

Genotoxic Effects of Dursban 4 in *Allium cepa*

Allium cepa'da Dursban 4'ün Genotoksik Etkileri

Research Article

Serap Topcu, Fadime Yılmaz, Aysun Ergene, Sema Tan*

Kırıkkale University, Department of Biology, Kırıkkale, Turkey

ABSTRACT

Genotoxic effects of Dursban 4 (Chlorpyrifos-Ethyl), which is used commonly in agricultural areas were evaluated in the root meristem cells of *Allium cepa*. The roots of the plants were treated with 600 ppm, 1200 ppm and 1800 ppm concentrations of Dursban 4. Root tips after having grown to a certain length were stained according to aceto-orcein squash procedure. Metabolic variations in response to Dursban 4 toxicity was measured using physiological parameters and antioxidant enzymatic activities. Chromosomal aberrations, mitosis abnormalities, mitotic index and micronucleus assay of applied pesticides on *Allium cepa* roots were determined.

Key Words

Allium cepa, Dursban 4, Genotoxicity, Chromosomal aberrations

ÖZET

Genellikle tarımsal alanlarda kullanılmakta olan Dursban 4'ün genotoksik etkileri, *Allium cepa*'nın kök meristem hücrelerinde görüldü. Bitkilerin kökleri 600 ppm, 1200 ppm ve 1800 ppm derişimlerde Dursban 4 ile muamele edildi. Kök uçları belli bir boya ulaştıktan sonra aceto-orcein yöntemine göre boyandı. Dursban 4 toksisitesine cevaptaki metabolik deęişimler fizyolojik parametreler ve antioksidan enzimatik aktiviteler kullanılarak ölçüldü. Pestisitlerle muamele edilen *Allium cepa* köklerinde kromozomal hatalar, mitoz anomalileri, mitotik indeks ve mikronükleus saptandı.

Anahtar Kelimeler

Allium cepa, Dursban 4, Genotoksisite, Kromozomal anomaliler

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Correspondence to: Sema Tan, Department of Biology, Yahşihan, 71450 Kırıkkale, Turkey

Tel: +90 0318 357 42 42 / 4018

Fax: +90 0 318 357 24 61

E-Mail: sematan2003@yahoo.com

INTRODUCTION

Plants constitute the basic food source of rapidly increasing world population. However, the amount of agricultural land used to feed the growing world population is decreasing by the day. 100,000 species of plant pathogens cause disease and more than 10,000 insect species reduce the amount and quality of the product obtained per unit area by entering the competition with plants. Different pesticides are used to prevent this kind of losses [1-4]. Pesticides, used to destroy or away such as microorganism, insects, rodents, weeds and fungi damaged agricultural products during production, consumption and storage of nutrients, caused product lost and used to regulate plant growth are chemical products [5,6]. Cytogenetic studies have shown that many insecticides affect cell division and induce mitotic and chromosomal abnormalities in crop plants. Mitotic index (MI) is considered a parameter that allows one to estimate the frequency of cellular division. All the experiments were carried out when the roots reached 2-2.5 cm in length. At this time the roots are in dynamic balance while the number of cells in the division phase being equal to the number of cells in the differentiation phase. The aim of this study is to investigate the effects of Dursban 4 on chromosome aberration and DNA damage induction in root cells and activity of the enzymatic antioxidant system (SOD, CAT, GSH-Px), carotenoid, chlorophyll a and b level in the leaves of *Allium cepa*.

MATERIALS and METHODS

Plant material and chemicals

Equal-sized bulbs (25-30 mm in diameter) of a commercial variety of *Allium cepa* (L. familia: *Liliaceae*, $2n = 16$) were used as the test plant. The Dursban 4 ($C_9H_{11}Cl_3NO_3PS$; CAS No. 39475-55-3) was obtained from Bayer Co.

Treatment solution and root preparation

We carried out tests with the Dursban 4 insecticide in several concentrations. The higher concentration of Dursban 4 (degree of purity 480 g/L) was obtained diluting the commercial product in tap water, according to the recommendation to use in the agriculture. Taking into consideration the amount of the active compound contained in the

commercial product, concentrations of 600 ppm; 1200 ppm; and 1800 ppm of Dursban 4 was tested.

