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Research Article

INVESTIGATION OF THE CYTOTOXIC, GENOTOXIC AND PHYSIOLOGICAL EFFECTS OF INSECTICIDE KORBAN 25 W WITH THE ALLIUM ASSAY

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ABSTRACT. Cytotoxic and physiological effects of the insecticide Korban 25 W [25% Chloropyrifos-Ethyl (0.0-diethyl 0-(3,5,6 trichloro 2-pyridyl) phosphorothrioate)] were investigated on *Allium cepa* L. The roots of the 5 days old seedlings were treated with 0 g/lt (control=tap water), 2 g/lt, 3 g/lt and 4 g/lt concentrations of Korban 25 W during 5 and 10 days.
Korban 25 W significantly decreased Mitotic Index (MI) and increased the chromosome aberrations (micronucleus, chromosome bridge, sticky metaphase) at all concentrations and treatment periods when compared with their controls. It was also determined that Korban 25 W enhanced the Malondialdehide (MDA) level by increasing the lipid peroxidation and caused changes in Superoxide dismutase (SOD) and Catalase (CAT) activities. All these changes were depending on dose and duration of applied insecticide.

Keywords: *Allium test, antioksidants, genotoxicity, insecticide, lipid peroxidation*

INTRODUCTION

Increasing nutrition requirements are one of the most important problem in the world. In agriculture, one of the first objective is increasing the yield per area. But during production if no precaution is taken aganist diseases and pests, there will be about 65% production losses. The most efficient protection method is using chemicals in these areas [1].

Pesticides are extensively used all over the world to protect agricultural products from diseases, weeds and insects (destroy or remove creatures such as microorganisms, insects, rodents, weeds and fungi). Worldwide, annual average pesticide use is 3 million tons, whereas it is around 33 thousand tons in Turkey, which consist of 47% herbicides, 29% insecticides, 19% fungicides and 5% of other groups [1].

Nowadays, it is known that increasing pesticide usage had genotoxic effects to non-target organisms and cause biochemical changes in metabolism. Besides, residues of pesticides lead to environmental pollution and pose threat to people and animals.

Allium test which is a rapid and sensitive assay to detect cytotoxicity of several genotoxics and mutagenic agents, has many advantages according to the standard protocol for the plant assays established by the International Program on Chemical Safety

(IPCS) and the World Health Organization. Lots of cytological studies have been made with Allium test to determine the harmful effects of various pesticides [2-5].

Insecticide is a type of pesticides that is used aganist the insects. Insecticides, whose active ingredient is chlorpyrifos ethyl, and termed different commercial names are widely used in agricultural areas. There are limited reports on genotoxic and biochemical effects of derivatives of chlorpyrifos ethyl [6] but no reports about effects of Korban 25 W on plants.

In this study, insecticide Korban 25 W (25% Chloropyrifos-Ethyl (0.0-diethyl 0-(3,5,6 trichloro 2-pyridyl) phosphorothrioate)) was used as test chemical. The cytotoxic (mitotic index (MI) in root cells), genotoxic (chromosomal aberrations (CA) in root cells) and physiological (SOD, CAT antioxidant enzymes activity and MDA content in the leaves) effects of the insecticide Korban 25 W were investigated on *Allium cepa* L.

MATERIALS & METHODS

Healthy and equal sized 30 seeds of *Allium cepa* were placed to plastic pans (20x14x6 cm) contained perlite and irrigated with tap water for a period of 5 days. After this period, the seedlings were treated with 0 g/lt (control=tap water), 2 g/lt, 3 g/lt and 4 g/lt concentrations of Korban 25 W solution. The plants were harvested and sampled for analyses at 5th and 10th days of treatment.

In performing cytotoxic and genotoxic analyses, embryonic roots were fixed in acetic alcohol (acetic acid and alcohol, 1:3) for 24 h. Root tips were stored at 70 % ethanol in the refrigerator at 4 °C. The roots were washed with distilled water three times, then hydrolyzed in 1N HCl for 12 min and stained with Feulgen. The squash technique was applied for the study of the mitotic index (MI) and chromosomal aberrations (CA). Minimum 1000 mitotic cells were examined from each slide. Three replicates were performed for each treatment and scoring was made for the three roots of each replicate so at least 3000 cells were scores for each concentration.

Mitotic index (MI) and chromosomal aberration frequency (CF) were determined by using the following equations:

MI = (Number of dividing cells/Total number of cells) × 100

CF = Total number of abnormal cells /total number of dividing cells.

