



Molecular docking assisted toxicity assessment of copper stress and protective role of triacontanol

Deniz Tiryaki¹ · Gokce Karadayi² · Yusuf Gulsahin¹ · Busra Tosun² · Hatice Ogutcu³ · Mehmet Karadayi¹ · Medine Gulluce¹

Received: 19 February 2025 / Revised: 4 May 2026 / Accepted: 5 May 2026 / Published online: 18 May 2026
© The Author(s) 2026

Abstract

Triacontanol (TRIA), a natural plant growth regulation has been used on millions of hectares to increase agricultural productivity. In our study, to investigate the effects of TRIA on the resistance to Copper (Cu) heavy metal, bread wheat (*Triticum aestivum* L. Ayyıldız) seeds were planted in petri dishes containing CuSO₄ (50, 100 and 200 µM). After germination, TRIA (10, 20 and 40 µM) was sprayed on the leaves on the 7th day. The plants were harvested on the 10th day. While some of the harvested plant root and leaf samples were first used for length measurements and fresh dry weight measurements, the remaining part was used to determine antioxidant enzyme activities such as catalase (CAT), peroxidase (POX), superoxide dismutase (SOD) and additionally lipid peroxidation (LPO) level and hydrogen peroxide (H₂O₂) amount. TRIA+Cu applications caused changes in enzyme activities depending on the doses. TRIA application, which caused an increase in Cu in malondialdehyde (MDA) content, decreased. A general decrease in H₂O₂ content was observed in leaf tissue. However, in root tissue, H₂O₂ content increased with Cu application, while it decreased with TRIA application. In addition, *in silico* molecular docking analyses were performed to explore potential interactions between Cu²⁺ ions and key physiological and antioxidant-related target proteins. The docking scores for WRKY4, catalase, peroxidase, and superoxide dismutase were 4.991, 4.055, 6.327, and 4.673, respectively, indicating stable Cu²⁺ binding through conserved coordination environments. These findings provide mechanistic support for the experimentally observed alterations in antioxidant enzyme activities. As a result, it was suggested that TRIA played an important role in the antioxidant mechanism in wheat exposed to copper stress.

Keywords Antioxidant enzymes · Copper stress · Molecular docking · Triacontanol · *Triticum aestivum*

Introduction

Exposure of agricultural soils to heavy metals negatively affects the development of plants grown in these soils, leading to heavy metal toxicity. This causes damaging effects

on the growth processes, development and epigenetic characteristics of agricultural plants. Therefore, the prohibition on heavy metals has become an important environmental problem on a global scale (Memon 2020). Plants, which are organisms that cannot move, are directly affected by soils contaminated with heavy metals (Ali and Perveen 2020). Plants are exposed to various abiotic stresses, including heavy metals (Viehweger 2014). Increasing heavy metal pollution in soils as a result of agricultural and industrial activities causes heavy metal stress (Yuce et al. 2024; Li et al. 2023). Heavy metals are important pollutants that cause environmental pollution and many can be toxic even at low concentrations. Copper (Cu) is a micronutrient that is vital for plant growth. Like other micronutrients, copper is an element that only needs to be taken up by plants in small amounts (Tanyolac et al. 2007). Although it is a physiologically important element, increased copper levels can be

Communicated by S. Esposito.

✉ Gokce Karadayi
gokce.karadayi@atauni.edu.tr

¹ Department of Biology, Faculty of Science, Atatürk University, 25240 Erzurum, Turkey

² Department of Molecular Biology and Genetics, Faculty of Science, Atatürk University, 25240 Erzurum, Turkey

³ Department of Biology Faculty of Science, Hacıbayram Veli University, 06100 Ankara Polatlı, Turkey

harmful to plants; because high copper levels create toxic effects in plants and cause disruptions in the enzyme activities of lipid and protein components in cell membranes and DNA structure (Lombardi and Sebastiani 2005; Alaoui-Sossé et al. 2004; Maksymiec 1997). Another important feature of copper stress is that it triggers the accumulation of reactive oxygen species (ROS), leading to oxidative damage in plants (Dai et al. 2016; Demirevska-Kepova et al. 2004). At the molecular level, excessive Cu^{2+} ions may interact directly with proteins and enzymes, particularly those involved in antioxidant defense and stress signaling pathways. Metal ions exert their effects by forming specific coordination environments with conserved amino acid residues, which may alter enzyme structure, catalytic activity, or regulatory function. In recent years, structure-based metal ion-binding site prediction approaches have been increasingly applied to investigate metal–protein interactions in the absence of experimentally resolved metal-bound complexes. Tools such as MIB and its updated version MIB2 predict metal-binding residues and model metal ion placement based on coordination geometry and structural features rather than performing classical ligand re-docking. These approaches are known to converge on a single metal-binding site when a well-defined and conserved coordination environment is present, reflecting the structural robustness of metal-binding pockets (Lin et al. 2016; Lu et al. 2022; Dürr et al. 2023). Triacntanol (TRIA) is a long-chain primary alcohol ($\text{C}_{30}\text{H}_{61}\text{OH}$) and a phytohormone naturally found in plants and was first obtained from the alfalfa plant. It has been reported that triacntanol has healing effects against abiotic stresses such as salinity, drought, and heavy metal toxicity by regulating various metabolic activities in plants (Naeem et al. 2012). It was also determined that TRIA had positive effects on growth parameters such as plant height, fresh and dry weight, leaf area and root nodulation. In this study, antioxidant enzyme activities (CAT, POX, and SOD), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents were evaluated in wheat seedlings treated with TRIA under copper stress. In addition, *in silico* molecular docking analyses were performed to elucidate the potential interactions between Cu^{2+} ions and key physiological and antioxidant-related target proteins, providing mechanistic insight into copper-induced alterations in enzyme activity.

Materials and methods

Experimental design

Bread wheat (*Triticum aestivum* L. Ayyıldız) seeds were first sterilized by soaking them in sodium hypochlorite for 5 min, 20 in each petri dish for heavy metal concentration

and control group. Afterwards, the seeds were washed with pure water for 15–20 min and placed in a beaker, autoclaved water was added and left to swell for 7 h in a dark environment at 24–25 °C. At the end of this period, the seeds were removed from the swelling medium and sown as 20 seeds each in 11 cm diameter petri dishes prepared with double-layered filter paper and wetted with 20 ml of autoclaved water (control group) and 50, 100 and 200 μM Cu (20 ml). The sown petri dishes were left to germinate in a dark climate cabinet at 24–25 °C. One week later, triacntanol (10, 20 and 40 μM) was sprayed on the leaves. Some of the plant root and leaf samples harvested on the 10th day were used fresh for length measurements and wet-dry weight measurements (Fig. 1), while the rest were frozen in liquid nitrogen and stored at -80 °C for experiments.

The Allium assay

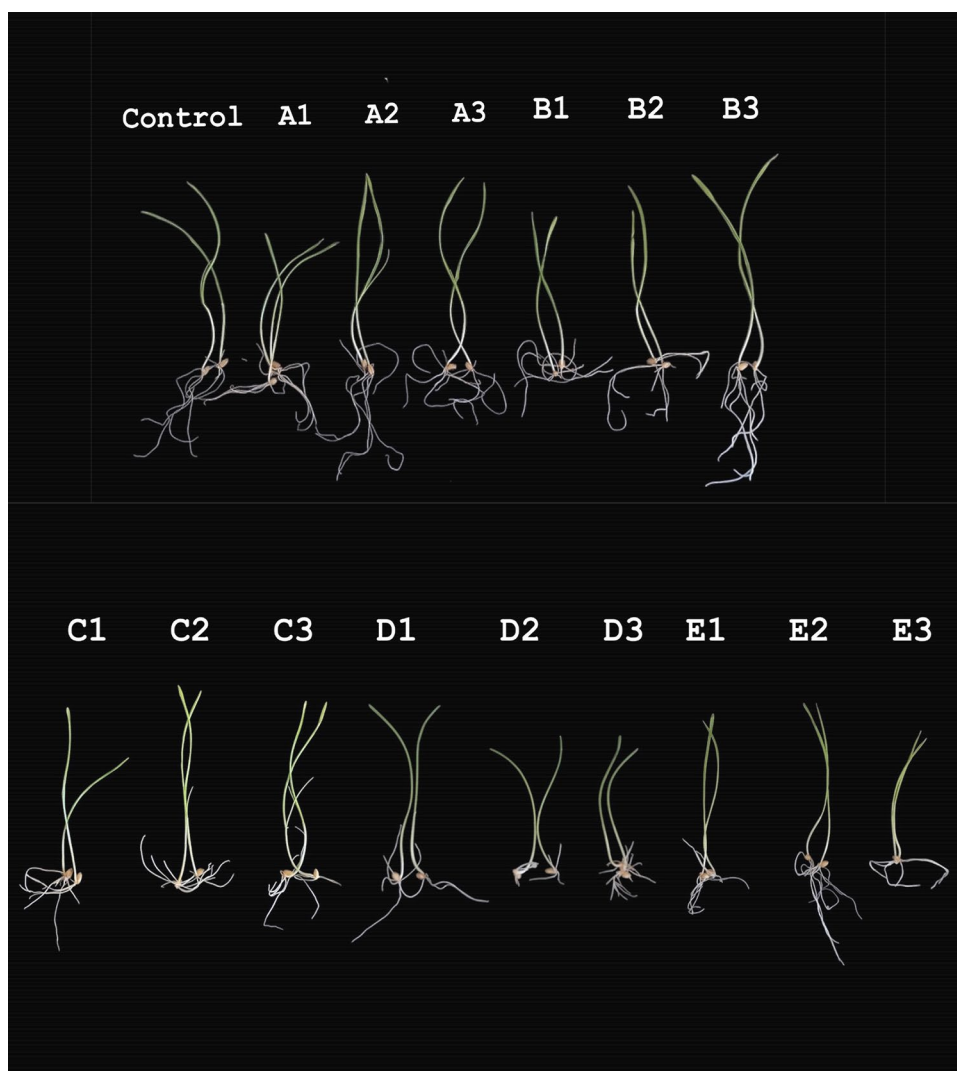
The Allium test was used to investigate the cytogenotoxic effect of varying concentrations of Cu (50, 100 and 200 μM), triacntanol (10, 20 and 40 μM) and the treatment groups. For this goal, *Allium cepa* L. bulbs were purchased from local markets in Erzurum. Uniform-sized healthy bulbs were selected for the cytogenotoxicity assays. Then, the yellow-brown outer scales of the bulbs were removed and the clean bulbs were immersed into distilled water (control group) and distilled water supplemented with the extract solutions (0.2–1 mg/ml). After 48 h incubation at 25 °C in the dark, the root tips were collected, fixed with 3 ml of a fixation/hydrolysis solution consisting of acetic acid (45%) and 1 N HCl (9:1) for 6 min at 52 °C. Then, the tips were stained with aceto-orcein for 1 min, gently squashed between slides, and examined by light microscopy (400 $\times$). For the calculation of mitotic index (MI) and chromosomal aberrations, 5,000 mitotic root cells were scored for each test group and the results were expressed as percentage (%) values, and statistically analysed Fisher's exact test and Linear trend ($p < 0.05$) (Ayдын et al. 2024).

