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Research Article

GENOTOXICITY AND ANTIOXIDANT ENZYME ACTIVITIES INDUCED BY THE CAPTAN FUNGICIDE IN THE ROOT OF BELL PEPPER (*Capsicum annuum* L. var. *grossum* L. cv. Kandil)

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Abstract: In this study, we investigated the toxic effects of the captan fungicide by using morphological, physiological and cytological parameters in bell pepper (*Capsicum annuum* L. var. *grossum* L. cv. Kandil) root tissue. The seeds of bell pepper were germinated in Petri dishes including different concentrations (0, 25 µM, 50 µM, 100 µM, 150 µM) of captan fungicide for 7 days. The germination rates and root lengths were significantly reduced in captan-treated seeds. All concentrations caused a significant decrease in mitotic index and increase in different types of chromosomal abnormalities such as c-mitosis and chromosome stickness in meristematic cells of bell pepper root. Captan treatment also induced oxidative stress by leading to membrane damage with an increase in root electrolyte leakage in 7 days-old bell pepper root. Catalase, glutathione reductase and total peroxidase activities increased under different concentrations as a response to oxidative stress. Our results showed that captan fungicide had negative effects on germination and growth in bell pepper seed.

Key words: Chromosomal abnormality, antioxidant enzyme activity, pesticide toxicity.

Özet: Bu çalışmada, dolmalık biberde (*Capsicum annuum* L. var. *grossum* L. cv. Kandil) kök dokusunda morfolojik, fizyolojik ve sitolojik parametreler kullanarak captan fungisitinin toksik etkisi araştırıldı. Dolmalık biber tohumları 7 gün boyunca farklı konsantrasyonlarda (0,25 µM, 50 µM, 100 µM, 150 µM) captan fungisit içeren petri kabında çimlenmiştir. Elde edilen sonuçlar, captanla muamele edilmiş tohumlarda çimlenme oranının ve kök uzunluğunun düştüğünü göstermiştir. Ayrıca, captan fungisitinin tüm konsantrasyonları, mitotik indekste önemli bir azalmaya ve biber kökünün meristematik hücrelerinde c-mitoz ve kromozom yapışkanlığı gibi farklı tipte kromozomal anormalliklerin artmasına neden olmuştur. Ayrıca, captan muamelesi, 7 günlük dolmalık biber kökündeki kök elektrolit sızıntısında bir artış ile membran hasarına yol açarak oksidatif stresi tetiklemiştir. Oksidatif stres ile başa çıkmak için katalaz, glutatyon redüktaz ve toplam peroksidaz aktivitelerinin dolmalık biber köklerinde farklı captan fungisit konsantrasyonu altında arttığı belirlenmiştir. Elde ettiğimiz sonuçlar captan fungisitinin dolmalık biber tohumundaki çimlenme ve büyümeyi olumsuz yönde etkilediğini göstermiştir.

Introduction

Pepper (*Capsicum annum* L.), a member of the Solanaceae family, is cultivated in different parts of the World and is an important vegetable with various fruit types known as bell-shaped, charleston, conic and long-green all which are consumed either as fresh, processed, pickled or powder (Dağistan *et al.* 2015). According to the production ratio, pepper is ranked the sixth among commonly grown vegetables in Turkey (Aytop *et al.* 2014). However, yield and quality of pepper is decreased by many diseases that cause great losses in its production (Abou-Zeid *et al.* 2016). Many soilborne fungal root rot and wilt pathogens such as *Rhizoctonia solani* Kühn, *Macrophomina phaseolina* (Tassi) Goid, *Fusarium oxysporum* Schlecht. *and F. solani* (Mart.) Sacc. have been reported to be widespread and attack pepper roots and stems causing severe losses in seed germination, plant growth and yield (Güney & Güldür 2018).

Fungicides are used to protect agricultural products against fungal infections in seed, root, shoot and leaves of plants. Members of the dicarboximide from the oldest groups of fungicides have been frequently used in these treatments since 1949s (Thomson 1997). Captan is a dicarboximide and phthalimide member non-systemic fungicide. It is one of the most commonly used fungicides in seed treatment and used to protect crops, vegetable and fruit from fungal diseases caused by pathogens such as *Phytophthora infestans* (Mont.) de Bary and *Botrytis cinerea* Pers.

