Genotoxic effects of herbicide Illoxan (Diclofop-Methyl) on Allium cepa L.

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Abstract: The genotoxic potential of the commercial herbicide Illoxan (containing 284 g/L diclofop-methyl) was determined by using chromosome aberrations in Allium cepa root tip cells. The EC50 value was determined as 150.00 mg/L using a root growth inhibition test and then the roots were treated with 37.50, 75.00, and 150.00 mg/L concentrations for 12, 24, and 48 h. The results indicated that Illoxan significantly increased the abnormal cell frequency at all concentrations and treatment periods when compared with their controls, and this increase was dose-dependent for the 24 and 48 h treatments. On the other hand, Illoxan significantly decreased the mitotic index (MI) in all treatments when compared with their controls. The decrease in the mitotic index was slightly dose-dependent for the 24 and 48 h treatments. Illoxan did not affect the percentage of mitotic stages. For the pretreated root tips, Illoxan (except 37.50 mg/L at 12 and 24 h) significantly increased the frequency of abnormal cells in a dose-dependent manner. This study indicates that Illoxan decreased the mitotic index and produced clastogenic and aneugenic types of abnormalities in Allium cepa root tip cells. The data obtained in this study showed that plant bioassays can be used as an important test battery to detect possible genotoxicity of chemicals.

Key words: Pesticide, herbicide, Illoxan, diclofop-methyl, genotoxic effects, Allium cepa

Illoksan herbisitinin Allium cepa L’da genotoksik etkileri

Özet: Bu çalışmada, illoksan herbisitinin ticari formunun (284 g/L diclofop-methyl içermektedir) genotoksik potansiyeli, Allium cepa kök ucu hücrelerinde kromozomal anormallikler kullanarak belirlenmiştir. EC50 değer, büyüme inhibisyon testi kullanarak 150,00 mg/L olarak belirlenmiş ve daha sonra kökler 37,50, 75,00 ve 150,00 mg/L konsantrasyonlar ile 12, 24 ve 48 saat muamele edilmiştir. Sonuçlar, illoksanın tüm muamele süreleri ve konsantrasyonlarda anormal hücre frekansını kontrole göre önemli düzeyde artırığı göstermektedir, bu artış 24 ve 48 saatlik uygulamalarda doza bağlıdır. Diğer taraftan illoksan mitotik indeksi (MI) tüm uygulamalarda kontrole göre anlamlı oranda düşüş göstermiştir. Mitotik indeks depremi 24 ve 48 saatlik uygulamalarda düşüş oranda doza bağlıdır. Illoksan mitotik safhaların frekansını etkilememiştir. Ön muameleli kök uçlarında, illoksan anormal hücre frekansını (12 ve 24 saatlik uygulamalarda 37,50 mg/L hariç) doza bağlı olarak önemli oranda artırılmıştır. Bu çalışmada illoksanın Allium cepa kök ucu hücrelerinde mitotik indeksi düşüşü, klastojenik ve anöjenik tip anormalliklere neden olduğu belirlenmiştir. Bu çalışmanın verileri, kimyasal maddelerin muhtemel genetiksik etkilerinin belirlenmesinde bitki deneme sistemlerinin önemli bir deneme sistemi olarak kullanılabileceğini göstermektedir.

Anahtar sözcükler: Pestisit, herbisit, illoksan, diclofop-methyl, genetoksik etki, Allium cepa
Introduction

Due to their widespread use in agriculture, pesticides are some of the compounds most frequently released into the environment. Despite the beneficial effects associated with the use of pesticides, many of these chemicals may pose potential hazards to humans and to nature. The genotoxic effects of some pesticides have been evaluated by several researchers through several test systems (1-5). Plant bioassays, which are considerably more sensitive and simple in comparison with animal bioassays, have been validated in international collaborative studies under the United Nations Environment Program (UNEP), World Health Organization (WHO), and US Environmental Protection Agency (US-EPA), and proven to be efficient tests for the genotoxicity monitoring of environmental pollutants (6-8).

In this study, an Allium test system was used in the evaluation of genotoxic activity of the herbicide Illoxan with the active ingredient diclofop-methyl. Diclofop-methyl is a selective systemic herbicide, absorbed primarily through leaves, with some absorption through roots in moist soil. Diclofop-methyl undergoes rapid transformation, which is translocated within the plant. This herbicide is a fatty acid synthesis inhibitor, and inhibits acetyl CoA carboxylase (ACCase). It destroys the cell membrane, prevents the translocation of assimilates to the roots, reduces the chlorophyll content, and inhibits photosynthesis and meristem activity. It is registered for use on the post-emergence control of wild oats, wild millets, and other annual grass weeds in wheat, barley, rye, and broad-leaved crops such as soybeans, sugar beet, legumes, sunflowers, clover, brassicas, carrots, lettuce, spinach, potatoes, cucumbers, peas, beans, tomatoes, alliums, and herbs (9). Diclofop-methyl has been classified as “likely to be carcinogenic to humans” by the laboratory studies based on the rats and mice (10).

