species [35, 36]. Cd concentration in the plant exceeds 10 ppm, resulting in a decline in plant growth, developmental disorders on leaves such as chlorosis, color changes in leaf tips, leaf rolling and root damage [37]. Differences in Cd concentrations between shoot and root could be considered as a way to protect photosynthesis metabolism of plants from great damage provoked by excess Cd [38]. This idea seems to be supported by [39], the existing root-shoot barrier for Cd in higher plants and thus prevention to move heavy metals to the top of plants.

Pigment Content

Carotenoid and total chlorophyll content diminished with two doses (50 and 100 µM Cd) significantly. In contrast to, chlorophyll a/b ratio of the tomato seedlings was significantly increased by the treatment of Cd (As shown in Table 3).

Table 3. *Effect of increasing Cd on pigment content*

Cd treatment (µM)	Carotenoid (mg/g FW)	Total Chl (mg/g FW)	Chl a/b
Control	0.79±0.05	3.00±0.11	1.68±0.15
5	0.72±0.06	2.85±0.18	1.75±0.26
25	0.60±0.02	2.52±0.06	2.36±0.05
50	0.58±0.02	2.45±0.07	2.37±0.20
100	0.51±0.04	2.10±0.17	2.76±0.17
$LSD_{0.05}$	0.07	0.22	0.31

In comparison with chlorophyll-a, applied Cd doses caused a relative higher decrease of chlorophyll-b. Similar results in chlorophyll-a and chlorophyll-b, has been explained in other studies also [40, 41]. Higher Cd concentrations provoked the degeneration of Chl b and therefore increased the Chl a/b ratio. Whole Cd doses affected carotenoid content negatively in a significant manner (As shown in Table 3). Chlorophyll and carotenoid amount of tomato seedlings reduced with an increase in Cd concentration. Chlorophyll concentration in Cd-treated leaves was also reduced in *Lycopersicon esculentum* [42] and *Lactuca sativa* [43]. Our results confirm that [44,45]. Heavy metal stress resulting in reduction of synthesis in chlorophyll and degeneration [46, 47, 48] and this is one of the first reactions to metal toxicity [49]. Furthermore, heavy metals cause oxidative destruction of membrane lipids by free radical formation, in this way chlorophyll degradation is increased and synthesis is inhibited.

MDA Content

Whole Cd applications increased MDA content but only the highest Cd dose (100 µM Cd), had a significant effect. Malondialdehyde formation in seedling exposed to Cd was higher than in non-threated seedlings (As shown in Fig. 2).

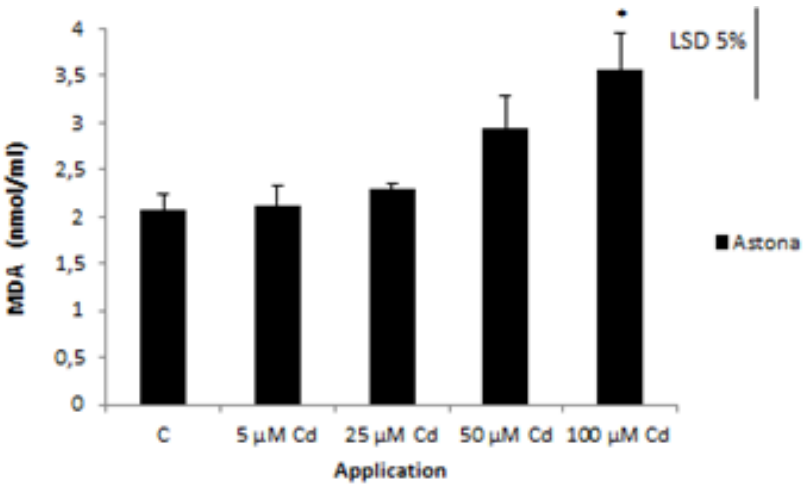


Fig 2. *Effect of Cd application on shoot MDA content*

Lipid peroxidation frequently used as a signal of oxidative stress [49,50] in this research, lipid peroxidase increased gradually in leaves following Cd treatment. Heavy metals are inducted many ways to activate ROS production that cause peroxidation of membrane lipids [51]. Increasing of H_2O_2 and other toxic substances may be involved in lipid peroxidation. These results suggest that studies in which the MDA content in different plants increased at Cd levels [52, 53].