Excessive use of pesticides not only poses risk for soil, water and air but also deleteriously affects non-target

organism including humans, animals and plants (Parween *et al.* 2016). Many studies showed that indiscriminate use of pesticides results in undesirable consequences one of which is induction of genetic damage on plant cells (Aktar *et al.* 2009). The most important effect of pesticides is their genotoxic, mutagenic or cytotoxic roles in non-target organism (Çavuşoğlu *et al.* 2011; Verma & Srivastava 2018). The alkylating abilities of pesticides break DNA and cause damages in DNA replication (Kaur *et al.* 2011). Pesticides also lead to mitotic disorders such as abnormal chromosomes, micronucleus formation, chromosomal bridges and polyploidy (Iqbal *et al.* 2019). Studies revealed that pesticides caused a wide range of genotoxic effects in *Allium cepa* L. (Türkoğlu 2012, Karaismailoğlu 2017), *Vicia faba* L. (Singh *et al.* 2013) *Helianthus annuus* L. (Karaismailoğlu 2014) and *Lycopersicon esculentum* Mill. (Akpınar 2014).

Pesticide toxicity can deleteriously affect various metabolic processes by inhibition of germination, retardation of growth, reduction in photosynthesis and alteration of nitrogen/carbon metabolism (Dias 2012). At cellular level, high pesticide concentrations induce oxidative damage which result in accumulation of reactive oxygen species, injury and increase ion leakage of the cell membrane (Parween *et al.* 2012). Plants are able to develope multiple complex enzymatic antioxidant system including catalase (CAT), peroxidase (POX) and glutathione reductase (GR) to protect themselves against harmful effects of oxidative stress.

In the present study, we investigated the potential genotoxic effect of the captan fungicide on bell pepper *Capsicum annuum* L. var. *grossum* L. cv. Kandil by determining mitotic index and chromosomal and mitotic aberrations in root meristems. The toxic effects of captan were also evaluated by considering the alterations in some growth parameters (root length, germination rate and protein content), root electrolyte leakage and antioxidant enzyme activities.

Materials and Methods

Experimental Design

The test substance Captan (N-triklorometilmercapto-4-siklohekzen-1)(IUPAC Name: $C_9H_8Cl_3NO_2S$, CAS No: 133-06-2, molecular weight: 300, 59 g/mol, purity of 99,6 %) was purchased from Pestanal (32054) (Steinheim, Germany) and prepared by ultrapure water. 500 µM stock solution was stored in the dark and under +4°C. The seeds of *Capsicum annuum* (2n = 24) were purchased from a seed company (Agrogen) operating in Tekirdağ, Turkey. All chemicals were obtained from Sigma Aldrich (St. Louis, MO).

The seeds were sterilized in 1% sodium hypochlorite and imbibed in deionized water for 6 hours. The imbibed seeds were kept on Petri dishes (9 cm diameter) containing different concentrations (25 µM, 50 µM, 100 µM, 150 µM) of captan solutions for 7 days at 25°C under dark conditions in an incubator. The seeds of the control group were kept in deionized water.

EC50 Determination

Bell pepper seeds were primarily treated with different concentrations of captan ranging from 12.5 µM to 200 µM for EC50 determination. Test concentrations which caused 50% reduction in root length in comparison with the control group were designated as EC50. EC50 values for captan was 25 µM and 150 µM. After EC50 determination, four different concentrations (25 µM, 50 µM, 100 µM, 150 µM) were selected for the test applications. In the selection of the experimental concentrations, the doses used by local farmers in agricultural fields were considered.

Germination Rates and Root Growth

The number of germinating seeds and the root length was determined at the end of the 7^{th} days following applications.

Mitotic Index and Assays

The root tips were randomly collected from each Petri plate in triplicate for cytological studies. Primary roots were fixed in freshly prepared Carnoy's solution (acetic-ethanol: 1:3, v/v) in separate vials. Squash preparations were made using 2% aceto-orcein. For each replicate, about 1000 cells were examined in roots and analyzed with respect to mitosis and chromosomal aberrations. For each treatment, mitotic index was calculated as a percentage by the ratio of dividing cell number to total cell number. Chromosomal abnormalities were counted in prophase, metaphase, anaphase and telophase stages and expressed as a percentage of the total number of abnormalities in the dividing cells.