$$\text{MI} = (\text{Number of dividing cells} / \text{Number of total cells}) \times 100.$$

Anatomical investigations

The Allium root tips grown in the control and test solutions were also used for determination of anatomical alterations in the plant roots. For this aim, the tips were collected and washed with distilled water. Then, cross-sections were taken with a sharp razor blade and stained with methylene blue (5%) for 2 min. Finally, cellular and structural damages were observed under the light microscope (LEICA DM 750) at 500 $\times$ magnification (Cavusoglu et al. 2021).

Fig. 1 Root and leaf lengths in *Triticum aestivum* seedlings exposed to CuSO_4 and TRIA



Determination of antioxidant enzyme activities

The activity of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and were determined spectrophotometrically from samples obtained from wheat leaves and roots. For antioxidant enzyme measurement, leaf and root tissues (0.5 g) were homogenized in 50 mM phosphate buffer at pH=7 in the presence of 1% polyvinylpyrrolidone. The reaction mixture for monitoring SOD activity by measuring its ability to reduce the photochemical reduction of nitroblue tetrazolium (NBT) should contain 50 mM KH_2PO_4 (pH: 7.8), 13 mM methionine, 63 μM NBT, 0.1 mM EDTA, 30 μl of enzyme extract and 13 μM riboflavin. One unit of SOD activity is defined as the amount of enzyme that inhibits the photoreduction of nitroblue tetrazolium chloride by 50% (Agarwal and Pandey 2004). CAT activity is measured by decreasing the absorbance value at 240 nm of the reaction mixture (3 ml) formed by adding 103 mM KH_2PO_4 buffer and 40

mM H_2O_2 substrate solution and 25 μl enzyme solution. One unit of CAT activity is defined as the amount of enzyme that uses 1 mmol H_2O_2 in one minute (Gong et al. 2001). To determine POX activity, guaiacol was used as a hydrogen donor and the rate of dissociation of H_2O_2 by the peroxidase enzyme at 470 nm was measured. One unit of POX activity is defined as the amount of enzyme that causes an increase in absorbance of 0.01 units per minute (Angelini and Federico 1989).

Determination of LPO

LPO change was obtained by measuring MDA content according to Heath and Packer (1968). For this, wheat leaves (0.5 g) and roots (0.5 g) were homogenized in 5 ml of cold 5% TCA and the resulting homogenates were centrifuged at $12,000\times g$ for 15 min at $+4^\circ\text{C}$. One ml of 0.5% thiobarbituric acid (TBA) (20%) prepared in TCA was mixed with 4 ml of supernatant in a clear tube and the resulting mixture

was incubated in boiling water for 30 min. The reaction was stopped by removing the incubated tubes from the ice bath. The tubes were then centrifuged again at $10,000\times g$ for 10 min. The absorbance of the obtained supernatant was measured at 532 nm and 600 nm. It was calculated with the formula $[(A_{532}-A_{600})/155000] \times 10^6$.

Determination of H_2O_2

Content Fresh leaf and root tissues (0.5 g) were homogenized in 10 ml cold acetone and then the homogenate was centrifuged at $+4^\circ C$ at $10,000\times g$ for 10 min. 0.15 ml of 5% $Ti(SO_4)_2$ and 0.3 ml of NH_4OH were added to the 1.5 ml of supernatant obtained after centrifugation. The mixture was centrifuged at $10,000\times g$ for 10 min and the supernatant was removed. Then the obtained pellet was dissolved in 3 ml of 2 M H_2SO_4 and the absorbance value was measured at 415 nm. These absorbance values were converted to the amount of H_2O_2 in nanograms by means of a standard graph. The obtained data were presented as the amount of H_2O_2 per g tissue (ng/g leaf) (He et al. 2005).

Statistical analysis

The analysis of each sample was carried out in three independent experiments performed in duplicate. Enzyme activity values and application groups were tested by using Kolmogorov-Smirnov analysis, and the data's skewness and kurtosis values were assessed. One-way analysis of variance (ANOVA) was applied to the numerical data of the research, which were suitable for normal distribution and the dependent variables were more than two (Mao et al. 2024). Levene's test statistic was used to check the homogeneity of variances, and multiple comparison tests were used to meet the homogeneity of variances condition. Bonferroni tests were employed to compare groups of more than three groups, and Duncan's tests were utilized to compare groups with the same sample size (Aybeke 2024). Research findings were analysed using IBM SPSS Statistics 23 software.

Cu^{2+} binding site prediction and molecular docking

The Metal Ion-Binding Site Prediction and Modeling Server (MIB2), freely accessible at <http://combio.life.nctu.edu.tw/MIB2/>, was used to predict Cu^{2+} binding regions and interacting residues of the associated target proteins. A WRKY transcription factor of *Arabidopsis thaliana*, a model plant for understanding molecular mechanism of action of phytotoxicity, was chosen as the target receptor for physiological parameters, and catalase, peroxidase and superoxide dismutase for antioxidant-oxidant dynamics. The predicted 3D structures of WRKY4 (Q9XI90), catalase (Q96528),

peroxidase (P0DI10) and superoxide dismutase (P24704) were obtained from UniProt, a universal resource of protein sequence and functional information accessible at <https://www.uniprot.org/>. The predictions on interactions between query receptors and Cu^{2+} ions were done by using the fragment transformation method via MIB2 server (Lu et al. 2006, 2022). The docking position and scores of Cu^{2+} ions were also calculated by the same server after the model prediction. Metal ion-specific docking parameters were not manually defined by the user. Instead, all Cu^{2+} -related parameters, including charge state, preferred coordination geometry, metal-ligand interaction distances, and interactions with conserved coordinating residues, were automatically incorporated by the MIB2 server during metal-binding site prediction and modeling. This automated approach minimizes user bias and is particularly suitable for metal ion docking studies. Active sites were not predefined prior to docking. Instead, blind docking was performed to allow unbiased exploration of potential Cu^{2+} binding regions across the entire protein surface. This approach is particularly suitable for metal ions, which may interact with multiple functional or regulatory regions rather than a single catalytic active site. The MIB2 server predicts metal-binding sites based on coordination geometry, conserved residues, and structural features, enabling identification of the most favorable Cu^{2+} binding environments without prior site restriction. For each target protein, the MIB2 server generated multiple Cu^{2+} -binding models, and the top ten ranked docking models were selected for structural analysis, interaction characterization, and RMSD-based validation. Docking validation was performed by calculating RMSD values among the top ten Cu^{2+} -binding models generated by the MIB2 server. RMSD calculations were carried out using PyMOL based on the spatial positions of Cu^{2+} ions. The prediction scores were summarized in tables and docking interactions were visualized by using Schrödinger Maestro version 13.9.138.

Results

The Allium test results revealed that high concentrations of Cu are highly cytotoxic and genotoxic on the mitotic cells of the root tips. Root elongation was completely inhibited at 100 and 200 μM concentrations. At 50 μM concentration, the mitotic index decreased significantly and the chromosome aberration rate increased compared to the control group (Table 1). Similar chromosomal aberrations including anaphase bridges (AB), chromosome breaks, chromosome laggards (CLag), chromosome loses (CLos), C-metaphases (CM), and stickiness (S) formed in both 50 μM Cu induced test group and the EMS induced positive control (Fig. 2). On the other hand, increasing TRIA concentrations (10, 20 and

Table 1 Mitotic index and chromosome aberrations in *A. cepa* roots exposed to CuSO₄ and TRIA

Test Groups (μ M)	Mitotic Index (%)	Chromosome Aberrations (%)							Statistical Testing	
		AB	CB	CLag	CLos	CM	St	T	Fisher's exact test	Linear trend
CuSO ₄	13.0	1.1	0.1	0.7	0.3	0.1	0.3	2.5	S	S
50	11.2	1.6	0.5	1.1	0.8	0.3	0.1	4.4	S	
100	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
200*										
Triacantanol	19.1	0.1	0.0	0.1	0.1	0.0	0.0	0.3	NS	NS
10	16.3	0.2	0.0	0.1	0.2	0.0	0.1	0.4	NS	
20	13.4	0.2	0.0	0.2	0.1	0.0	0.1	0.3	NS	
40										
CuSO ₄ - Triacantanol	15.9	0.9	0.3	0.4	0.2	0.1	0.5	2.4	S	S
200–10	18.2	0.2	0.2	0.3	0.1	0.0	0.1	0.9	NS	
200–20	19.4	0.1	0.1	0.1	0.0	0.0	0.2	0.5	NS	
200–40										
Control Groups	19.1	0.1	0.1	0.1	0.0	0.0	0.1	0.4		
Negative control: ddH ₂ O	11.7	2.2	0.2	0.9	0.6	0.4	1.4	5.7		
Positive control: EMS (25 mM)										

AB: anaphase bridge, CB: chromosome break, CLag: chromosome laggard, CLos: chromosome lose, CM: C-metaphase, St: stickiness, T: total rate of chromosome aberrations, N/A: not applicable, S: significant and NS: not statistically significant compared to the negative control ($p < 0.05$)