Preparation of material

Allium cepa were used as plant material in this study. First the onion bulbs are weighed. Dursban 4 produced by Bayer Company was added at appropriate concentrations to the cultivation media of *Allium cepa*. For germination onion bulbs in the control group is prepared in tap water, onion bulbs in the application group is prepared in tap water by using 600, 1200 and 1800 ppm pesticide solutions for a week at room temperature.

The yellow shallows and the root primordia of the bulbs were removed before the onions were placed in the test solutions. Bulbs of *Allium cepa* were placed in test cup (size 25 mm) with their basal ends dipped in test solutions and rooted at room temperature for a week. The roots from each bundle were cut off on the seventh day and the length of each root was measured to the nearest mm in all groups. Experiments were carried out in triplicate.

Mitotic Index, Micronucleus Assay and Chromosomal Aberrations

Root tips cut from meristematic region 1.5 to 2 mm and fixed with Clarke Agent 3: 1 (glacial acetic acid/distilled water). Samples were washed in 96% ethanol; root tips were stored with 70% ethanol at +4°C until use. Then the root tips were hydrolyzed with 1 N HCl at 60°C. After hydrolysis root tips were washed with 45% acetic acid at 25°C. Acid treated root tips were stained with aseto-orsein in the dark for a night. The root tips washed with 45% acetic acid to remove excess stain. The squash technique was applied for the study of the mitotic index (MI) and chromosomal aberrations minimum 5000 mitotic cells were counted from each of the slides. The MI was calculated for each treatment as a number of dividing cells/100 cells. It was taken photographs of control group and chromosomal aberrations prepared with Crushing-smear Method with light microscope.

For micronucleus (MN) analysis, 1000 cells were scored for each slide. For the score of MN the following criteria were adopted from Fenech et al. [7]. These: (i) the diameter of MN should be tenth of the main nucleus, (ii) MN should be separated from

or marginally overlap with main nucleus as long as there is clear identification of the nuclear boundary, (iii) MN should have similar staining as the main nucleus.

Enzyme extraction and protein determination

To obtain the enzyme extract, 500 mg of fresh leaves or roots were homogenized in 2.5 mL of cold potassium phosphate buffer (50 mM, pH 7.6). The homogenate was centrifuged for 15 min at 15,000 rpm at 4°C and supernatant was used analyzing SOD, CAT and GSH-Px. Protein content was determined according to Biuret Method [8] using bovine serum albumin for calibration.

SOD (EC 1.15.1.1) activity was measured using the photochemical method described by Deschamps and Fridovich [9] with some modifications in the supernatant. In the presence of SOD, the photochemical reduction of NBT (n-nitrobluetetrazolium) is inhibited and the level of inhibition is used to quantify the enzyme activity. One unit of SOD activity is defined as the amount of enzyme required to achieve a 50% inhibition of the rate of NBT reduction measured at 560 nm.

CAT (EC 1.11.1.6) activity was measured by spectrophotometer by measuring the consumption of H₂O₂ at 310 nm, according to Beer and Sizer [10], in 1.9 mL potassium phosphate buffer (50 mM, pH 6.5) containing 0.5 mL H₂O₂ and 1.5 mL enzyme extract in a final volume of 3.9 mL. The reaction was started by adding H₂O₂.

GSH-Px (EC 1.11.1.9) activity the reaction mixture, consisted of 2.85 mL 50 mM potassium phosphate buffer and 2-methoxy phenol (1:1) (pH 7.2), 50 µL H₂O₂ (0.003%) and 100 µL enzyme extract. Enzyme activity was measured by monitoring the increase in absorbance at 470 nm [11].

Photosynthetic Pigments Content

For the determination of leaf pigments, an experiment was carried out 5 days with the Dursban 4 concentrations of 600, 1200 and 1800 ppm. Chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids were estimated according to Wellburn [12]. About 50 mg of leaves from control and all Dursban 4 treated (for consecutive 72 h) groups were cut into segments and kept in 10 mL

frozen methanol in a test tube. Test tubes were immediately wrapped with aluminum foil to avoid degradation of pigments by light. The contents of Chl a, Chl b, and carotenoids were measured using a spectrophotometer at 666, 653 and 470 nm, respectively.

Statistics

Analysis of variance of the data was performed by using SPSS 10.0 for Windows software. Statistically, significant differences between the groups were compared using one-way analysis of variance and Duncan's multiple range tests.