Electrolyte Leakage

Electrolyte leakage from fine roots (REL) was determined using the relative conductivity method of Wilner (1955). Roots were washed three times with deionized water to remove surface ions. Each root sample was put into 28 mL glass bottles containing 16 mL deionized water of a known conductivity. The sealed bottles were left at room temperature for 24 h after shaking. The conductivity of the solution in the bottle which was shaken again was measured using a conductivity probe with in-built temperature compensation. After samples were autoclaved at 110°C for 10 mins, it was cooled to room temperature and the total conductivity was measured for each sample. The 24 h conductivity was expressed as a percentage (%).

Analysis of Antioxidant Enzyme Activities

Root samples were extracted in 0.05 mM potassium phosphate buffer (pH: 7.8) containing 1 mM EDTA and 2% PVPP (Polyvinylpyrrolidone). The homogenate was centrifuged at 14.000×*g* for 30 min at +4°C, and the obtained supernatant was used in determination of protein and enzyme activity. All spectrophotometric analyses were conducted on Epoch 2 Microplate Spectrophotometer (United States). The soluble protein content was determined by Bradford (1976) method using bovine serum albumin as a standard. Total peroxidase activity was

assayed by following the increase in absorbance by oxidation of 3,3-diaminobenzidine tetrahydrochloride (DAB) at 465 nm, according to the method of Herzog & Fahimi (1973). Catalase (CAT, EC 1.11.1.6) activity was analyzed by measuring the rate of decomposition of H_2O_2 at 240 nm, as described by Bergmeyer (1970). Glutathione reductase (GR, EC 1.8.1.7) activity was measured by following the change in 340 nm as oxidised glutathione (GSSG)-dependent oxidation of NADPH, according to the method of Foyer & Halliwell (1976).

Statistical Analysis

All experimental data were analyzed using the mean ± standard deviation values of at least 5-10 replicates. The data is suitable for normal distribution according to Shapiro-Wilk test. The significance of differences between the mean values were determined by a one-way ANOVA followed by Tukey Post Hoc Test analysis. All analyzes were performed on GraphPad Prism version 5.2 for Windows (GraphPadSoftware, San Diego, CA).

Results

The Effect of Captan Fungicide on Seed Germination and Root Growth

The effect of captan fungicide on germination percentage and root length were given in Table 1. 25, 100 and 150 µM concentrations caused approximately 32, 55 and 66 % reduction in seed germination, respectively. On the other hand, 50 µM captan treatment significantly increased germination rate when compared to the control. The root length values significantly decreased by approximately 40, 59, 72 and 74 % with 25, 50, 100 and 150 µM/L captan treatments, respectively. 150 µM/L treatment caused the highest inhibition on germination and root length in bell pepper (Table 1).

The Effect of Captan Fungicide on Genotoxicity

The genotoxic effects of the captan fungicide were evaluated by the mitotic index and the percentage of chromosomal abnormalities in root meristems. The results of the mitotic index (%) and chromosome abnormality under fungicide treatments were shown in Table 2. Mitotic activity in root meristem cells gradually decreased with increasing captan concentrations. The highest inhibition of mitotic activity (38%) was observed in 150µM captan treatment. The microscopic investigations revealed that captan treatments induced various chromosomal aberrations in mitosis phase in root meristem cells (Fig. 1). The fungicide treatments reduced cell division frequency in comparison with the control and the maximum inhibition was determined in 150 µM treatment. The treatments also significantly affected the anaphase among other phases. The total abnormality frequency increased by 35, 35, 49 and 59% at 25, 50, 100 and 150 µM/L treatments, respectively. The types of chromosome abnormalities were indicated according to sticky chromosome, lagging chromosome, multipolarity, fragment, c-mitosis, chromosome bridge and binucleated (Table 2). When compared to the control, sticky chromosome and multipolarity were commonly observed in dividing cells following captan treatments (Table 2).

The Effect of Captan Fungicide on Membrane Damage

The root electrolyte leakage (REL) increased significantly, when compared to the control, by 37, 57, 94, 105 and 111,08 % in 25, 50, 100 and 150 µM captan treatments, respectively (Fig. 2).