Diclofop-methyl tested negative for genotoxic effects in a bacterial reverse mutation test in *Salmonella typhimurium*, an in vitro mammalian cell gene mutation test with Chinese hamster V79 cells, an in vitro mammalian chromosomal aberration test in primary human lymphocytes, an in vivo cytogenetic test in bone marrow cells of the Chinese hamster, unscheduled DNA synthesis in primary rat hepatocytes and in A549 human lung carcinoma in

vitro, a dominant lethal test in male NMRI mice, a mouse bone marrow micronucleus test, and mutagenic activity with *Saccharomyces cerevisiae* (11). Although there are studies showing the negative effects of diclofop-methyl, there has been no study on the effects of this pesticide in plant test systems used for scoring the effects of chemicals.

The present investigation was conducted using chromosome aberrations, detecting clastogenic activity qualitatively and quantitatively, in an *Allium cepa* test system in order to assess the genotoxic potential of the commercial herbicide Illoxan (containing 284 g/L of diclofop-methyl). We have chosen *Allium cepa* as a test organism and a commercial formulation as a test compound for the following reasons: 1) Some pesticides have negative effects on some organisms as indicated above while showing positive effects in other organisms (12). 2) Plant and animal assays are differentially responsive to pesticides. Unlike animals, some chemicals, including pesticides, are metabolically activated by plant peroxidases and may give different responses than those of mammalian cytochromes P-450 (5). 3) Many studies report the importance of evaluating the potential toxicities of complete formulations, rather than just evaluating the toxicities of the active components, because it is the commercial formulation that has its application in commercial agriculture (5). 4) There has been no study, to our knowledge, on the genetic effects of Illoxan or diclofop-methyl in plant test systems.

Materials and methods

Illoxan belongs to the diphenylether/chlorophenoxy group of herbicides. Its chemical abstract name is methyl 2-[(4-(2,4-dichlorophenoxy) phenoxy) propanoate or 2-[(4-(2,4-dichlorophenoxy) phenoxy) methyl – propanoate. Its molecular weight is 341.2. Its chemical formula is C_{16}H_{14}Cl_{2}O_{4}.

![Figure 1. The structural formula of Illoxan.](image-url)
Approximately equal-sized onion bulbs (*Allium cepa* L., 2n = 16) were used as the test material. The outer scales of the bulbs were carefully removed and the dry plates were scraped away without destroying the root primordia. The growth inhibition test was carried out as described by Fiskesjö (13), with minor modifications. Five onion bulbs were set up in 10, 25, 50, 75, 100, 125, 150, 200, 250, and 300 mg/L concentrations. A set of 5 bulbs was also set up in distilled water. After 4 days, the mean value of 10 measurements of each onion, i.e. 50 roots for each concentration, was expressed as a percent of the control value. This measurement was used to calculate the EC$_{50}$ value, which is the concentration where root growth is reduced by 50% compared with the control. The EC$_{50}$ value of Illoxan was determined as 150.00 mg/L and then the root tips were treated with 37.50 (EC$_{50}$/4), 75.00 (EC$_{50}$/2), and 150.00 (EC$_{50}$) mg/L concentrations.

Bulbs were placed over the test tubes filled with distilled water at room temperature (20 ± 2 °C). When the roots reached 1.5-2 cm, they were treated with each concentration of Illoxan for 12, 24, and 48 h. Twelve bulbs were used for each concentration, treatment period, and control group. Following all the treatments, the root tips were fixed, macerated, stained, and squashed as described earlier by Yüzbaşioğlu et al. (3). The mitotic index (MI) was found by observing 1000 cells and counting the stages of mitotic cells and mitotic abnormalities. Slides were prepared from different onions; 1000 cells were screened from each of 10 onions yielding 10,000 cells for each concentration and duration of time. In addition, in pretreated roots, 100 metaphase cells were scored and aberrations were recorded for each of the concentrations and treatment times (14). Using the z-distribution test method, the data obtained for the mitotic index were statistically analyzed for the frequency of mitotic phases and mitotic disturbance. Metaphase aberrations were analyzed using a χ$^2$ test.

**Results**

Table 1 shows the mitotic index in *Allium cepa* root tip cells. The mitotic index significantly decreased in all treatments when compared with their controls.