RESULTS and DISCUSSION

Mitotic Index, Micronucleus Assay and Chromosomal Aberrations

The cytotoxicity level of a test compound can be determined based on the increase or decrease in the mitotic index (MI), which can be used as a parameter of cytotoxicity in studies of environmental biomonitoring [13]. Micronucleus (MN) has been considered by many authors as the most effective and simplest endpoint to analyze the mutagenic effect promoted by chemicals. This is due to the fact that MN results from damages, not or wrongly repaired, in the parental cells, being easily observed in daughter cells as a similar structure to the main nucleus, but in a reduced size [14].

Based on the analysis carried out in roots from *Allium cepa*, it was observed, after treatment with distinct concentrations of the Dursban 4, inhibition of mitotic index and the presence of the several cells with micronuclei, chromosomal aberrations, such as unequal separation of chromosomes and chromosomal losses.

The results obtained, after analysis of the mitotic index in the roots treated with the Dursban 4 for 7 day, revealed a significant decrease ($p < 0.05$) of cell division index at concentrations of 600, 1200 and 1800 ppm (Table 1).

Table 2 shows induction of chromosome aberrations in the root meristem cells of *Allium cepa* exposed to Dursban 4. *Allium cepa* showed concentration-related increase in the frequencies of chromosome aberrations, however, significant

Table 1. Effects of Dursban 4 on the MI and MN level in the root meristem cells of *Allium cepa*.

Compounds	Concentrations (ppm)	MI (%)	MN Frequency
Control	-	49	1.45 ± 0.01
Dursban 4	600	37	15.2 ± 0.01
	1200	31	22.4 ± 0.03
	1800	29	25.8 ± 0.001

Table 2. Chromosome aberrations obtained for the root meristem cells of *Allium cepa* treated with different concentrations of Dursban 4.

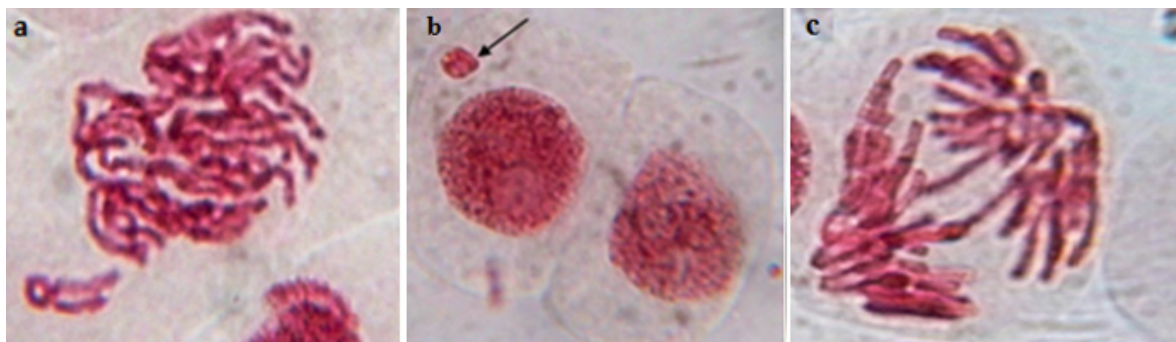
Compounds	Concentrations (ppm)	Aberration (%)			
		Bridge	Stickiness	Fragment	Disturbed chromosome
Control	-	0.30 ± 0.48	1.60 ± 0.93	2.50 ± 0.88	3.20 ± 1.10
Dursban 4	600	3.30 ± 1.03	3.30 ± 1.03	4.50 ± 0.82	5.50 ± 0.88
	1200	3.70 ± 0.68	3.70 ± 0.68	4.80 ± 1.03	6.10 ± 1.22
	1800	3.80 ± 1.65	3.80 ± 1.65	5.10 ± 1.23	6.40 ± 1.07

* Data obtained from 5000 cells and expressed as mean ± S.E. from the three replicates.

percentage of aberrations was observed at 1800 ppm in *Allium cepa*. Chromosome breaks and fragments were observed to be frequent aberrations in *Allium cepa*. Significant frequencies of chromosome aberrations were still observed in the root meristems exposed to the highest concentrations (1800 ppm) of Dursban 4.

Both pesticides induced similar type of aberrations such as chromosome breaks, stickiness, multipolar anaphase, chromosomal bridges, lag gards, unequal separation of chromosomes and micronucleated cells (Figure 1).