Chromosomal aberrations in each treatment were also recorded and the most frequent chromosome aberrations were presented with micrographs. The aberrations were characterized and classified in to the following categories: sticky chromosomes, anaphase bridge, disturbed chromosomes and micronucleus. Photographs were taken by an Olympus CX41 microscope.

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in 1 g leaf fresh weight according to [7]. The concentration of MDA was calculated from the absorbance at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecific turbitidity) by using extinction coefficient of 155 $mM^{-1}.cm^{-1}$. For the determination of SOD and CAT enzyme activity, leaf samples were frozen in liquid nitrogen and extracted with ice-cold 50 mM phosphate buffer including 0,1 mM Na-EDTA. All enzymes assays were based on the method of Çakmak and Marshner [8,9].

Each experiment was carried out with three replications. Data presented are mean ± standard errors (SE). Variance analysis of the results were tested using SPSS statistical

programme The differences between the treatments compared with least significant differences (LSD) at 5% level.

RESULTS AND DISCUSSION

The increase or decrease in the mitotic index (MI) shows the cytotoxicity of the test compound, thus can be used as a parameter of cytotoxicity in studies of environmental biomonitoring [10].

The effects on MI and the frequency of mitotic phases and chromosome aberrations are given in Table 1 for the treatments with Korban 25W. Exposure to different concentrations (2g/l, 3g/l, 4g/l) of insecticide dose dependently inhibited the mitotic index in the root tip cells of *Allium cepa*. In the present study, at all treatment periods, the highest concentration (4g/l) of Korban 25W decreased mitotic activity more than other concentrations (2g/l and 3g/l). This insecticide can be accepted as toxic agent while it decreased MI in the root tip cells of *A. cepa*. The decrease of mitotic index was time and dose dependent at all concentration periods. When compared to the controls, Korban 25 W caused significantly ($P<0.05$) decrease in MI at 5 and 10 days of all treatments.

Table 1. *Cytogenetic analysis of Allium cepa root tips exposed to different concentrations of Korban 25W for different periods (MI, type and percentage of mitotic phases and chromosome aberrations)*

Time of treatment (days)	Doses (g/l)	Examined total cells	%Prophase	% Metaphase	% Anaphase	% Telophase	Mitotic Index (±SE)	MI % inhibition	% Sticky chromosomes	% Anaphase-bridge	Disturbed	c- metaphase	Micronucleus	% Total CA
5	control	3035	45,54	22,54	15,96	15,96	6,93±0,94	-	-	-	-	-	-	-
	2g/l	3240	30,77	27,69	20,00	21,54	3,67*±0,09	47	9,24	15,13	-	1,68	19,33	45,38
	3g/l	4302	53,06	14,28	7,14	25,52	3,16*±0,24	58	19,18	4,79	1,37	0,68	6,85	32,87
	4g/l	3646	37,97	25,32	13,93	22,78	2,68*±0,12	65	10,32	6,45	-	4,76	15,87	37,4
10	control	3983	34,88	30,82	16,28	18,02	4,13±0,17	-	-	-	-	-	-	-
	2g/l	4003	41,38	10,35	13,79	34,48	3,01*±0,16	27	9,82	16,07	-	1,79	20,54	48,22
	3g/l	3261	48,84	16,28	13,95	20,93	2,36*±0,82	43	15,73	12,36	-	5,62	17,98	51,69
	4g/l	3032	50,00	3,34	30,00	16,66	1,90*±0,17	54	6,45	6,45	4,84	16,13	17,75	51,62

*Means significantly different at the $p<0.05$ level compared with compared with control for MI.

Several other pesticides have been reported to inhibit mitosis [2, 11,12] . Mitotic index is an acceptable measure of cytotoxicity for all living organisms [13,14]. The cytotoxicity level can be determined by the decreased rate of mitotic index [5,15]. Panda and Sahu

[16] state that, if decrease of mitotic index below 50% usually has lethal effects. According to Antonsie-Wiez [17], the decrease of mitotic index more than 22% of control, that it causes sub lethal effects on test organism. Also in our study, MI inhibition is 27% to 65%. Korban 25 W has lethal effect on test organism (*Allium cepa*).

Korban 25W increased chromosome aberrations at all concentrations and treatment periods in mitotic division of *A. cepa* roots when compared with the control. In the present study, stickiness, anaphase- bridge, disturbed metaphase, c-mitosis and micronucleus formation were the most frequently observed chromosomal aberrations, otherwise CA's were not observed in the control. Chromosome aberrations due to inhibition of spindle formation such as c-mitosis, stickiness reflects highly toxic effects of mutagen [10,18-21].