* 200 μ M concentration of CuSO₄ completely inhibited the seedling growth

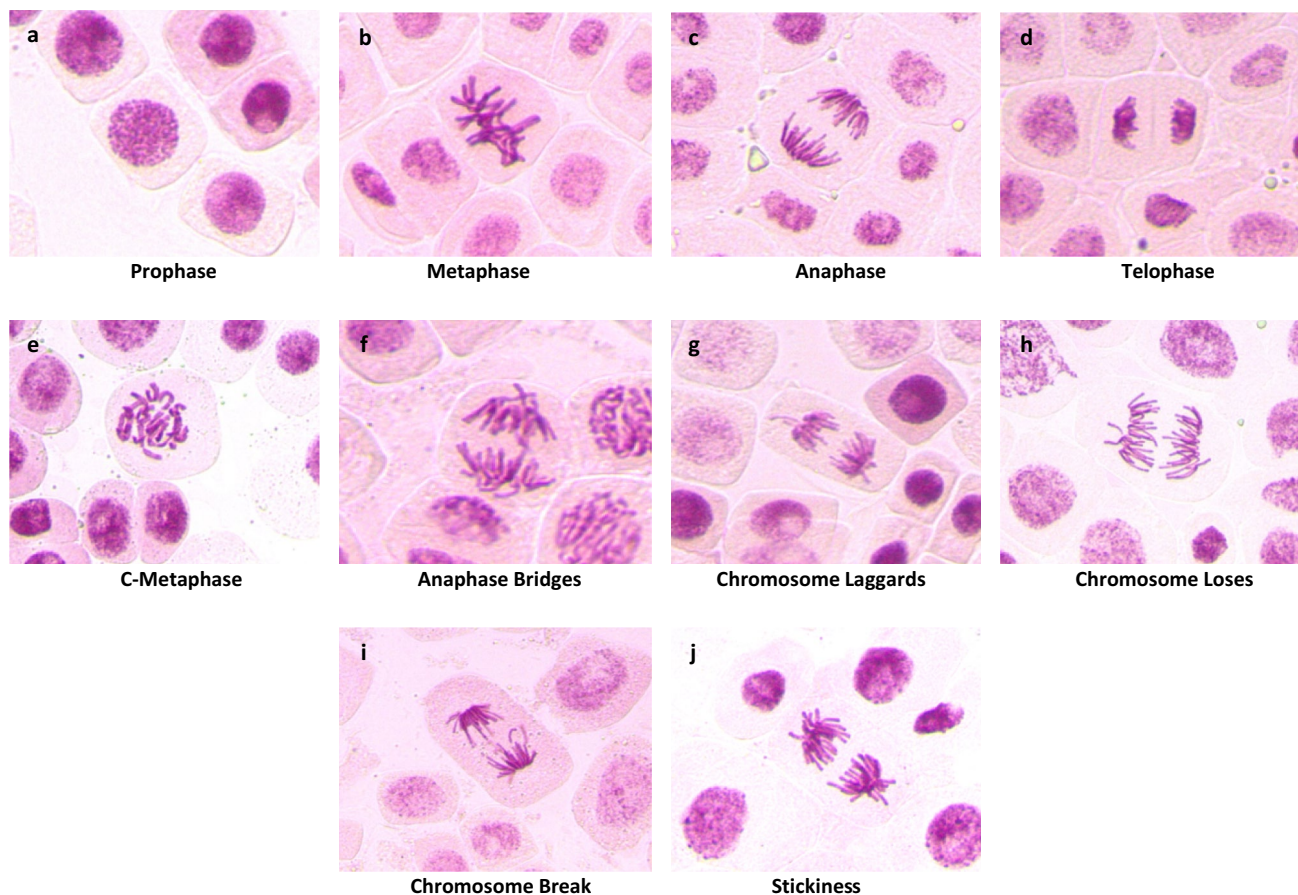


Fig. 2 Typical stages of mitosis (a–d) and chromosomal aberrations (e–j) induced by EMS in mitotic *A. cepa* root cells. The images are representative of observations obtained from three independent experi-

ments, each performed in duplicate ($n=6$), with 5000 cells analyzed per treatment group

Table 2 Alleviating role of TRIA against meristematic cell injuries induced by Cu²⁺

Groups	ECD	CCD	TCCW	FCN	UVT
Control	–	–	–	–	–
TRIA 10 µM	–	–	–	–	–
TRIA 20 µM	–	–	–	–	–
TRIA 40 µM	–	–	–	–	–
CuSO ₄ 50 µM	+	+	+	+	–
CuSO ₄ 100 µM	++	++	++	++	+
CuSO ₄ 200 µM	+++	+++	+++	+++	++
CuSO ₄ 200 µM+TRIA 10 µM	+	+	+	+	+
CuSO ₄ 200 µM+TRIA 20 µM	+	+	+	+	–
CuSO ₄ 200 µM+TRIA 40 µM	–	–	–	–	–

ECD: epidermis cell damage, CCD: cortex cell damage, TCCW: thickening of the cortex call wall, FCN: flattened cell nucleus, UVT: unclear vascular tissue, (+++) severe damage, (++) moderate damage, (+) slight damage, and (–) no damage

40 µM) caused significant reduction in mitotic index of root tips in a dose dependent manner, but did not increase the chromosomal aberration rate for any test group. Moreover, increasing TRIA treatments together with Cu significantly reduced the toxicity, resulting in a strong recovery in the mitotic index of meristematic cells, and the chromosomal aberration rate decreased in all groups.

The anatomical investigations on the *Allium* root tips showed that increasing concentrations of Cu significantly induces cell and tissue alterations including cellular damages in epidermis, cortex and transmission tissues, thickening of the cell walls, nuclear vacuolization and formation of micronuclei. However, increasing concentrations of TRIA did not cause any anatomical alteration. Also, triacotanol cotreatments (10 µM TRIA+200 µM Cu, 20 µM TRIA+200 µM Cu and 40 µM TRIA+200 µM Cu) significantly reduced the rate of Cu induced anatomical alterations in a dose dependent manner (Table 2; Fig. 3).

Antioxidant enzymes decrease under abiotic stress conditions. In our study, SOD, CAT and POX antioxidant enzyme activities were studied in leaf and root tissues (Tables 3 and 4). When we look at the SOD enzyme activity in leaves, it was found that only Cu and TRIA applications decreased compared to the control. However, it was observed that SOD enzyme activity generally increased in the concentrations where these molecules were applied together. 200 µM Cu+10 µM TRIA concentration increased the activity by approximately 83% compared to the 200 µM Cu dose. In general, it was determined that 10 µM TRIA dose increased activity significantly compared to TRIA concentrations. In root tissues, 20 µM TRIA and 40 µM TRIA doses together with Cu concentrations increased SOD activity more than other applications. In addition, the highest SOD activity was determined at 20 µM TRIA+100 µM Cu dose, while the lowest was determined at 40 µM TRIA dose. CAT enzyme activity in leaf tissue increased only with TRIA applications compared to Cu applications. Moreover, 200 µM Cu application increased with 10 µM TRIA, 20 µM TRIA and 40

µM TRIA doses activity, 31%, 37% and 56%, respectively. When CAT enzyme activity was examined in root tissue, a significant increase was observed in TRIA single applications compared to control, while a significant decrease in activity was observed in Cu single application doses. When POX enzyme activity was examined, only Cu application in leaf tissue decreased activity, while only TRIA application increased activity compared to control, especially 40 µM TRIA, it was observed that there was a 44% increase in activity. When only Cu application was compared with Cu and 10 µM TRIA and 20 µM TRIA applications, it was determined that TRIA increased activity. When we looked at POX activity in root tissue, it was seen that there was a significant increase especially in 40 µM TRIA compared to control, and a significant decrease was observed in 200 µM Cu application. When Cu applications applied together with TRIA were examined, it was seen that POX activity increased significantly. In fact, it was determined that only Cu applications caused a significant increase in activity compared to TRIA+Cu applications. It was observed that the MDA content in the leaf decreased when only TRIA applications were compared with the control. However, the MDA content increased in only Cu applications. The doses applied together with Cu and TRIA decreased the MDA content compared to only Cu applications. When 100 µM Cu dose was applied with the highest dose of TRIA, the MDA content decreased by 56%. Again, it was determined that 50 µM Cu application decreased by 67% compared to 10 µM TRIA application. When the root tissue was examined, it was determined that the MDA content increased 3 times when 200 µM Cu dose was compared with the control. However, it was observed that the same dose decreased the MDA content almost at the same rate as the control when 10 µM TRIA was applied. However, when only 50 µM Cu dose was applied together with 10 µM TRIA, it was observed that TRIA decreased the MDA content 4 times compared to TRIA. When the amount of H₂O₂ in the leaf was examined, it was seen that all doses of TRIA decreased