The Effect of Captan Fungicide on Antioxidant Enzyme Activities

Captan fungicide negatively affected protein content in bell pepper root tissues. The increasing treatment concentrations decreased protein content (Fig. 3) and the reduction was significant for 100 and 150 µM/L treatments. We investigated POX, CAT and GR activities in roots treated with different fungicide concentrations (Fig. 4). POX activity increased by 26, 29, 68 and 80% at 25, 50, 100 and 150 µM/L treatments, respectively. CAT and GR activities significantly increased with only 150 µM/L treatment by 17 and 32%, respectively (Fig. 4).

Table 1. The effect of different concentrations of captan fungicide on germination rate (%) and root length (cm) in bell pepper. Data are means (±) standard deviations (SD). (*) represent adjusted p values ($p<0.0001$) for statistically significant differences from the control as revealed by one-way ANOVA analysis followed by Tukey Multiple Comparison Test.

Treatment	Germination (%)	Root Length (cm)
Control	15.66±0.54	1.62±0.34
25 µM/L	10.63*±0.55	0.98*±0.28
50 µM/L	58.1*±0.47	0.67*±0.11
100 µM/L	7.10*±0.51	0.46*±0.13
150 µM/L	5.33*±0.49	0.42*±0.12

Table 2. Mitotic index and the frequency of chromosome abnormalities in the root meristematic cells in bell pepper treated with different concentrations of captan (S=Sticky chromosome; L=Lagging chromosome; M=Multipolarity; F=Fragment; C=C-mitosis; B=Bridge; BN=Binucleated). Data are means (±) standard deviations (SD). (*) represent adjusted p values ($p<0.0001$) for statistically significant differences from the control by one-way ANOVA analysis followed by Tukey Multiple Comparison Test.

Concentration	MI (%)	Dividing Cell	Abnormality							
			S	L	M	F	C	B	BN	Total Abnormality Frequency (%)
Control	12.55 ± 0.74	127	2	-	-	-	-	-	-	1.64 ±1.17
25 µM	9.6* ± 1.09	107	20	-	9	1	4	-	3	34.58* ± 2.21
50 µM	9.53* ± 1.62	102	14	6	12	1	-	2	1	35.29* ± 3.12
100 µM	7.82* ± 0.97	81	19	2	5	2	3	1	8	49.38* ± 2.42
150 µM	7.73* ± 1.99	78	21	3	11	1	5	-	5	58.97* ± 3.53