The presence of sticky chromosomes (Fig. 1a) is higher at 3 g/l at 5 and 10 days than the other consentrations and control (Table 1). Türkoğlu (2009) mentioned that sticky chromosomes might have resulted from increased chromosome contraction and condensation or possibility from depolymerization of DNA[21]. Chromosome stickiness reflects highly toxic effects of mutagen, usually of an irreversible type probably leading to death (10,21).

C-mitosis (Fig. 1b) was observed as 16,13% higher than the control at 10 days period (Table 1). C-mitosis was first reported by Levan (1938) in root meristems of *Allium cepa* L. as disruption of the spindle fibers leading to the random scattering of the condensed chromosomes[22]. Mann (1977) stated that C-mitosis is one of the consequences of inactivation of spindle apparatus connected with delay in the division of centromere[23].

The induction of micronucleus formation (Fig. 1c) was observed in all treatments (Table 1). The induction of MN is commonly used to detect genetic damages derived from exposure to mutagenic chemicals. The presence of MN detect genetic damages derived from exposure to mutagenic chemicals. Micronucleus can be a formed as a result of acentric fragments or entire chromosomes not incorporated to the main nucleus during the cell cycle.

Anaphase bridges (Fig. 1d) were observed at 2 g/l at both concentrations of Korban 25 W (Table 1). Grover et al. (1999) suggested that chromosome bridges result from chromosome or chromatid breakage and fusion, whereas laggard chromosomes increase the risk for aneuploidy [24]. According to Gömürgen (2000), chromosome bridges may have occurred due to the chromosomal stickiness and subsequent failure of free anaphase separation or may be attributed to an unequal translocation or inversion of chromosome segment [2].

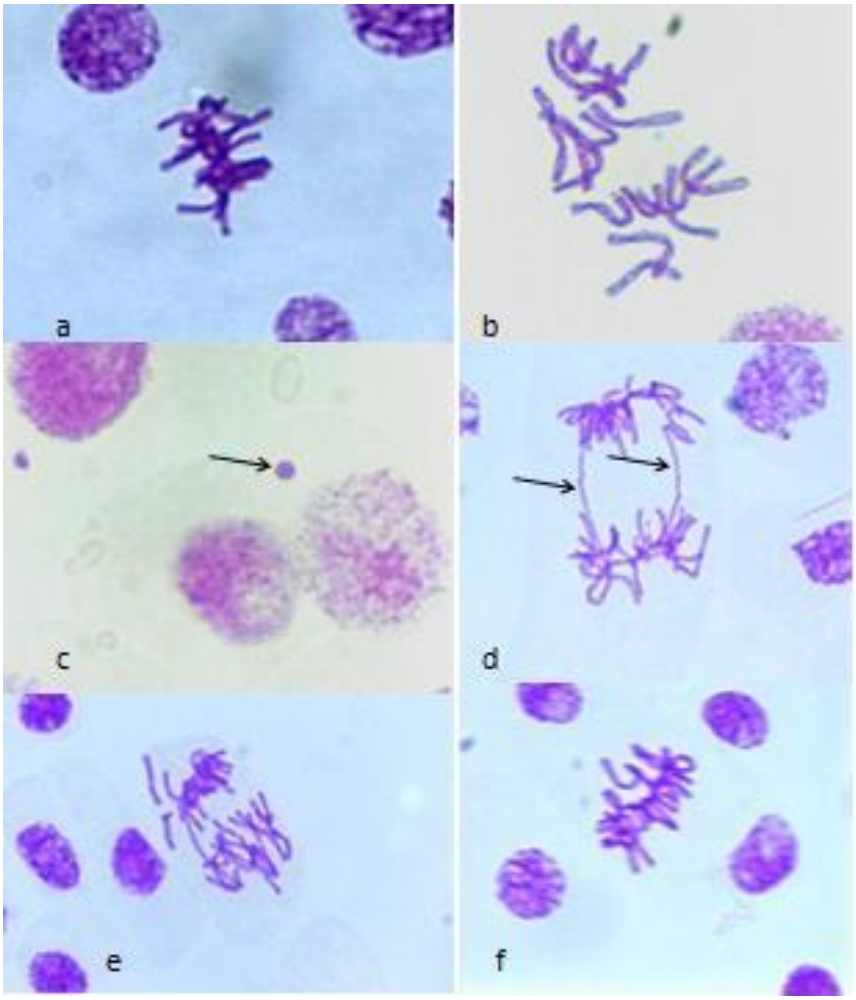

***Figure 1**. a. Sticky chromosomes, b. C-mitosis, c. Micronucleus, d. Anaphase bridge, e. Disturbed anaphase, f. Disturbed metaphase*

Disturbed chromosomes (Fig. 1e and f) were observed at 3 g/l and 4 g/l at 5 and 10 days treatments respectively (Table 1).