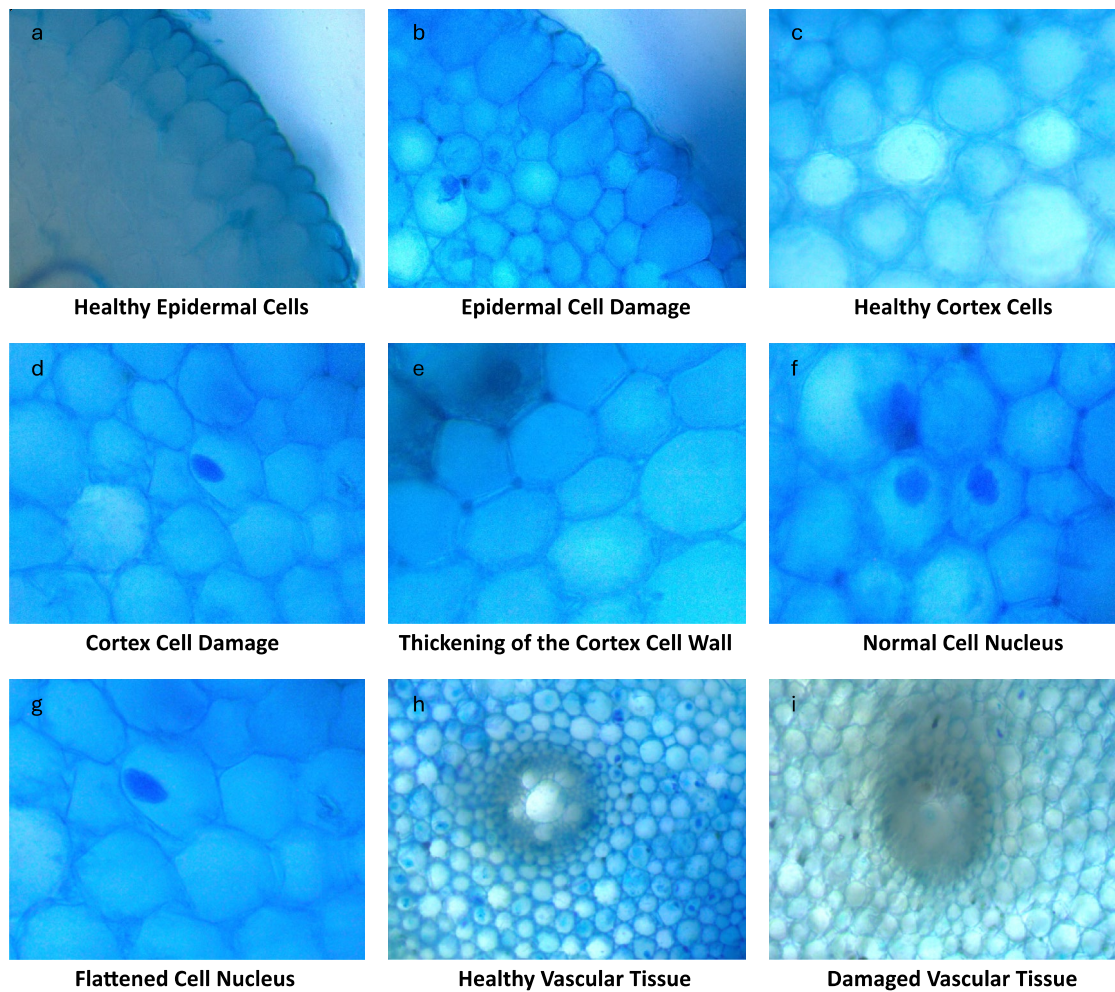


Fig. 3 Typical appearance of plant tissues (a, c, f, h) and anatomical alterations (b, d, e, g, i) in *A. cepa* roots. The images are representative of observations from three independent experiments conducted in

duplicate ($n=6$). In total, approximately 5000 cells were evaluated per treatment group

Table 3 Antioxidant enzyme activities and oxidative stress markers in *Triticum aestivum* roots under CuSO_4 and TRIA treatments. Data are presented as mean $\pm$ SE ($n=6$; three independent experiments performed in duplicate). Statistical analysis was performed using one-way ANOVA followed by Duncan's post hoc test. Different letters (a–k) indicate statistically significant differences between groups ($p<0.05$)

Treatment Groups	CAT (EU/mg) $\pm$ SE	POX (EU/mg) $\pm$ SE	SOD (EU/g) $\pm$ SE	MDA (nmol/g) $\pm$ SE	H_2O_2 ($\mu\text{g/g}$) $\pm$ SE
Control	20,1306 $\pm$ 0,467 ^{de}	6,2988 $\pm$ 0,338 ^b	17,5046 $\pm$ 1,429 ^{ab}	0,3611 $\pm$ 0,108 ^{abcd}	105,52 $\pm$ 0,501 ⁱ
TRIA 10 μM + CuSO_4 50 μM	14,1689 $\pm$ 0,92 ^{ab}	11,5777 $\pm$ 0,431 ^d	25,5712 $\pm$ 0,853 ^{fg}	0,2738 $\pm$ 0,031 ^a	88,5909 $\pm$ 0,449 ^d
TRIA 20 μM + CuSO_4 50 μM	12,4496 $\pm$ 0,153 ^a	18,3177 $\pm$ 0,088 ^h	25,2125 $\pm$ 0,451 ^{fg}	0,5794 $\pm$ 0,030 ^{gh}	88,9561 $\pm$ 0,075 ^d
TRIA 40 μM + CuSO_4 50 μM	12,3023 $\pm$ 0,153 ^a	9,55 $\pm$ 0,95 ^c	25,4644 $\pm$ 0,677 ^{fg}	0,3283 $\pm$ 0,072 ^{abc}	90,0247 $\pm$ 0,963 ^c
TRIA 10 μM + CuSO_4 100 μM	12,6572 $\pm$ 0,082 ^a	13,9358 $\pm$ 0,714 ^f	22,7776 $\pm$ 0,443 ^c	0,4333 $\pm$ 0,030 ^{de}	94,5819 $\pm$ 0,626 ^g
TRIA 20 μM + CuSO_4 100 μM	12,7515 $\pm$ 0,152 ^a	13,86 $\pm$ 0,387 ^f	31,9334 $\pm$ 2,697 ^h	0,3234 $\pm$ 0,041 ^{abc}	92,4219 $\pm$ 0,452 ^f
TRIA 40 μM + CuSO_4 100 μM	11,7222 $\pm$ 0,237 ^a	18,0474 $\pm$ 0,752 ^h	27,0085 $\pm$ 0,314 ^g	0,4084 $\pm$ 0,067 ^{cde}	98,7866 $\pm$ 0,704 ⁱ
TRIA 10 μM + CuSO_4 200 μM	12,5042 $\pm$ 0,082 ^a	12,2192 $\pm$ 0,963 ^{de}	18,9184 $\pm$ 0,777 ^{bc}	0,4509 $\pm$ 0,051 ^{ef}	76,2114 $\pm$ 0,273 ^a
TRIA 20 μM + CuSO_4 200 μM	18,4007 $\pm$ 1,319 ^{cd}	13,1966 $\pm$ 0,601 ^{ef}	22,716 $\pm$ 0,981 ^c	0,8264 $\pm$ 0,020 ⁱ	94,5428 $\pm$ 0,505 ^g
TRIA 40 μM + CuSO_4 200 μM	17,6092 $\pm$ 0,691 ^{cd}	11,3615 $\pm$ 0,1 ^d	23,3143 $\pm$ 0,59 ^e	0,5234 $\pm$ 0,035 ^{fg}	86,2 $\pm$ 0,200 ^c
CuSO_4 50 μM	16,6462 $\pm$ 0,212 ^{bc}	6,6822 $\pm$ 0,506 ^b	23,9612 $\pm$ 0,553 ^{ef}	0,8827 $\pm$ 0,069 ^{ij}	93,0038 $\pm$ 1,005 ^f
CuSO_4 100 μM	17,5925 $\pm$ 0,414 ^{cd}	8,7222 $\pm$ 0,038 ^c	24,3001 $\pm$ 1,04 ^{ef}	0,6364 $\pm$ 0,038 ^h	120,5733 $\pm$ 0,515 ⁱ
CuSO_4 200 μM	21,9567 $\pm$ 4,32 ^e	4,2403 $\pm$ 0,125 ^a	20,9264 $\pm$ 0,202 ^d	0,9306 $\pm$ 0,010 ^j	111,8171 $\pm$ 0,742 ^k
TRIA 10 μM	24,6214 $\pm$ 0,462 ^f	6,2733 $\pm$ 0,598 ^b	19,5938 $\pm$ 1,138 ^{cd}	0,3494 $\pm$ 0,040 ^{abcde}	87,0742 $\pm$ 1,008 ^c
TRIA 20 μM	24,9884 $\pm$ 1,4 ^f	5,9722 $\pm$ 0,847 ^b	16,7189 $\pm$ 0,491 ^a	0,3132 $\pm$ 0,035 ^{ab}	96,52 $\pm$ 0,501 ^d
TRIA 40 μM	24,8736 $\pm$ 3,908 ^f	15,1066 $\pm$ 1,516 ^g	16,9821 $\pm$ 0,31 ^a	0,3866 $\pm$ 0,020 ^{bcde}	84,6533 $\pm$ 0,566 ^b

Table 4 Antioxidant enzyme activities and oxidative stress markers in *Triticum aestivum* leaves under CuSO₄ and TRIA treatments. Data are presented as mean ± SE (*n* = 6; three independent experiments performed in duplicate). Statistical analysis was performed using one-way ANOVA followed by Duncan's post hoc test. Different letters (a–k) indicate statistically significant differences between groups (*p* < 0.05)