Ascorbic Acid and SH Content

Cadmium concentrations had a rising effect on ascorbic acid content, however only low Cd doses (5 and 25 µM Cd) effects were found significant (As shown in Fig. 3). Increase in ascorbate content with Cd application may indicate that ascorbate is an important component of responding to heavy metal stress.

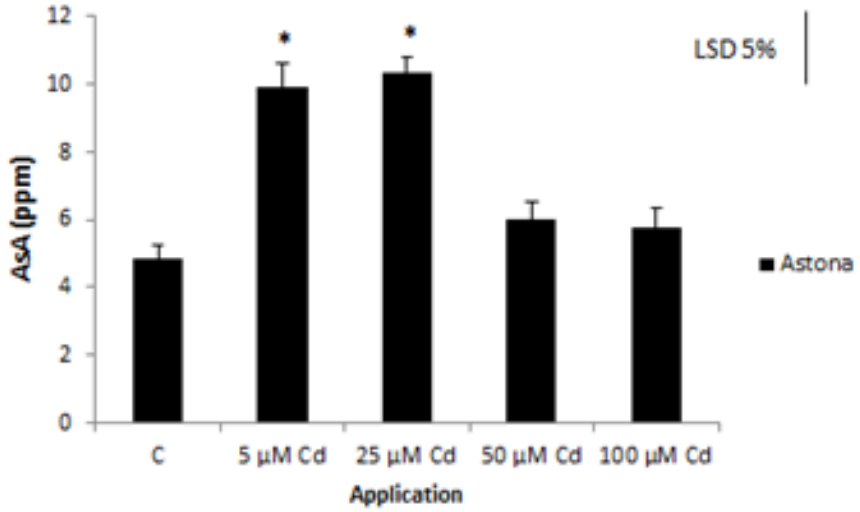


***Fig 3**. Effect of Cd application on shoot AsA content*

Ascorbate is known to have enzymatic (converting H2O2 to H2O with the aid of ascorbate peroxidase enzyme) and non-enzymatic (recreation of α-tocopherol, a membrane-dependent antioxidant, scavenging of peroxyl radical and singlet oxygen) effects on oxidative stress in plant cells [54]. It is known that ascorbate has a considerable assignment in the scavenging of free oxygen radicals, in this way has active antioxidant function in plants [55]. Whole Cd doses increased SH compounds, but it was not found significant at the highest Cd concentration (100 µM Cd) (As shown in Fig. 4).