Chromosomal aberrations in cells which are caused to the cytotoxic effects of pesticides on plants can be considered as an indicator of genetic damage [15,16]. For this reason, *Allium cepa* test are often preferred in toxicity measurements. Investigation of plant root tip cells is rapid and sensitive method for determination of environmental impacts. Because root tips are the first structures exposed to chemicals in the soil and water involved in. In this study, the cytotoxic effect of Dursban 4 pesticide on *Allium cepa* root cells was investigated. It was seen that micronucleus formations, mitotic abnormalities and chromosome damages increased

**Figure 1.** *Allium cepa* meristematic cells exposed to Dursban 4 carrying MN and CAs. (a) Vagrant chromosome (1200 ppm Dursban 4), (b) MN of small size (1800 ppm Dursban 4), (c) Double-bridge formation (1800 ppm Dursban 4)

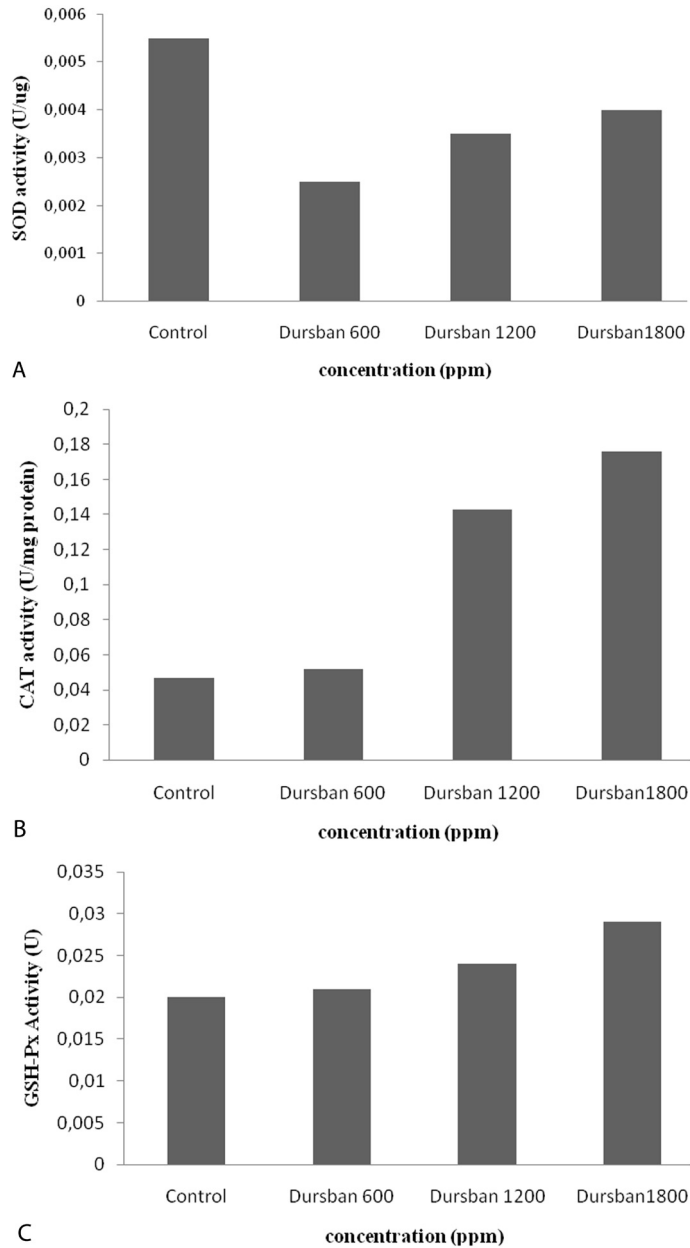


Figure 2. Activities of SOD, CAT and GSH-Px in the leaves of *Allium cepa*.

All values are means of three independent experiments (three replications each) means followed by the same letter to that of the control were not significantly different at $p \leq 0.05$.

but the rate of mitotic index decreased according to the control group in *Allium cepa* root tip cells germinated with different Dursban concentrations.

Antioxidant Enzymes

With higher Dursban 4 concentrations, SOD activity in the *Allium cepa* leaves increased [Figure 2 (a)]. Maximum increase in SOD was observed on 8th day at 1800 ppm Dursban 4 exposure. The CAT and

Table 3. The contents of leaf pigments at different concentrations of Dursban 4

C (ppm)	Chl a	Chl b	Carotenoids
Control	0,048 ± 0,01	0,016 ± 0,01	0,050 ± 0,29
Dursban 4			
600	0,023 ± 0,002	0,006 ± 0,04	0,025 ± 0,06
1200	0,018 ± 0,003	0,005 ± 0,02	0,022 ± 0,05
1800	0,016 ± 0,002	0,004 ± 0,03	0,021 ± 0,05

GSH-Px activities also increased with increasing Dursban 4 concentration [Figure 2 (b), (c)].