<table>
<thead>
<tr>
<th>Conc. (mg/L)</th>
<th>No. of dividing cells</th>
<th>MI±SE</th>
<th>% Abnormalities</th>
<th>% Total abnormalities</th>
<th>% Abnormal interphase cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stickness</td>
<td>Laggards</td>
<td>C-mitosis</td>
<td>Bridges</td>
</tr>
<tr>
<td>12 h</td>
<td>Control</td>
<td>707</td>
<td>7.07±0.25</td>
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<td>0</td>
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<tr>
<td></td>
<td>37.5</td>
<td>427</td>
<td>4.27±0.20a</td>
<td>2.34</td>
<td>6.56</td>
</tr>
<tr>
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<td>75</td>
<td>270</td>
<td>2.70±0.16a</td>
<td>12.22</td>
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<tr>
<td></td>
<td>150</td>
<td>475</td>
<td>4.75±0.21a</td>
<td>4.84</td>
<td>2.74</td>
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<tr>
<td>24 h</td>
<td>Control</td>
<td>668</td>
<td>6.68±0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>37.5</td>
<td>401</td>
<td>4.01±0.19a</td>
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<tr>
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<td>75</td>
<td>336</td>
<td>3.36±0.18a</td>
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<td>3.27</td>
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<tr>
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<td>150</td>
<td>431</td>
<td>4.31±0.20a</td>
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<td>3.71</td>
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<td>0.16</td>
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<td>75</td>
<td>321</td>
<td>3.21±0.18a</td>
<td>2.49</td>
<td>0</td>
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<tr>
<td></td>
<td>150</td>
<td>362</td>
<td>3.62±0.18a</td>
<td>14.92</td>
<td>4.97</td>
</tr>
</tbody>
</table>

Frequency of abnormalities (%) 40.30, 24.73, 21.53, 8.32, 2.99, 2.13.

a Significantly different from the control P < 0.001 (z test)
* Significantly different from the control P < 0.01 (χ$^2$ test)
** Significantly different from the control P < 0.001 (χ$^2$ test)
This decrease was slightly dose dependent at 24 h ($r = -0.56$) and 48 h ($-0.69$). However, Illoxan had no significant effect on the percentage of the mitotic stages. Illoxan significantly increased the frequency of abnormal cells in all concentrations and in all treatment times when compared with their controls (Table 1). This increase was dose dependent in the 24 h and 48 h treatments ($r = 0.84$, $r = 0.99$ respectively). Six types of aberrations were recorded: stickiness, laggards, C-mitosis, bridges, multipolarity, and fragments (Figure 2). Stickiness was the most common abnormality. Illoxan also induced micronucleated and bi-nucleated cells at interphase. However, these aberrations were not significant when compared to the controls.

The results of metaphases obtained from α-monobromonaphthalene pretreated root tip cells are summarized in Table 2. This herbicide induced four types of structural aberrations in metaphase cells: fragments, sister union, chromosome, and chromatid breaks (Figure 3). This herbicide also induced polyploidy, a numerical aberration (Figure 3). Illoxan significantly increased the frequency of abnormal cells (except 37.50 mg/L at 12 and 24 h) in a dose-dependent manner ($r = 0.76$ at 12 h, $r = 0.87$ at 24 h, $r = 0.87$ at 48 h).

**Discussion**

This study investigated genotoxicity of the pesticide Illoxan in *Allium cepa* root tip cells. The commercial form of the pesticide was tested because this is the form that is utilized in agriculture and introduced into the environment. *Allium cepa* was used as the test system because pesticides are applied on plants in agriculture and plants may produce unique genotoxic metabolites.

In this study, Illoxan decreased the mitotic index in *Allium cepa* root tip cells. This decrease was significant in all concentrations when compared to the control. The decreasing of the MI was slightly dose dependent at 24 h and 48 h. These results show that concentrations of Illoxan used were cytotoxic in *Allium*. A similar result in mitosis has been observed from the treatment of the herbicides racer (3), atrazine (12), and arsenic (15). There are some possible mechanisms for chemically decreased mitotic index in plant cells. The first is that a decrease in MI could be due to blocking of G₁ suppressing DNA synthesis (16). The second possible mechanism is a blocking of G₂ preventing the cell from entering mitosis (17). The lowering of the mitotic index might have been achieved by the inhibition of DNA synthesis at the S-phase (18). Another possible mechanism is explained by Chand and Roy (19).

These authors proposed that 2, 4-dinitrophenol inhibits DNA and RNA synthesis by reducing the oxidative phosphorylation in plants, resulting in lower levels of ATP.