Treatment Groups	CAT (EU/mg) ± SE	POX (EU/mg) ± SE	SOD (EU/g) ± SE	MDA (nmol/g) ± SE	H ₂ O ₂ (μg/g) ± SE
Control	21,611 ± 1,195 ^{bcd}	5,842 ± 0,101 ^d	33,547 ± 2,176 ^{cd}	9,433 ± 0,179 ^e	112,327 ± 1,149 ^h
TRIA 10 μM + CuSO ₄ 50 μM	22,848 ± 0,326 ^{de}	8,416 ± 0,036 ^g	41,935 ± 3,584 ^e	5,18 ± 0,132 ^b	98,316 ± 1,14 ^{de}
TRIA 20 μM + CuSO ₄ 50 μM	19,378 ± 0,995 ^b	2,639 ± 0,087 ^a	39,808 ± 1,806 ^e	11,608 ± 0,171 ^g	96,836 ± 0,767 ^{cd}
TRIA 40 μM + CuSO ₄ 50 μM	22,901 ± 0,24 ^{de}	4,393 ± 0,005 ^b	32,360 ± 2,398 ^{cd}	12,264 ± 0,079 ^h	96,767 ± 1,569 ^{cd}
TRIA 10 μM + CuSO ₄ 100 μM	20,023 ± 0,536 ^{bc}	5,313 ± 0,408 ^{cd}	33,744 ± 0,476 ^d	10,266 ± 0,275 ^f	96,904 ± 0,861 ^{cd}
TRIA 20 μM + CuSO ₄ 100 μM	23,959 ± 0,839 ^{df}	7,651 ± 0,747 ^f	27,939 ± 2,241 ^{ab}	11,333 ± 0,375 ^g	96,365 ± 0,633 ^c
TRIA 40 μM + CuSO ₄ 100 μM	24,642 ± 0,246 ^{efg}	4,004 ± 0,487 ^b	26,928 ± 0,306 ^a	7,56 ± 0,268 ^d	97,169 ± 1,042 ^{cd}
TRIA 10 μM + CuSO ₄ 200 μM	21,625 ± 0,929 ^{bcd}	6,888 ± 0,255 ^e	55,849 ± 1,605 ^f	11,226 ± 0,126 ^g	92,592 ± 0,524 ^a
TRIA 20 μM + CuSO ₄ 200 μM	22,407 ± 0,872 ^{cde}	5,536 ± 0,17 ^{cd}	33,167 ± 1,015 ^{cd}	11,333 ± 0,441 ^g	94,611 ± 0,535 ^b
TRIA 40 μM + CuSO ₄ 200 μM	25,888 ± 1,839 ^{fg}	5,057 ± 0,173 ^c	33,825 ± 0,563 ^d	9,453 ± 0,28 ^e	96,767 ± 0,681 ^{cd}
CuSO ₄ 50 μM	19,977 ± 1,39 ^{bc}	5,236 ± 0,55 ^{cd}	33,260 ± 2,186 ^{cd}	15,406 ± 0,405 ⁱ	97,348 ± 1,168 ^{cd}
CuSO ₄ 100 μM	26,479 ± 1,124 ^f	5,589 ± 0,331 ^{cd}	31,801 ± 0,566 ^{cd}	16,436 ± 0,424 ^k	101,72 ± 0,628 ^f
CuSO ₄ 200 μM	16,553 ± 0,688 ^a	5,769 ± 0,562 ^d	30,628 ± 1,038 ^{bc}	13,133 ± 0,152 ⁱ	104,304 ± 1,13 ^g
TRIA 10 μM	35,781 ± 1,376 ^h	6,781 ± 0,302 ^e	26,007 ± 0,115 ^a	6,563 ± 0,056 ^c	93,027 ± 0,063 ^a
TRIA 20 μM	36,795 ± 0,352 ^h	5,656 ± 0,153 ^{cd}	26,776 ± 0,31 ^a	6,383 ± 0,406 ^c	98,384 ± 0,666 ^{de}
TRIA 40 μM	30,373 ± 3,691 ^g	9,017 ± 0,524 ^g	27,645 ± 0,243 ^a	4,356 ± 0,166 ^a	99,051 ± 0,089 ^e

Table 5 Physico-chemical interactions of Cu²⁺ ions docked with physiological and antioxidant-oxidant property associated target receptors

Protein	Docking Score	Residue (ID)	Atom (donor)	Distance (Å)	Interaction type
WRKY4 (Q9X190)	4.991	Arg429 (R429)	NE	1.56	Cu–N coordination
		Arg429 (R429)	NH1	2.02	Cu–N coordination
		Tyr431 (Y431)	OH	3.11	Weak polar contact / borderline H-bond
Catalase (Q96528)	4.055	His444 (H444)	ND1	3.63	No direct coordination (second-shell)
		Asp254 (D254)	OD1	1.67	Cu–O coordination
		Asp254 (D254)	OD2	2.21	Cu–O coordination
		His201 (H201)	ND1	3.86	No direct coordination (second-shell)
		Ser258 (S258)	OG	4.28	No direct coordination (second-shell)
Peroxidase (P0DI10)	6.327	Arg62 (R62)	NH2	1.25	Strong Cu–N coordination
		Arg62 (R62)	NH1	1.45	Strong Cu–N coordination
		Arg62 (R62)	NE	2.20	Cu–N coordination
		His66 (H66)	NE2	2.84	Weak/borderline Cu–N coordination
		His119 (H119)	NE2	1.53	Strong Cu–N coordination
Superoxide dismutase (P24704)	4.673	His45 (H45)	ND1	2.12	Cu–N coordination
		His62 (H62)	NE2	2.82	Weak/borderline Cu–N coordination
		His47 (H47)	NE2	3.28	No direct coordination (second-shell)

the amount compared to the control. It was seen that only Cu applications caused a significant increase in the amount of H₂O₂ compared to Cu+TRIA applications. In the root tissue, it was determined that the amount of H₂O₂ increased with 100 μM Cu and 200 μM Cu doses and decreased with all other applications. Moreover, it was seen that the 200 μM Cu + 10 μM TRIA dose decreased the amount of H₂O₂ by 32% compared to the 200 μM Cu dose.

Ion-binding site prediction results revealed that 36 residues of WRKY4 transcription factor, 64 residues of catalase, 24 residues of peroxidase, and 21 residues of superoxide dismutase are higher than the threshold, predicted as Cu²⁺ binding residues. Then, the most probable docked position of the Cu²⁺ ion for each target protein was determined. The docking scores for WRKY4, catalase, peroxidase and

superoxide dismutase were 4.991, 4.055, 6.327 and 4.673, respectively (Table 5; Fig. 4).

Distance-based analysis of the docked complexes revealed that Cu²⁺ binding was primarily stabilized through metal–ligand coordination and hydrogen bonding with polar and charged residues. Strong coordination was observed particularly for Arg429 in WRKY4, Asp254 in catalase, Arg62 in peroxidase, and His119/His45 in superoxide dismutase, with Cu²⁺–ligand distances ranging from 1.25 to 2.21 Å. Several residues suggested by the MIB2 server, such as Tyr431 in WRKY4 and His201 in catalase, were located outside the direct coordination range, indicating second-shell or indirect contributions to the metal-binding environment. No direct hydrophobic interactions were detected in any of the docked complexes.

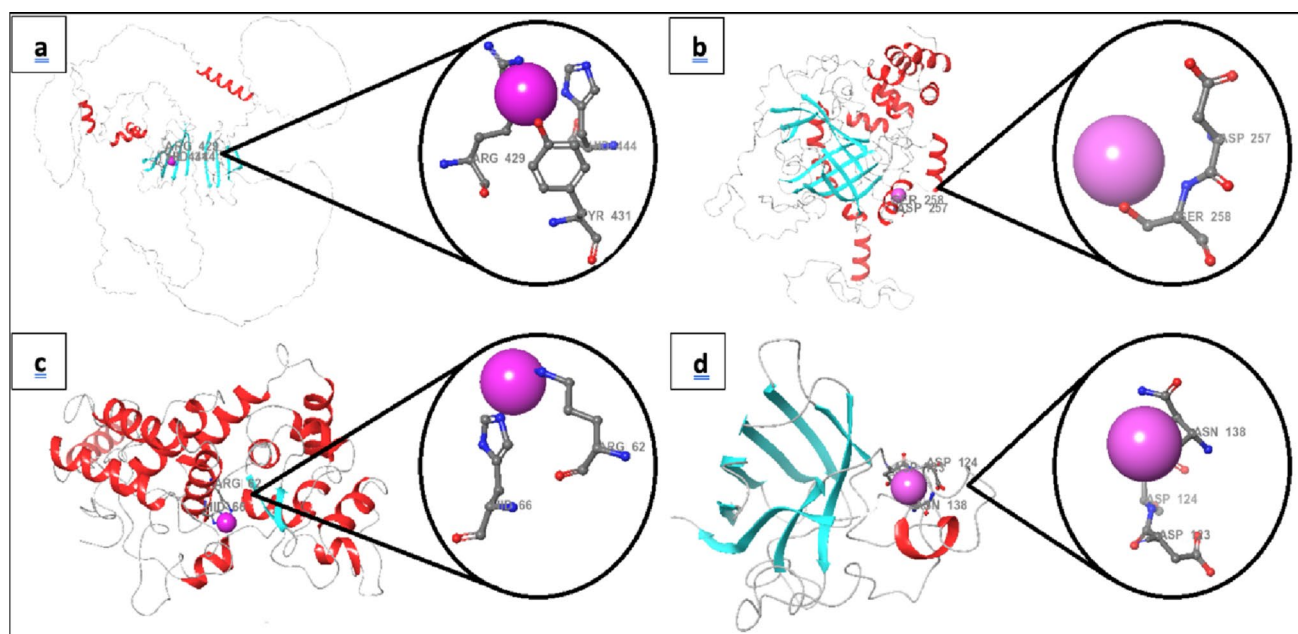


Fig. 4 Molecular docking interactions of Cu^{2+} with the physiological observations and antioxidant-oxidant dynamics associated target receptors: (a) Cu^{2+} - WRKY4, (b) Cu^{2+} - Catalase, (c) Cu^{2+} - Peroxidase and (d) Cu^{2+} - Superoxide dismutase

Since MIB2 predicts metal-binding sites rather than performing classical ligand re-docking, RMSD validation was carried out by comparing the spatial consistency of Cu^{2+} positions among the top ten predicted models. All models showed identical Cu^{2+} coordinates (RMSD=0.0 Å), indicating a highly stable and reliable Cu^{2+} binding site prediction. Such convergence is expected for metal-ion binding site prediction servers when a highly conserved coordination environment is identified.

Discussion

The present study revealed that increasing concentration of Cu cause significant reductions in MI values and inductions in CAs in the root tip cells of *A. cepa*. However, high concentrations of TRIA reduced only MI. The Allium test is an efficient, cost effective, and sensitive assay system to investigate the cytogenotoxic effects of heavy metal exposure at high concentrations on higher plants (Tzima et al. 2022). Formerly, various reports involving the Allium test showed that Cu^{+2} ions reduce the mitotic index, inhibit cell division and induce chromosomal aberrations (Liu et al. 1994, 2009; Jiang et al. 2001; Tzima et al. 2022). In this regard, the results of this study on cytogenotoxicity of Cu^{+2} ions are well correlated with the literature. On the other hand, the results of previous studies regarding the effects of TRIA on MI and CAs are conflicting. A pioneering study reported that exogenous TRIA application promoted cell division during germination under stress free conditions (Hangarter

et al. 1978). However, Tabur and Demir (2008) claimed that TRIA application reduced cell division in barley root meristems under optimal growth conditions. A most recent study also showed that TRIA reduced MI in barley root meristems at 10 μM concentration under optimal growth conditions, but did not affect CAs rates (Tabur et al. 2024). Similar trends that include low concentration promotes MI while high concentration inhibits, were previously reported for other plant growth regulators (Hu et al. 2000; Ismailoglu et al. 2004; Kartal et al. 2009; Tabur and Demir 2009, 2010; Maraklı et al. 2014; Özmen et al. 2022). In this context, our findings were in line with the literature, increasing concentrations of TRIA (10–40 μM) decreased the MI in meristematic cells of *A. cepa* under stress-free growth conditions. On the other hand, co-treatment of TRIA together with 200 μM Cu significantly reduced the metal toxicity on the root tips meristematic cells, resulting in a strong recovery in the MI, and a significant reduction in the CAs rate. To our knowledge, several studies have investigated the protective potential of triacontanol against various physical and chemical stress conditions (Chandra and Roychoudhury 2020; Islam and Mohammad 2020; Verma et al. 2022; Jahan et al. 2023). However, this is the first report on the alleviative and protective role of TRIA against Cu^{+2} -induced cytogenotoxicity in *A. cepa* meristematic cells.