Many investigators accepts that, chromosome aberrations due to inhibition of spindle formation such as c-mitosis, stickiness reflects highly toxic effects of mutagen [10,18-21].

Cytological observations indicate that all the tested concentrations of Korban 25 W insecticide cause chromosome abnormalities mostly during metaphase, anaphase and telophase stages. The result of this study reveals that Korban 25 W insecticide decreased the mitotic index. Furthermore, different treatments of Korban 25 W insecticides caused chromosomes abnormalities such as stickiness, disturbance, chromosome bridges on anaphase stage. Decrease in the mitotic index and increase in the chromosomal aberration frequency indicates that they had a cytotoxic effect on cells division; chromosomes abnormalities point out that insectcide has a clastogenic property that leads to genotoxic effects.

On the other hand, it is known that the environmental stresses including chemical toxicity lead to enhanced formation of reactive oxygen species (ROS) such as superoxide,

hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen (O_2^-) in plants due to impairment of cellular homeostasis which is called "oxidative stress"[25,26]. High concentrations of ROS are extremely harmful to organism by causing peroxidation of lipids, oxidation of proteins, damage to cellular components, nucleic acids, inhibition of enzymes, disrupts the physiological and biochemical life process [27,28].

Lipid peroxidation is often measured as Malondialdehyde (MDA) which is an oxidized product of membrane lipids, and commonly considered as a general indicator of lipid peroxidation as well as stress level [7,26,29-32].

The effects of Korban 25 W on MDA level in *A. cepa* leaves after 5 and 10 days of treatment are shown in Fig. 2a. When compared to the controls, Korban 25 W caused significantly ($P<0.05$) increase in MDA content at 5 and 10 days of all treatments. In the leaves treated with 2, 3 and 4 g/l Korban 25 W, at 5 days level of MDA was about 2.3, 6.2 and 8.1 times; at 10 days 2.2, 2.55 and 2.51 times higher than their control groups, respectively. At 5 days both of increasing of MDA content to control and between the treatments is significant ($P<0.05$). Althought, increasing in MDA content to the control was significant ($P<0.05$) differences between treatments were insignificant at 10 days. Maximum increase in MDA content was observed on 5th days at 4 g/l Korban 25 W exposure.

These observations are also in agreement with results reported by previous authors on the generation of lipid peroxidation products in plant tissues under various pesticides stresses such as glyphosate, paraquat, cypermethrin, thiamethoxam and Dursban 4 [6, 33-36]. These researchers reported a significantly increase in the MDA content in the root and leaves treated with different doses of these pesticides.

Chemical (such as pesticide)-induced oxidative stress has been observed in various researches [6, 34-36]. The presence of ROS in the cell environment can cause continuous oxidative damage to cell structure and function. Scavenging or detoxification of excess ROS is achieved by an efficient antioxidative system such as non enzymatic antioxidants and enzymatic antioxidants to mitigate the effects of oxidative stress [28,37]. Among the antioxidant enzymes SOD which is called the cell's first line of defense against ROS decompose O_2^- to H_2O_2 which later is scavenged by CAT and APOX enzymes and decomposed to H_2O and O_2^- [38,39].

In this study, the effects of different concentrations of Korban 25 W on the activity of the enzymatic antioxidant system (SOD, CAT) in the leaves were determined (Figs. 2b and c). Effects of Korban 25 W on SOD activities of *Allium cepa* leaves varied with the different concentrations of Korban 25 W and the duration of treatment. The SOD activities were generally higher than the control in the different concentrations of Korban 25 W, except in significant decrease on 5 th day at 3 g/l tratment. Maximum increase in SOD activity was observed on 5 th day at 4 g/l concentration. In the leaves treated with 2, 3 and 4 g/l Korban 25 W, the level of SOD was about 199, 8.9, 164 % higher than the control group at 10 days, respectively (Fig. 2b). Compared with controls, increasing SOD activities were significant ($P<0.05$) at 5th days only 4 g/l, at 10th days 2 g/l and 4 g/l Korban 25 W treatment.

CAT (Catalase is one of the H_2O_2-capturing enzymes) activities increased in leaves with increasing concentrations of Korban 25 W. CAT activities are significantly higher than the control (Fig. 2c). CAT activity showed a significant increase ($P<0.05$) at 5 days of treatments except 3g/l Korban 25 W. Maximum increase in CAT activity was observed on 5th days at 4 g/l Korban 25 W exposure. In the leaves treated with 2, 3 and 4 g/l Korban 25 W, the level of CAT was about 105, 80.9 and 118.9 % higher than the control group

at 5 days. At 10 days only 4 g/l Korban 25 W treatment significantly increased CAT activity when compared to the control.