Illoxan increased the percentage of abnormal cells in *Allium cepa*. This increase was significant in all concentrations applied when compared to the control and was dose dependent. Common abnormalities were stickiness, laggards, and C-mitosis. Fiskesjö (13) reported that sticky chromosomes indicate highly toxic chemical effects that are usually not reversible and will probably lead to cell death. These results were reported by many investigators following treatment with some pesticides (2, 20). Generally, a mitotic poison causes disturbance of the spindle apparatus, resulting in a C-mitosis effect, which means the complete absence of a spindle. A weak C-mitotic effect produces lagging chromosomes that do not attach to the spindle apparatus. The formation of C-mitosis, lagging chromosomes and multipolarity may be due to the disturbance in the spindle formation which was effected by the herbicide (1, 21). Pentachlorophenol (PCP), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2-chloro-2,6-diethyl-N-(butoxymethyl) acetanilide (butachlor) induced chromosome aberrations occur at a statistically significant level. These herbicides induce breaks, bridges, stickiness, and laggards in *Allium cepa* root tips (20). Illoxan induced abnormalities in interphase cells. However, the frequency was not high or significant when compared to the control.

Illoxan also induced a significant increase of chromosomal aberrations at the metaphase stage in pretreated root tip cells in all concentrations and treatment periods when compared with their respective controls. While this increase was significant in only 75 and 150 mg/L concentrations at 12 h and 24 h treatments, the increase was significant in all concentrations at 48 h. This herbicide induced
Figure 2. Different types of aberrations induced by Illoxan in *Allium cepa* root tips. a) Stickiness b) C-mitosis c) Chromosome fragment d) Chromosome bridge e) Lagging chromosome f) Multipolarity.
fragments, sister union, chromosome and chromatid breaks as structural aberrations and poliploidy as a numerical aberration in metaphase cells in this study. Sister union is the breakage followed by reunion of both sister chromatids at an identical site (22). This abnormality resulted from terminal deletion. Terminal deletions cause sticky chromatid tips and then these sticky tips may join to each other (23). The herbicide flurochloridone induced a significant increase in chromosomal aberrations at the metaphase stage in all concentrations and treatment periods when compared to the control groups. These abnormalities manifested as chromosome breaks, fragments and sister union (3). Çelik et al. (4) reported that dinocap induced a significant increase in chromosomal aberrations in pretreated root tips when compared to control groups. The four types of abnormalities recorded were chromosome breaks, fragments, tetraploidy, and sister union at the metaphase stage.

Although diclofop-methyl has been classified as “likely to be carcinogenic to humans” based on laboratory studies in rats and mice (10), the diclofop-methyl tested negative for genotoxic effects in a bacterial reverse mutation test, an in vitro mammalian cell gene mutation test, an in vitro mammalian chromosomal aberration test, an in vivo cytogenetic test, and a mouse bone marrow micronucleus test as mentioned in the introduction. However, the results of this study indicate that Illoxan decreased the mitotic index and produced clastogenic and aneugenic types of abnormalities in *Allium cepa* root tip cells.

Some compounds, such as triazines, maleic hydrazide, and sodium azide (24), appear to be plant specific, i.e. they are activated by genotoxic metabolites that are not formed in mammals (25, 26). More recently, the group of Gomez-Arroyo conducted experiments with extracts from *Vicia faba* roots and

<table>
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<tr>
<th>Conc. (mg/L)</th>
<th>Total cells scored</th>
<th>Aberrations</th>
<th>Fragments</th>
<th>Sister union</th>
<th>Chromosome breaks</th>
<th>Polyploidy</th>
<th>Chromatid breaks</th>
<th>% Total aberrations</th>
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*Significantly different from the control P < 0.05 ($\chi^2$ test)

**Significantly different from the control P < 0.01 ($\chi^2$ test)
cultured human lymphocytes (27). They found that sister chromatid exchange (SCE) induction occurred with 2 herbicides (molinate and butylate), which per se did not cause positive effects in human lymphocytes. These results show that plants can metabolize chemicals that are not genotoxic per se to mammalian cells. These findings are of crucial importance in the case of pesticides that give negative results in routine genotoxicity testing. If these compounds are utilized on crop plants and consequently convert to DNA-reactive metabolites, ingestion of the plants could pose a threat to human health. These kinds of metabolites may also be mutagens in bacteria as well (28).

In conclusion, plant models such as micronucleus (MN), chromosomal aberration (CA), and sister chromatid exchange (SCE) assays have been found to be highly sensitive in the detection of hazards arising from pesticides, industrial contamination, and heavy metals. The data obtained here support these findings and indicate that plant bioassays can be used as an important and integral part of test batteries for the detection of genotoxic effects of chemicals used in the environment.
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References