Stress factors that cause a decrease in the growth, development and productivity of plants cause serious damage to plants and can even stop the vital functions of the plant. Various methods are used to cope with these factors, which are important factors that reduce agricultural productivity.

In this study, it was aimed to evaluate the growth, development, cytogenetic, anatomical, physiological and in silico properties of exogenously applied TRIA by creating a response to copper stress, as copper stress can affect the growth or physiology of wheat. According to the statistical analyzes, it was determined that Cu stress affects the growth, weight and antioxidant defense system of the wheat plant. Heavy metal toxicity negatively affects seedling growth (Seneviratne et al. 2019). In this study, significant changes were observed in root and leaf growth of wheat and also in root and leaf dry weight. Compared to the control group in Tables 2 and 3, Cu caused a decrease in root and leaf growth, while it caused an increase in some TRIA doses. When we look at the relative water content, only copper applications caused a decrease in water amount, while TRIA applications increased it. TRIA+Cu applications also generally increased water content. In a study, it was reported that TRIA increased plant growth by regulating adenosine biosynthesis (Ries et al. 1990). Moreover, Cu application in leaf and root tissues was observed to reduce dry and fresh weight as well as growth, while this situation was significantly alleviated with salicylic acid (Zengin 2014). In pepper plants, Cu stress significantly reduced the fresh and dry weight of roots and leaves compared to control, while an increase was observed with chrysin doses (Yuce et al. 2024). Melatonin application in basil seedlings with heavy metal content (150 μM Cu+100 μM Zn) caused an increase in fresh weight, dry weight, shoot length and root length compared to seedlings that did not receive melatonin (Oloumi et al. 2024). TRIA applications have shown that it improves growth and biomass production in *Erythrina variegata*, *Coriandrum sativum* L. and *Brassica juncea* plants under metal stress (Ahmad et al. 2021a, b). The current study is parallel to the literature and has proven that Cu toxicity has a significant negative effect on the growth and development processes of seedlings, while TRIA regulates these processes. Copper stress triggers the formation of harmful reactive oxygen species (ROS) that cause irreversible damage to macromolecules (Lombardi and Sebastiani 2005). Antioxidant enzymes, which are the basic components of the plant defense system, play an important role in clearing ROS accumulated in cells (Tiryaki et al. 2019; Apel and Hirt 2004). In our study, SOD activity in leaves decreased with alone Cu and TRIA applications compared to the control, but it was generally observed to increase when applied together. 200 μM Cu+10 μM TRIA concentration increased the activity significantly compared to 200 μM Cu dose. It was observed that 10 μM TRIA dose increased the activity significantly compared to other TRIA concentrations. In roots, it was observed that 20 μM TRIA and 40 μM TRIA doses increased SOD activity together with Cu concentrations more than other treatments. In addition, the highest SOD

activity was determined in 20 μM TRIA+100 μM Cu dose, while the lowest SOD activity was determined in 40 μM TRIA dose. When POX enzyme activity was examined, Cu application alone decreased the activity in leaf tissue, while TRIA application increased the activity compared to the control. 44% increase in activity was observed in 40 μM TRIA. When only Cu application was compared with Cu+10 μM TRIA and 20 μM TRIA applications, it was determined that TRIA increased the activity. When the root tissue was examined, it was seen that POX activity caused a significant increase especially in 40 μM TRIA compared to the control, while a significant decrease was observed in 200 μM Cu application. When TRIA+Cu applications were examined, it was seen that POX activity increased significantly. In fact, it was determined that only Cu applications caused a significant increase in activity when compared to TRIA+Cu applications. CAT enzyme activity in leaf tissue increased only TRIA applications compared to Cu applications. When CAT enzyme activity was examined in root tissue, a significant increase was observed in TRIA alone applications compared to the control, while a significant decrease in activity was observed in Cu alone application doses. It has been determined that TRIA applied to wheat leaves increases the enzyme activities of SOD, CAT and POX. The balance of the activities of SOD, CAT and POX, which are the main antioxidant enzymes, is of great importance in terms of preventing the harmful effects of O_2^- and H_2O_2 by keeping them at stable levels (Esim 2014). There are studies in the literature on the effect of TRIA on SOD, CAT and POX activities with exogenous applications. Seedlings developed from coriander seeds treated with TRIA alleviated the oxidative stress caused by Cd by improving the antioxidant activity including superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) enzymes (Sardar et al. 2022). In another study, exogenous TRIA applications improved the performance of hot pepper under salinity and has been seen clearly more effective in reducing the lethal effect of salt stress by controlling the increase in antioxidant enzyme activities (SOD, CAT, POX) (Sardar et al. 2022). The ability of TRIA to reduce Cd toxicity was examined in canola (*Brassica napus* L.) plants and it was observed that it reduced oxidative stress by increasing the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (GPX) (Karam et al. 2017). Although studies conducted with Cu and TRIA were not found in the literature, the results of our study were consistent with different stress conditions. Analysis of ROS levels and antioxidant system activities provides important information about stress responses of plants (Apel and Hirt 2004; Noctor and Foyer 1998, 2). As with other stress factors, heavy metal stress increases the production of ROS such as H_2O_2 (Esim 2014;

Baek and Skinner 2012). The increase of this molecule causes significant cell damage (Esim 2014). According to the results of our study, it was observed that H_2O_2 levels in leaves decreased in all doses of TRIA compared to the control. However, it was observed that only Cu applications caused a significant increase in H_2O_2 levels compared to Cu+TRIA applications. It was determined that H_2O_2 levels in root tissue increased with 100 μM Cu and 200 μM Cu doses, while decreasing with all other applications. In addition, it was determined that 200 μM Cu + 10 μM TRIA dose reduced H_2O_2 levels by 32% compared to 200 μM Cu dose. In another study, it was observed that TRIA application reduced the H_2O_2 content in canola plants exposed to Cd heavy metal stress (Karam et al. 2017). TRIA applied to *Coriandrum sativum* L. (coriander) plants exposed to Cd reduced the amount of H_2O_2 (Sardar et al. 2022). Cu (150 μM) applied to basil seedlings caused a decrease in H_2O_2 content, while melatonin application caused an increase in H_2O_2 content (Oloumi et al. 2024). Our findings are parallel to the literature and support that TRIA can reduce oxidative stress in wheat plants during Cu stress. Increased antioxidant enzyme activities with TRIA application significantly reduced the amounts of reactive oxygen species such as H_2O_2 , and the continuity of plant growth rate was ensured by activating the stimulating signal role of H_2O_2 in the plant defense system. LPO is formed as a result of oxidative degradation of cell membrane lipids by free radicals including reactive oxygen species. It leads to the degradation of cell membranes by a free radical chain reaction. Therefore, membrane lipids are very sensitive to oxidative damage. The end products of LPO are very reactive aldehydes such as MDA (Tiryaki et al. 2019; Baek and Skinner 2012). MDA content is an indicator of membrane integrity and lipid peroxidation. MDA content was measured to analyze lipid peroxidation to evaluate membrane damage caused by copper. In the study, it was observed that Cu applications alone increased the MDA content in wheat exposed to Cu stress, but TRIA applications decreased the MDA content. When Cu and TRIA applications were examined together, it significantly decreased the MDA content compared to Cu applications alone. It was determined that 50 μM Cu application decreased compared to 10 μM TRIA application. When the root tissue was examined, it was determined that 200 μM Cu dose increased the MDA content 3 times compared to the control. However, it was observed that the same dose, when applied with 10 μM TRIA, decreased the MDA content almost at the same rate as the control. However, when the application of only 50 μM Cu dose together with 10 μM TRIA was compared, it was observed that TRIA reduced the MDA content by 4 times. Reducing the MDA content with TRIA applications is important for improving Cu tolerance. Considering all the results, it can be concluded

that exogenously applied TRIA prevents cell membrane damage by reducing the lipid peroxidation (MDA) level that increases with Cu application. As far as we can see, although there is no study in the literature that shows that the amount of MDA and therefore the lipid peroxidation level decreases with TRIA application, there are some different studies. In one study, MDA content increased in Cu-treated bean leaves and roots and decreased with salicylic acid application (Zengin 2014). Compared with the control, Cd-treated pea plants showed higher lipid peroxidation, and pre-soaking the seeds with SA before Cd application was observed to reduce MDA levels (Popova et al. 2009). In the study conducted on basil seedlings, MDA content increased with 150 μM Cu application and decreased with melatonin application (Oloumi et al. 2024). It was observed that the results of the current study were compatible with the application of methods against heavy metal application. The activation of SOD, CAT and POX enzymes, which play a role in the cleaning of ROS, is important for the plant to develop tolerance to oxidative stress. However, the activity of these enzymes alone may not be sufficient. In order to provide tolerance to oxidative stress, antioxidant enzymes are expected to be activated and the amount of ROT and LPO levels are expected to decrease. The TRIA concentrations we used in the study increased enzyme activities while at the same time decreasing the amount of ROT and LPO levels. The increase in antioxidant enzyme activities occurs due to the increase in ROT, thus helping the plant adapt to various stresses. Therefore, it is very important that TRIA causes increases in enzyme activities. Thus, TRIA can increase the resistance of wheat plants exposed to Cu stress and facilitate adaptation to Cu stress.