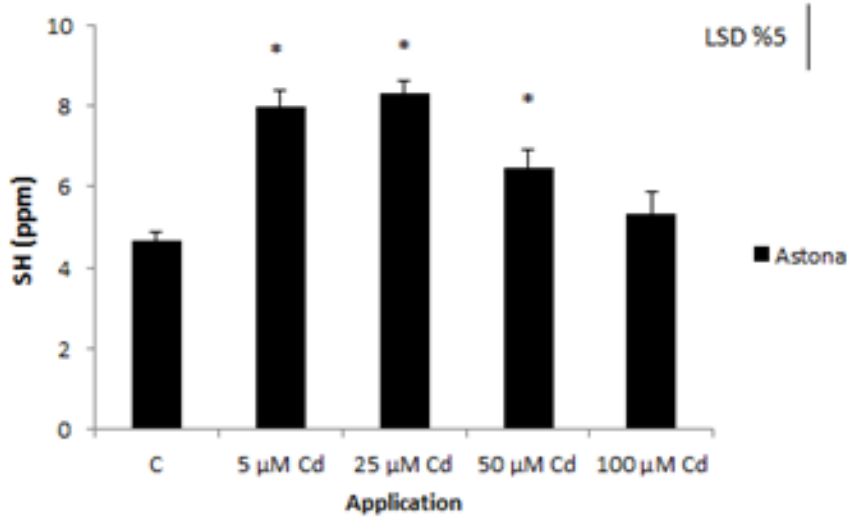


Fig 4. *Effect of Cd application on shoot SH compounds amount*

Plants improve some tolerance mechanisms for detoxification such as the rise in the synthesis of sulfur-containing compounds (glutathione, phytochelatins, metallothionein) under heavy metal effect. These mechanisms have a vital role in plant survival and metal tolerance [56]. Amount of -SH groups increases on stress activated by heavy metals such as Cd has been reported in plants [5]. Increasing of non-protein SH groups may be associated with stimulation of the sulfate reduction pathway and decreasing of non-protein SH groups may originate from consumption of non-protein-SH groups to synthesis of glutathione and phytochelatins [57].

Protein Content

Protein content increased by 25 µM Cd application (As shown in Fig. 5).

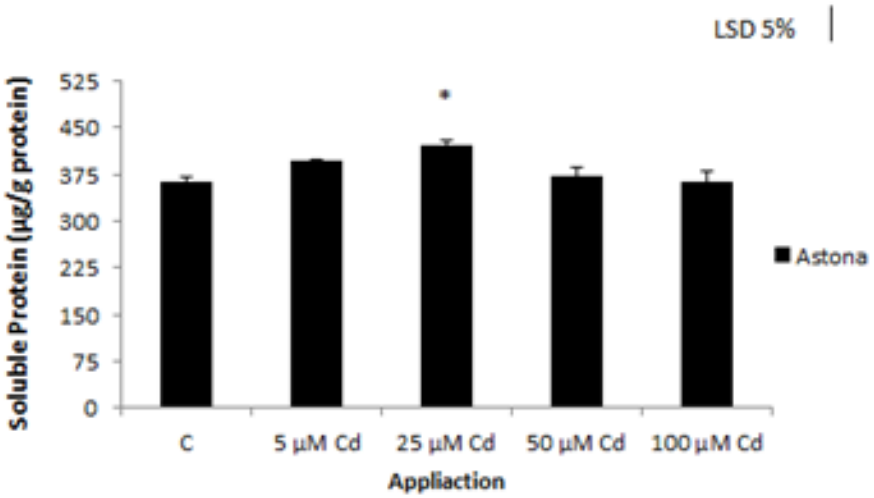


Fig 5. *Effect of Cd application on shoot soluble protein amount*

This was the sole serious effect against Cd on protein amount. Protein amount in Cd-treated seedlings of tomato was slightly increased up to 25 µM Cd; mean that heavy metals may take a vital role in the generation of stress proteins [5]. Also, some scientists reported that increased protein synthesis in some plant species with heavy metal effect [58, 59].

Antioxidative Enzyme Activity

The plants are equipped with enzymatic and non-enzymatic mechanisms to protect from oxidative harm induced by heavy metals [60]. Antioxidant defense system manages the ROS content in plant tissues [61]. Therefore, in the case of tomato plants under the heavy metal toxicity (Cadmium or Lead) the level of antioxidants could increase.

SOD activity increased by Cd applications and highest SOD activity was determined at 25 µM Cd dose (As shown in Fig. 6).