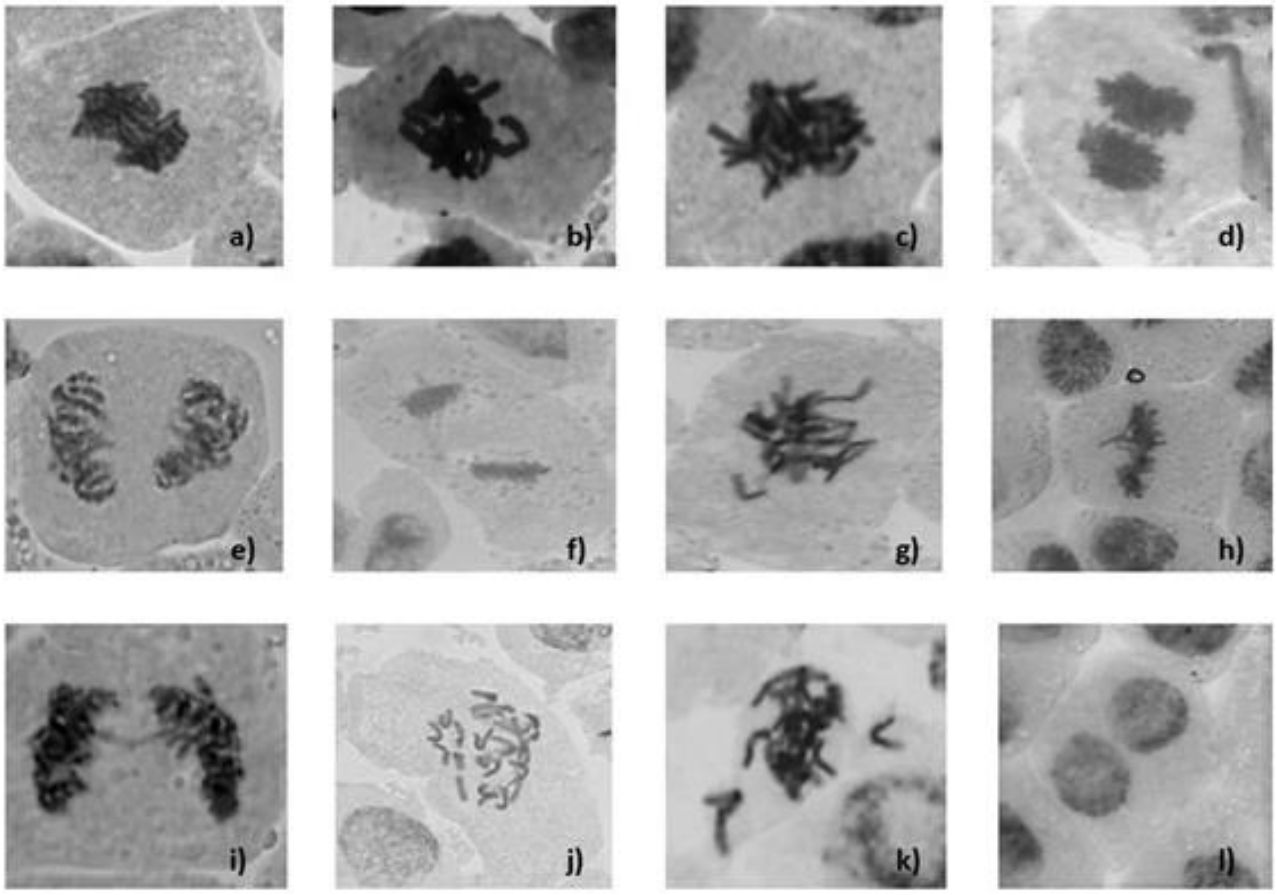

Fig. 1. Some chromosomal abnormalities which were seen in the root meristem cells in bell pepper following captan treatments of different concentrations. a-d) Stickiness; e-f) Multipolarity; g-h) Lagging Chromosomes; i) Bridge; j) C-mitosis; k) Fragmentation; l) Binucleated cell.

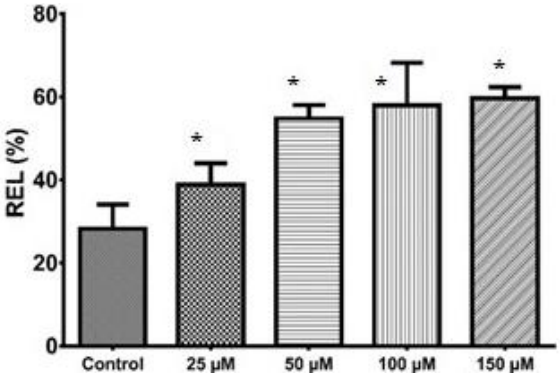

Fig. 2. The effects of different concentrations of captan fungicide on REL (%). Bars represent standard deviations (SD). (*) represent adjusted p values ($p<0.0001$) for statistically significant differences from the control as revealed by one-way ANOVA analysis followed by Tukey Multiple Comparison Test.

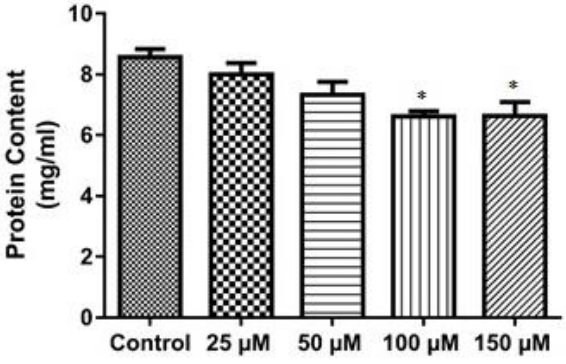

Fig. 3. The effects of different concentrations of captan fungicide on soluble protein content in bell pepper root. Bars represent standard deviations (SD). (*) represent adjusted p values ($p<0.0001$) for statistically significant differences from the control as revealed by one-way ANOVA analysis followed by Tukey Multiple Comparison Test.