Observations on anatomical alterations in the plant tissues serve as a useful tool for monitoring hazardous or beneficial potency of test compounds (Çavuşoğlu et al. 2021; Macar et al. 2022; Cakir et al. 2023). In this regard, the anatomical investigation results were closely correlated with cytogenotoxicity findings, increasing Cu^{2+} concentrations caused significant anatomical alterations including cellular damages in epidermis, cortex and transmission tissues, thickening of the cell walls, nuclear vacuolization and formation of micronuclei while increasing triacontanol concentrations did not affect the anatomical parameters. Furthermore, increasing concentrations significantly reduced Cu^{2+} -induced alterations in the co-treatment groups, indicating protective potential of triacontanol against heavy metal toxicity in *A. cepa* roots.

In silico approaches including binding site prediction algorithms and molecular docking are widely used in the investigation of interactions between query molecules and target proteins (Lin et al. 2016; Lu et al. 2022). In this regard, the molecular interactions of Cu^{2+} ions with

WRYK4 transcription factor, catalase, peroxidase, and superoxide dismutase enzymes were investigated by MIB2, a comprehensive web-based tool for the reliable prediction of metal ion-binding sites and metal docking, to provide new molecular insights into the understanding of the mechanism of Cu^{2+} phytotoxicity observed in vitro experiments. The MIB2 results indicated that Cu^{2+} ions had strong interactions with mainly hydrophilic and acidic amino acids in the target proteins. The negatively charged nature of these interacting amino acids enhances the formation of the ionic bonds between Cu^{2+} ions and the residues (Tan et al. 2024). Moreover, the distance-based analysis of Cu^{2+} –protein interactions (Table 5) revealed that metal binding was primarily stabilized through coordination and hydrogen bonding with polar and charged residues, which is consistent with the well-established principles of metal–protein interactions. In particular, Cu^{2+} coordination distances observed for Arg429 in WRKY4 (1.56–2.02 Å), Asp254 in catalase (1.67–2.21 Å), Arg62 in peroxidase (1.25–2.20 Å), and His119/His45 in superoxide dismutase (1.53–2.12 Å) fall within the typical range reported for Cu^{2+} –ligand interactions in proteins. Histidine- and aspartate-mediated Cu^{2+} coordination has been widely documented, especially in antioxidant enzymes such as catalase and superoxide dismutase, where metal binding plays a crucial role in enzymatic activity and redox regulation. Several residues suggested by the MIB2 server, including Tyr431 in WRKY4 and His201 and Ser258 in catalase, were located outside the direct coordination range (> 3.2 Å). These residues are therefore likely to function as second-shell contributors, indirectly stabilizing the metal-binding pocket rather than directly coordinating the Cu^{2+} ion. Such second-shell interactions have been reported to influence metal-binding geometry and protein stability without forming direct coordination bonds. Importantly, no direct hydrophobic interactions between Cu^{2+} and nonpolar residues were detected, which is consistent with the charged nature of Cu^{2+} and further supports the validity of the docking results (Bertini 2007; Dudev and Lim 2014). These results further suggested that Cu ions exerts phytotoxic effect on the seedling growth parameters via interacting with WRYK4 transcription factor in higher plant species, and on the oxidant/antioxidant dynamics via interacting with catalase, peroxidase, and superoxide dismutase enzymes.

When the results are evaluated, it is suggested that TRIA can be used to increase the resistance of this plant exposed to Cu stress by increasing the antioxidant activities of the wheat plant and decreasing the H_2O_2 and lipid peroxidation levels. For these reasons, our study can provide ideas for future studies by showing the usability of TRIA to increase the resistance of plants against environmental stresses.

Author contribution DT, GK, BT, HO took part in plant cultivation, homogenization, enzyme activities, lipid peroxidation, hydrogen per-

oxide amount determination and article writing. MG, YG, MK took part in allium assay, anatomical investigations, molecular docking studies and article writing. All authors read and approved the final manuscript.

Funding Open access funding provided by the Scientific and Technological Research Council of Türkiye (TÜBİTAK).

Declarations

Conflict of interest The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Agarwal S, Pandey V (2004) Antioxidant enzyme responses to NaCl stress in *Cassia angustifolia*. *Biol Plant* 48:555–560. <https://doi.org/10.1023/B:BIOP.0000047152.07878.e7>
- Ahmad A, Shahzadi I, Mubeen S, Yasin NA, Akram W, Khan WU, Wu T (2021a) Karrikinolide alleviates BDE-28, heat and Cd stressors in *Brassica alboglabra* by correlating and modulating biochemical attributes, antioxidative machinery and osmoregulators. *Ecotoxicol Environ Saf* 213:112047. <https://doi.org/10.1016/j.ecoenv.2021.112047>
- Ahmad J, Ali AA, Al-Huqail AA, Qureshi MI (2021b) Triacantanol attenuates drought-induced oxidative stress in *Brassica juncea* L. by regulating lignification genes, calcium metabolism and the antioxidant system. *Plant Physiol Biochem* 166:985–998. <https://doi.org/10.1016/j.plaphy.2021.07.009>
- Alaoui-Sosse' B, Genet P, Vinit-Dunand F, Toussaint ML, Epron D, Badot PM (2004) Effect of copper on growth in cucumber plants (*Cucumis sativus*) and its relationships with carbohydrate accumulation and changes in ion contents. *Plant Sci* 166:1213–1218. <https://doi.org/10.1016/j.plantsci.2003.12.032>
- Ali HMM, Perveen S (2020) Effect of foliar applied triacantanol on wheat (*Triticum aestivum* L.) under arsenic stress: a study of changes in growth, yield and photosynthetic characteristics. *Physiol Mol Biol Plants* 26(6):1215–1224. <https://doi.org/10.1007/s12298-020-00831-0>
- Angelini R, Federico R (1989) Histochemical evidence of poliamin oxidation and generation of hydrogen peroxide in the cell wall. *J Plant Physiol* 135:212–217. [https://doi.org/10.1016/S0176-1617\(89\)80179-8](https://doi.org/10.1016/S0176-1617(89)80179-8)
- Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55:373–399. <https://doi.org/10.1146/annurev.arplant.55.031903.141701>
- Aybeke M (2024) Interplay of Melatonin and Jasmonic Acid in Modulating Wheat (*Triticum aestivum* L.) Physiology and Molecular