It is widely accepted that pesticide toxicity represents an oxidative stress in plants by inducing formation of ROS [27,28]. In this study enhanced SOD activities may indicate oxidative stress. Similar results were reported that SOD initially increased as a result of the formation of superoxide radical. Increased SOD activity produced H_2O_2. Also H_2O_2 is eliminated by CAT decomposing it down directly to water and oxygen under the other pesticide treatments [6,34,35].

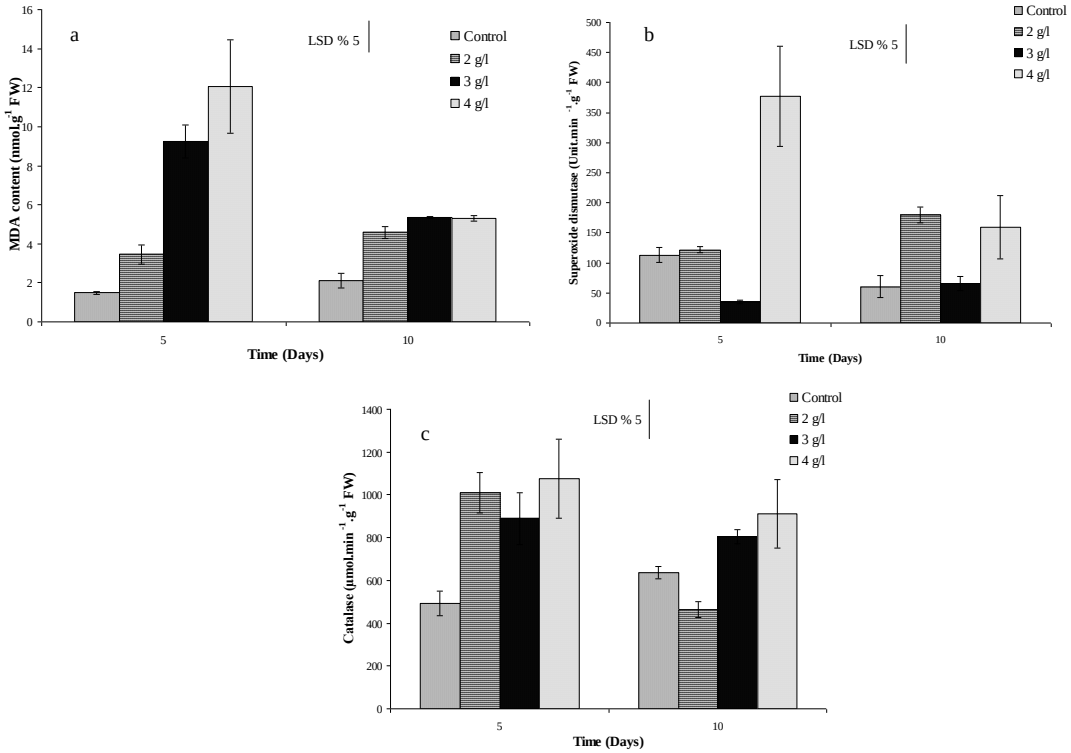

Fig. 2. *The effects of Korban 25W on MDA content (a), SOD actvity (b) and CAT actvity (c) of A. cepa leaves. Values were the means of three replicates and vertical bars represent the standart errors.*

In our study Korban 25 W treatment increased the activities of antioxidative enzymes and the MDA contents in all treatments. Similarly, Özen et al. (2011) have observed that paraquat induce lipid peroxidation and increase the activity of antioxidant enzymes such as superoxide dismutase and catalase [34].

All tested consentrations of Korban 25 W insecticide cause chromosome abnormalities such as stickiness, disturbance, chromosome bridges on anaphase stage, decreased the mitotic index. Chromosome abnormalities indicate that insectcide has a clastogenic feature that leads to genotoxic effects, while increase in the chromosomal aberration frequency shows that they had a cytotoxic effect on division of cells. Besides physiological results showed that Korban 25 W cause increases in MDA contents, SOD ve CAT activities.

Consequently, the results of this study show that antioxidant defence system works however could not prevent toxic effects of used concentrations of Korban 25 W. In summary, these results indicated that Korban 25 W should be regarded as a cytotoxic

agent for plants. Hence, the use of this insecticide should be under control at these concentrations.

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