Discussion

Random, excessive and unconscious use of pesticides have recently been an important pollutant for the environment (Parween *et al.* 2016). The soil is the first compartment affected by the pesticide toxicity, which in turn leads plant root sytems to be directly or indirectly influenced from pesticide related pollution. Thus, it is important to understand effects of pesticides on root systems in plant development. In the first step, pesticide toxicity causes an inhibition on seed germination and reduction on growth and development. In this study, we showed that the captan fungicide negatively influenced seed germination and root growth in bell pepper. This finding revealed that the fungicide had a toxic effect for bell pepper germination and root growth when used at concentrations from 25 to 150 µM. The reduction in

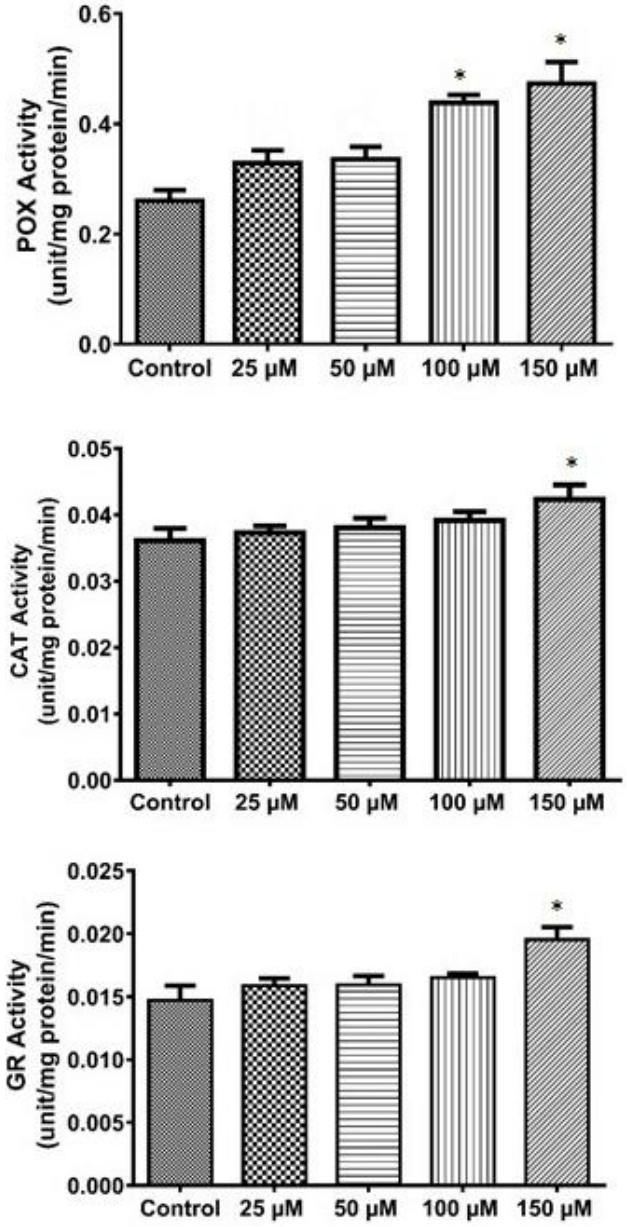

Fig. 4. The effects of different concentrations of captan fungicide on POX, CAT and GR activities in bell pepper root. Bars represent standard deviations (SD). (*) represent adjusted p values for statistically significant differences from the control as determined by one-way analysis of ANOVA followed by Tukey Multiple Comparison Test. POX activity (*): $p < 0.001$, CAT activity (*): $p < 0.01$, GR activity (*): $p < 0.006$.

germination may be explained by inhibition of important enzymes like amylase and protease, inhibition of imbibition of water and inhibition of mobilization of sugar (Gange *et al.* 1992). You & Barker (1997) showed that root fresh weight of tomato plants were decreased by 47% after 6 days of glufosinate herbicide treatment. Karaismailoğlu & İnceer (2017) determined that the insecticide deltamethrin decreased root growth in sunflowers (*Helianthus annuus*). Another study revealed that increasing concentration of tricyclazole and thiabendazole fungicides caused inhibition of germination in the tropical crop plant *Trigonella foenum - graecum* L (Mahapatra *et al.* 2019).