- Pathways Under Salt Stress. *J Plant Growth Regul.* <https://doi.org/10.1007/s00344-024-11567-6>
- Aydın B, Alkuyruk SB, Tekman E, Yuca H, Karadayı M, Gülşahin Y, Bona M, Karakaya S (2024) Investigating the phytochemical profile, biological potentials, morphological, and anatomical characteristics of *Cyclotrichium origanifolium* (Labill.) Manden. & Scheng. (Lamiaceae) from Turkey. *Protoplasma* 1–20. <https://doi.org/10.1007/s00709-024-01992-5>
- Baek KH, Skinner DZ (2012) Production of reactive oxygen species by freezing stress and the protective roles of antioxidant enzymes in plants. *J Agric Chem Environ* 34–40. <https://doi.org/10.4236/jacen.2012.11006>
- Bertini I (ed) (2007) Biological inorganic chemistry: structure and reactivity. University Science Books
- Cakir F, Kutluer F, Yalcin E, Cavusoglu K, Acar A (2023) Deep neural network and molecular docking supported toxicity profile of prometryn. *Chemosphere* 340:139962. <https://doi.org/10.1016/j.chemosphere.2023.139962>
- Cavusoglu D, Yalcin E, Cavusoglu K, Acar A, Yapar K (2021) Molecular docking and toxicity assessment of spirodiclofen: Protective role of lycopene. *Environ Sci Pollut Res* 28:57372–57385. <https://doi.org/10.1007/s11356-021-14748-y>
- Chandra S, Roychoudhury A (2020) Penconazole, paclobutrazol, and triacontanol in overcoming environmental stress in plants. Protective chemical agents in the amelioration of plant abiotic stress: biochemical and molecular perspectives, 510–534. <https://www.researchgate.net/publication/341839220>
- Dai H, Xu Y, Zhao L, Shan C (2016) Alleviation of copper toxicity on chloroplast antioxidant capacity and photosystem II photochemistry of wheat by hydrogen sulfide. *Braz J Bot* 39:787–793. <https://doi.org/10.1007/s40415-016-0250-6>
- Demirevska-Kepova K, Simova-Stoilova L, Stoyanova Z, Holzer R, Feller U (2004) Biochemical changes in barley plants after excessive supply of copper and manganese. *Environ Exp Bot* 52:253–266. <https://doi.org/10.1016/j.envexpbot.2004.02.004>
- Dudev T, Lim C (2014) Competition among metal ions for protein binding sites: determinants of metal ion selectivity in proteins. *Chem Rev* 114(1):538–556. <https://doi.org/10.1021/cr4004665>
- Dürr SL, Levy A, Rothlisberger U (2023) Metal3D: a general deep learning framework for accurate metal ion location prediction in proteins. *Nat Commun* 14(1):2713. <https://doi.org/10.1038/s41467-023-37870-6>
- Esim N, Atici Ö (2014) Nitric oxide improves chilling tolerance of maize by affecting apoplastic antioxidative enzymes in leaves. *Plant Growth Regul* 72:29–38. <https://doi.org/10.1007/s10725-013-9833-4>
- Gong Y, Toivonen PMA, Lau OL, Wiersma PA (2001) Antioxidant system level in 'Braeburn' apple in related to its browning disorder. *Bot Bull Acad Sin* 42:259–264
- Hangarter R, Ries SK, Carlson P (1978) Effect of triacontanol on plant cell cultures in vitro. *Plant Physiol* 61:855–857. <https://doi.org/10.1104/pp.61.5.855>
- He Y, Liu Y, Cao W, Huai M, Xu B, Huang B (2005) Effects of salicylic acid on heat tolerance associated with antioxidant metabolism in Kentucky bluegrass. *Crop Sci* 45:988–995. <https://doi.org/10.2135/cropsci2003.0678>
- Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplast I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys* 25:189–198. [https://doi.org/10.1016/0003-9861\(68\)90654-1](https://doi.org/10.1016/0003-9861(68)90654-1)
- Hu Y, Bao F, Li J (2000) Promotive effect of brassinosteroids on cell division involves a distinct CycD3-induction pathway in Arabidopsis. *Plant J* 24:693–701. <https://doi.org/10.1046/j.1365-3113.2000.00915.x>
- Islam S, Mohammad F (2020) Triacontanol as a dynamic growth regulator for plants under diverse environmental conditions. *Physiol Mol Biol Plants* 26:871–883. <https://doi.org/10.1007/s12298-020-00815-0>
- Ismailoglu I, Unal M, Palavan-Unsal N (2004) Effects of spermidine, spermine and cyclohexylamine on mitotic activity of 2X, 4X and 6X wheats. *J Cell Mol Bio* 3:83–88
- Jahan S, Rautela S, Chishti AS, Shankhdhar D, Shankhdhar SC, Srivastava A, Garg SK (2023) Triacontanol is a potent alleviator of stress induced by salt and heavy metal contamination in plants. *Rhizosphere* 28:100822. <https://doi.org/10.1016/j.rhisph.2023.100822>
- Jiang W, Liu D, Hou W (2001) Hyperaccumulation of cadmium by roots, bulbs and shoots of garlic (*Allium sativum* L.). *Bioresour Technol* 76:9–13. [https://doi.org/10.1016/S0960-8524\(00\)00086-9](https://doi.org/10.1016/S0960-8524(00)00086-9)
- Karam EA, Maresca V, Sorbo S, Keramat B, Basile A (2017) Effects of triacontanol on ascorbate-glutathione cycle in *Brassica napus* L. exposed to cadmium-induced oxidative stress. *Ecotoxi Environ Saf* 144:268–274. <https://doi.org/10.1016/j.ecoenv.2017.06.035>
- Kartal G, Temel A, Arican E, Gözükmızı N (2009) Effects of brassinosteroids on barley root growth, antioxidant system and cell division. *Plant Growth Regul* 58:261–267. <https://doi.org/10.1007/s10725-009-9374-z>
- Li R, Zhou Z, Zhang T, Su H, Li J (2023) Overexpression of LSU1 and LSU2 confers cadmium tolerance by manipulating sulfur metabolism in Arabidopsis. *Chemosphere* 334:139046. <https://doi.org/10.1016/j.chemosphere.2023.139046>
- Lin YF, Cheng CW, Shih CS, Hwang JK, Yu CS, Lu CH (2016) MIB: metal ion-binding site prediction and docking server. *J Chem Inf Model* 56:2287–2291. <https://doi.org/10.1021/acs.jcim.6b00407>
- Liu D, Jiang W, Lu C, Zhao FM, Hao YQ, Guo L (1994) Effects of copper sulfate on the nucleolus of *Allium cepa* root-tip cells. *Hereditas* 120:87–90. <https://doi.org/10.1111/j.1601-5223.1994.00087.x>
- Liu D, Jiang W, Meng Q, Zou J, Gu J, Zeng M (2009) Cytogenetical and ultrastructural effects of copper on root meristem cells of *Allium sativum* L. *Biocell* 33:25–32. <https://doi.org/10.32604/biocell.2009.33.025>
- Lombardi L, Sebastiani L (2005) Copper toxicity in *Prunus cerasifera*: growth and antioxidant enzymes responses of in vitro grown plants. *Plant Sci* 168:797–802. <https://doi.org/10.1016/j.plantsci.2004.10.012>
- Lu CH, Lin YS, Chen YC, Yu CS, Chang SY, Hwang JK (2006) The fragment transformation method to detect the protein structural motifs. *Proteins Struct Funct Bioinform* 63:636–643. <https://doi.org/10.1002/prot.20904>
- Lu CH, Chen CC, Yu CS, Liu YY, Liu JJ, Wei ST, Lin YF (2022) MIB2: metal ion-binding site prediction and modeling server. *Bioinformatics* 38:4428–4429. <https://doi.org/10.1093/bioinformatics/btac534>
- Macar O, Macar TK, Yalcin E, Cavusoglu K, Acar A (2022) Molecular docking and spectral shift supported toxicity profile of metaldehyde mollucide and the toxicity-reducing effects of bitter melon extract. *Pest Biochem Physiol* 187:105201. <https://doi.org/10.1016/j.pestbp.2022.105201>
- Maksymiec W (1997) Effect of copper on cellular processes in higher plants. *Photosynthetica* 34:321–342. <https://doi.org/10.1023/A:1006818815528>
- Mao W, Guo L, Sun T (2024) Exogenous Application of Silicon and Spermidine Alleviate Cadmium Toxicity by Modulating Endogenous Polyamines Level and Glutathione Metabolism in *Salix matsudana* Koidz. Seedlings. *J Plant Growth Regul* 1–17. <https://doi.org/10.1007/s00344-024-11570-x>
- Maraklı S, Temel A, Gözükmızı N (2014) Salt stress and homobrassinosteroid interactions during germination in barley roots. *Not Bot Horti Agrobot Cluj-Nap* 42:446–452
- Memon AR (2020) Heavy metal-induced gene expression in plants. *Contaminants Agriculture: Sources Impacts Manage* 143–173. https://doi.org/10.1007/978-3-030-41552-5_7

- Naeem M, Khan A, Masroor M, Moinuddin (2012) Triacantanol: A potent plant growth regulator in agriculture. *J Plant Interact* 7:129–142. <https://doi.org/10.1080/17429145.2011.619281>
- Noctor G, Foyer CG (1998) Ascorbate and glutathione; keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* 49:249–279. <https://doi.org/10.1146/annurev.arplant.49.1.249>
- Oloumi H, Zamani A, Ghotbzadeh S, Mozaffari H (2024) Zinc and copper toxicity in basil (*Ocimum basilicum* L.) seedlings: Role of melatonin in mitigating stress. *Plant Stress* 11:100365. <https://doi.org/10.1016/j.stress.2024.100365>
- Ozmen S, Tabur S, Oney-Birol S, Ozmen S (2022) Molecular responses of exogenous polyamines under drought stress in the barley plants. *Cytologia* 87:7–15. <https://doi.org/10.1508/cytologia.87.7>
- Popova LP, Maslenskova LT, Yordanova RY, Ivanova AP, Krantev AP, Szalai G, Janda T (2009) Exogenous treatment with salicylic acid attenuates cadmium toxicity in pea seedlings. *Plant Physiol Biochem* 47:224–231. <https://doi.org/10.1016/j.plaphy.2008.11.007>
- Ries S, Wert V, o'leary NFD, Nair M (1990) 9-f-L(+) Adenosine: A new naturally occurring plant growth substance elicited by triacantanol in rice. *Plant Growth Regul* 9:263–273. <https://doi.org/10.1126/science.195.4284.1339>
- Sardar R, Ahmed S, Akbar M, Yasin NA, Li G (2022) Alleviation of cadmium phytotoxicity in triacantanol treated *Coriandrum sativum* L. by modulation of physiochemical attributes, oxidative stress biomarkers and antioxidative system *Chemosphere* 295:133924 <https://doi.org/10.1016/j.chemosphere.2022.133924>
- Seneviratne M, Rajakaruna N, Rizwan M, Madawala HMSP, Ok YS, Vithanage M (2019) Heavy metal-induced oxidative stress on seed germination and seedling development: a critical review. *Environ Geochem Health* 41:1813–1831. <https://doi.org/10.1007/s10653-017-0005-8>
- Tabur S, Demir K (2008) Effects of triacantanol pretreatment on mitotic index and chromosome abnormalities under salt stress. *Res J Biol Sci* 1:11–15
- Tabur S, Demir K (2009) Cytogenetic response of 24-epi-brassinolide on the root meristem cells of barley seeds under salinity. *Plant Growth Regul* 58:119–123. <https://doi.org/10.1007/s10725-008-9357-5>
- Tabur S, Demir K (2010) Role of some growth regulators on cytogenetic activity of barley under salt stress. *Plant Growth Regul* 60:99–104. <https://doi.org/10.1007/s10725-009-9424-6>
- Tabur S, Ergun SBY, Özmen S (2024) Role of ten different exogenous plant growth promoters in regulating cytotoxic and genotoxic processes in barley exposed to high temperature stress. *Caryologia* 77:21–37. <https://doi.org/10.36253/caryologia-2409>
- Tan P, Yang J, Yi F, Mei L, Wang Y, Zhao C, Wang J (2024) Strontium attenuates LPS-induced inflammation via TLR4/MyD88/NF-κB pathway in bovine ruminal epithelial cells. *Biol Trace Elem Res* 202:3988–3998. <https://doi.org/10.1007/s12011-023-03992-7>
- Tanyolac D, Ekmekci Y, Unalan S (2007) Changes in photochemical and antioxidant enzyme activities in maize (*Zea mays* L.) leaves exposed to excess copper. *Chemosphere* 67:89–98. <https://doi.org/10.1016/j.chemosphere.2006.09.052>
- Tiryaki D, Aydın I, Atici O (2019) Psychrotolerant bacteria isolated from the leaf apoplast of cold-adapted wild plants improve the cold resistance of bean (*Phaseolus vulgaris* L.) under low temperature *Cryobiology*. 86:111–119. <https://doi.org/10.1016/j.cryobiol.2018.11.001>
- Tzima CS, Banti CN, Hadjikakou SK (2022) Assessment of the biological effect of metal ions and their complexes using *Allium cepa* and *Artemia salina* assays: a possible environmental implementation of biological inorganic chemistry. *J Biol Inorg Chem* 27:611–629. <https://doi.org/10.1007/s00775-022-01963-2>
- Verma T, Bhardwaj S, Singh J, Kapoor D, Prasad R (2022) Triacantanol as a versatile plant growth regulator in overcoming negative effects of salt stress. *J Agric Food Res* 10:100351. <https://doi.org/10.1016/j.jafr.2022.100351>
- Viehweger K (2014) How plants cope with heavy metals. *Bot Stud* 55(1):1–12. <https://doi.org/10.1186/1999-3110-55-35>
- Yuce M, Ekinici M, Turan M, Agar G, Murat A, İlhan E, Yıldırım E (2024) Chrysin mitigates copper stress by regulating antioxidant enzymes activity, plant nutrient and phytohormones content in pepper. *Sci Hort* 328:112887. <https://doi.org/10.1016/j.scienta.2024.112887>
- Zengin F (2014) Exogenous Treatment with Salicylic Acid Alleviating Copper Toxicity in Bean Seedlings *Proc Natl Acad Sci India Sect. B Biol Sci* 84:749–755. <https://doi.org/10.1007/s40011-013-0285-4>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.