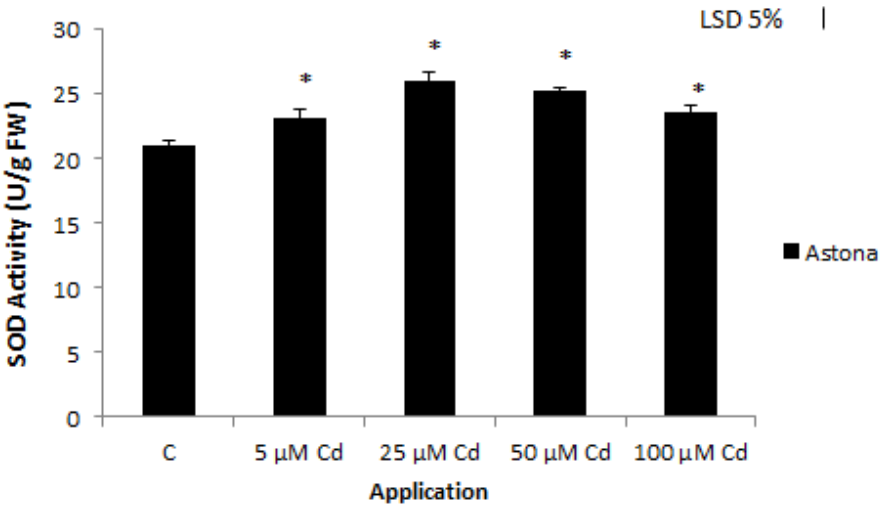


Fig 6. *Effect of Cd application on shoot SOD activity*

Cadmium may increase the activities of some antioxidant enzymes (SOD, APX, GR, POD and CAT) in plants [62, 63, 64]. Our findings show that changes in SOD in tomato leaves under Cd stress appeared to be due to reflecting an increase in the production of toxic ROS. [65] reported that SOD plays an important role in catalyzing of dismutation of superoxide radicals into H2O2 and O2. Increased SOD activity following Cd exposure has also been reported in *Miscanthus sinensis* [66] and *Brassica juncea* [40]. The changes in SOD activities in the leaves of Cd-treated tomato seedlings, especially at the 25 µM Cd, expressed that the scavenging role of SOD was impaired by Cd stress [20]. On the contrary, the slight reduction of SOD activity might be due to the damage of chloroplast or metabolic changes related to oxidative stress including lipid peroxidation.

In the present study, low Cd levels induced a slight increment in GR activity, whereas the decline in the activity starts at 25 µM Cd level. Excluding lowest Cd dose (5 µM Cd), whole applications had rising effect on GR activity significantly. Similarly,

SOD activity, highest GR activity was measured at 25 µM Cd dose. The increase with respect to control value was highest at 5 and 25 µM Cd (As shown in Fig. 7).

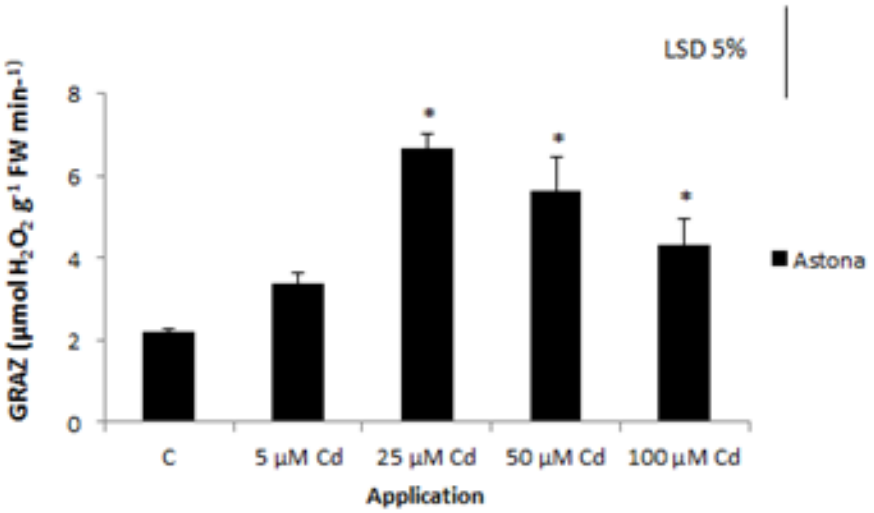


Fig 7. *Effect of Cd application on shoot GR activity*

Our results consistent with that of [67]. Glutathione reductase levels in shoots increased up to 25 µM Cd level and declined at 50 µM Cd and 100 µM Cd following Cd exposure. [35] have been verified that increased GR activity in plants under Cd stress.

CONCLUSION

In this study, the effects of different levels of Cd on tomato plants were investigated. According to our investigations, we can conclude that Cd stimulates oxidative stress, in terms the accumulation of lipid peroxidation products, ascorbic acid, SH-groups and protein amount in tomato seedlings.

Cadmium is a significant pollutant in the ecosystem and is harmful to living things. Also, it seems impossible to avoid from the Cd toxicity of people in the modern societies. For this reason, researches should seek a solution to Cd pollution with present studies, breeding Cd tolerant cultivars and make farmers conscious of Cd.

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