Photosynthetic Pigments

The leaf pigments of *Allium cepa* were significantly affected by Dursban 4 treatment, which represented in Table 3. From the table, with increased Dursban 4 concentrations, the reduction of chlorophyll a and b contents of leaves could be detected. Under Dursban 4 stress, the chlorophyll b content of *Allium cepa* was more affected than the chlorophyll a content. Total chlorophyll a content of *Allium cepa* decreased approximately by 90% at the highest Dursban 4 concentration, while for chlorophyll b, the decrease was around 60%. The carotenoid contents of *Allium cepa* decreased with Dursban 4 treatment, but the reduction rate of carotenoids was slower than chlorophyll contents.

DISCUSSION

MN can be a result of acentric fragments or entire chromosomes not incorporated to the main nucleus during the cell cycle. According to Ma et al. [17], among all the assessable endpoints, the MN are the most effective and simplest indicator of cytological damages, which makes the analysis of this parameter more efficient to evaluate environmental contamination. The results from the present study indicate that the Dursban 4 treatment presents mutagenic activity. This effect can be related to its ability to promote alterations in the root cells from *Allium cepa*, such as cells bearing chromosomal losses, polyploid metaphases, irregular nuclei, nuclear buds, micronuclei and mini cells. Significant increase in the frequencies of chromosome breaks and fragments as observed in our study. Our results are in agreement with the earlier studies reported similar aberrations in *Allium cepa* root meristem cells [18,19]. *Allium cepa* cells treated with Dursban 4 carrying MN with a small and large size together indicates Dursban 4 have clastogenic and aneugenic effects. And also MN frequency was increased with Dursban 4 increasing concentrations and the maximum MN frequency was observed at 1800 ppm. There is no published data available on the Dursban 4 effects on the formation of MN in *Allium cepa*. The Dursban 4 (Chlorpyrifos ethyl) group can be attached to tubulin molecules and prevent its polymerization and, consequently, the

formation of microtubules. Amongst microtubules, the fuses seem to be the most sensitive structure to Dursban 4 action, due to their constant reorganization during cell division events [20]. Constraints in the microtubule polymerization might be closely related to cell abnormalities such as polyploid metaphases, polyploid cells and chromosomal losses. Polyploid metaphases and polyploid cells can occur due to the cytokinesis process disruption, which can promote difficulties in the fragmoplast formation [21]. Oxidative damage can be provided by antioxidant enzymes such as SOD and CAT. The effects of Dursban 4 on the activity of the enzymatic antioxidant system (SOD, GSH-Px, CAT) in the leaves were studied. The SOD activities were generally higher than the control in the different concentrations of Dursban 4. Same result reported that SOD initially increased as a result of the formation of superoxide radical under stress conditions (Fatima and Ahmad [22] CAT and GSH-Px are H₂O₂-capturing enzymes. Both CAT and GSH-Px activities increased in leaves with increasing concentrations of Dursban 4. GSH-Px and CAT activities are significantly higher than the control. In our study, comparison of the three enzyme activities showed that the lower response of SOD activity to Dursban 4 in *Allium cepa* might be compensated by the increased activities of GSH-Px and CAT, showing that the three enzymes were functioning concurrently to remove H₂O₂ in the different parts of the plant. In pigment studies the chlorophyll a and b pigments of the *Allium cepa* leaves decreased significantly with increasing Dursban 4 concentrations. Similar studies shown chlorophyll content decreased in response to increasing stress conditions [23]. The carotenoid contents decreased with Dursban 4 treatments. This reduction in carotenoid content showed that Dursban 4 not only induce the degradation of pigments but also reduced the biosynthesis mechanism.

The *Allium cepa* test is a fast and sensitive assay to detect environmental genotoxics and mutagens. The results showed toxic effect on the MI and the chlorophyll in the cells of *Allium cepa*. The SPSS demonstrated a positive correlation of time and concentration, the effect of the latter being more drastic. The present results, together with earlier reports on the cytogenetic effects of pesticides,

weigh against the wide application of these chemicals in weed control and point out the need for mutagenicity testing of agricultural chemicals before they are released for application.

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