Mitotic index is commonly known as an indicator for determination of cytogenetic damage under stressful conditions. In this study, different concentration (25, 50, 100 and 150 µM) of captan fungicide significantly reduced the mitotic index in the root meristem of bell pepper as compared to their controls (Table 2). Pesticides may cause to inactivation of the cell cycle specific proteins and inhibition of DNA synthesis enzymes such as DNA polymerase and hindering in the G2-phase of the cell cycle, preventing the cell from entering mitosis (Mahapatra *et al.* 2019). Singh *et al.* (2013) determined that mitotic index and induction of chromosomal abnormalities increased under application of different concentration of alphamethrin and endosulfan insecticides in the meristematic cells of *V. faba* roots. Gill & Shaukat (2000) observed that mitotic index reduced by application of 5, 10, 20 and 40 ppm captan fungicide in meristematic cells of *A. cepa*.

Agrochemical toxicity directly leads to genotoxicity in many plant species. In recent studies, *A. cepa* and *V. faba* have commonly been used for indicating the genotoxic effect of pesticide usage. These studies showed a decrease in mitotic index and an increase in chromosomal abnormalities (Bonciu *et al.* 2018). The structural changes in the chromosomes are explained by alteration in the organization of histone and other proteins. These negative effect of protein organization cause changes on structure and adhesiveness of nuclear chromatin (Kurás 2004). Besides, stickiness can be formed by reaction of pesticides with DNA or proteins (Aksoy & Deveci 2012). Our result indicated that the captan fungicide had a harmful effect on chromosome structure by enhancement of sticky and multipolarity in meristematic cells of bell pepper root. The stickiness of chromosomes may cause incomplete separation of sister chromosomes as a result of cross-linkage chromoproteins (Aksoy & Deveci 2012). Gill & Shaukat (2000) indicated that captan fungicide led to chromosomal aberrations including chromosome stickiness, anaphasic bridges, and distribution of prophase in *A. cepa* cells. Aksoy & Deveci (2012) reported that Pomarsol Forte WP 80 fungicide triggered an increase of chromosomal abnormalities in soybean (*Glycine max* L.).

Excessive use of pesticides has triggered the induction of oxidative stress due to the production of reactive oxygen species (ROS) in many plant species (Yüzbaşıoğlu & Dalyan 2019). ROS accumulation damage cell membrane structure via changing the composition of the lipid bilayer and its result in leakage of potassium so electrolyte leakage is associated with membrane damage and potassium efflux in the cells (Demidchik *et al.* 2014). The present study revealed that different concentrations of captan induced oxidative stress by enhanced REL in bell pepper root. Similarly, different concentrations of monocrotophos insecticide causedan increase in electrolyte leakage in *Azolla microphylla* Kaulfuss (Raja *et al.* 2012).

Plants can response to oxidative stress via induction of antioxidant enzyme systems including POX, CAT and GR. These enzymes play a role for scavenging of the member of highly toxic ROS such as H_2O_2 and superoxide radical (Yüzbaşıoğlu *et al.* 2017). Our result showed that CAT, POX and GR were induced in 150 µM/L captan-treated root of bell pepper. In addition, all concentrations of captan enhanced the activity of POX enzyme in bell pepper root. POX enzyme is highly sensitive to pesticide application in plants because it plays a role in response to pesticides by detoxifying the pesticides and eliminating H_2O_2 in plants (Yüzbaşıoğlu & Dalyan 2019). Our previous study determined that thiram fungicide treatment caused oxidative stress. Further, it increased the antioxidant enzyme activities including CAT, POX and GR in tomato seedlings (Yüzbaşıoğlu & Dalyan 2019). Another study has indicated that mancozeb and chlorpyrifos increased CAT and POD activities in *A. cepa* seedling in a time and concentration-dependent manner (Fatma *et al.* 2018).

Conclusion

Despite the fact that use of a wide range of fungicides have risen productivity of crops, fungicides have also harmful effects on plant growth and development. As revealed by our results, the captan fungicide caused genotoxic effects in meristematic cells of bell pepper. Also, its toxicity has affected the membrane permeability and antioxidant enzyme activities in bell pepper root. These findings can contribute to increasing knowledge about the side effects of pesticides and it may help to develop a new perspective to minimize the destructive effects of pesticides in plant growth, development, and yield.

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