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Research Article

CYTOPHYSIOLOGICAL IMPACTS OF TRIBENURON METHYL HERBICIDE ON *Allium cepa*

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ABSTRACT. Tribenuron-methyl is sulfonylurea herbicide generally used in agricultural weed control for cereal crops. This herbicide kills weeds by stopping cell division and growth. *A.cepa* test is widely used as an environmental monitoring tool to detect cytotoxicity. The aim of the present work is to determine the cytotoxic, genotoxic and physiological effects of the tested herbicide. Cytotoxic effects of the herbicide Tribenuron-methyl were investigated by using Allium test. Also physiological effects (length of root and green leaf, chlorophyll and malondialdehyde contents) of the herbicide were examined on *Allium cepa*. Four days old seedling roots were treated with control group (distilled water), 37,5 mg/L, 75 mg/L, 150 mg/L and 300 mg/L concentrations of Tribenuron during 24, 48 and 72 hours. Tribenuron significantly decreased the Mitotic Index and increased chromosome aberrations (Anaphase bridge, sticky metaphase, disturbed anaphase, c-metaphase and micronucleus) at all treatment durations and concentrations compared to the control groups. It was found that tested herbicide increased malondialdehyde levels, decreased root and green leaf length and caused changes in chlorophyll content. Based on these results, tribenuron-methyl should be considered as a cytotoxic agent for plants. The use of this herbicide should be kept under control, considering it may pose an ecological risk as it may adversely affect the genomes of non-target organisms.

Keywords: *Allium test, antioxidants, genotoxicity, cytotoxicity, phytotoxicity, herbicide, lipid peroxidation*

INTRODUCTION

Pesticide use is an important part of agriculture in both developed and developing countries. Chemical pesticides are a considerable group of environmental pollutants despite being widely used in modern agriculture therefore the residues of these chemicals pose a risk for local and global biocenosis. Increasing pesticide usage will cause damage to the ecosystem. The use of chemical pesticides to protect plants against pests is a common tool and unfortunately pesticide toxicity is not limited to target organisms, but can also affect many non-target plants and animals [1, 2]. Herbicides control or kill weeds by various mechanisms, such as; inhibition of cell division, mitosis, enzyme function, photosynthesis, root growth, leaf formation or interference with the synthesis of proteins, DNA or pigments [3]. The use of herbicides may cause stress conditions that affect growth, development and productivity and cause phytotoxicity. The negative effects of the resulting stress are usually mediated by lipids, proteins, pigments and nucleic acids, resulting in lipid peroxidation, membrane damage, and oxidative damage that causes the enzymes to become inactive [4]. As a result of stress, damage to cell membranes with lipid peroxidation disrupts the physiological and metabolic processes of plants. In order

to determine suitable herbicides, it is important to carefully evaluate the changes in oxidative stress parameters as well as phytotoxic symptoms that occur in plants.

Tribenuron-methyl (C15H17N5O6S) (TBM) is a member of the sulfonylurea herbicide family which is generally used in agricultural weed control for cereal crops. This herbicide kills weeds by stopping cell division and growth. Due to the high phytotoxicity on susceptible plants, the susceptibility to environmental biodegradation and the proportion of sulfonylureas have increased. Previous studies with this herbicide have shown that even 1% of the recommended administration dose might adversely affect the growth, morphology and reproduction of non-target organisms [5, 6]. TBM prohibits aceto lactate synthesis (ALS) activation, which is a key enzyme in the biosynthesis of branched chain amino acids by disturbing the process of cell division leading to plant death [5, 7] stated that tribenuron-methyl herbicide has great phytotoxicity even at low concentrations. Numerous reports indicate that in some sensitive crops efficiency decreases due to phytotoxicity which is caused by the use of TBM [8].

The in vitro genotoxicity tests for chemicals and pharmaceuticals industries were recommended by authoritative international guidelines, one of these tests is the Allium test, which is used to determine their genotoxic effects on plants [9]. It is an efficient cytological model for chromosome aberration and mitotic activity assay of different environmental pollutants. The Allium test is a direct method to measure damage in systems exposed to mutagens or potential carcinogens by observing chromosomal changes [10]. In this context, the objective of this work was to evaluate the hazardous potential of the Tribenuron methyl herbicide on *Allium cepa*, particularly the genotoxic, cytotoxic (with Allium test) and physiological effects (with length of root and green leaf, chlorophyll and MDA contents). The test material (*Allium cepa*) was used for Allium test which has low cost [11], fast test and also presents correlation with other test models and systems, including animal models [12].

MATERIALS AND METHODS

In this study, commercially available sulfonylurea herbicide Tribenuron methyl (FİRMA CAS no), which contains 75% active ingredient, was used. In previous studies [3, 4, 5, 7, 8, 14, 15] aimed at demonstrating pesticide toxicity, the determination of the concentrations tested is generally based on the commercial formulation of the specified chemical. The brownish scales (*A. cepa*) of commercial bulbs purchased from the store were carefully removed without damaging the root primordia. Healthy and equally sized (1.5-2.0 cm diameter) allium bulbs at room temperature (20 ± 2 ° C) were placed in plastic containers filled with distilled water. When the roots reached 1.5-2 cm, 18 bulbs with good root growth were selected and treated with four test concentrations of TBM (37.5 mg / L, 75 mg / L, 150 mg / L and 300 mg / L). Plant samples were harvested at the end of the 24, 48 and 72 hours.

For cytotoxic and genotoxic analysis, root tips were fixed for 24 hours in acetic alcohol (acetic acid and alcohol, 1: 3), root tips were kept in 70% ethanol at 4°C. Onion root tips were washed three times with distilled water. They were hydrolyzed in 1N HCl at 60°C for 12 min and stained with Feulgen and squash preparations of root tips were made for the study of the mitotic index (MI) and chromosomal aberrations (CAs). All slides were examined blindly. At least 1000 cells from each slide were examined. Three replicates were made for each procedure as a result at least 3000 cells were scored for each

concentration. MI was calculated by dividing the number of cells by the total number of cells. Chromosomal aberrations (CAs) were determined according to chromosomal abnormalities such as chromosome stickiness, anaphase bridge, c-metaphase and disturbed anaphase. The frequency of chromosome aberrations percentage was estimated based on the number of aberrant cells per total cells calculated at each concentration. CAs at each treatment were also recorded and the most frequent chromosome aberrations were presented with micrographs. Photographs were taken by a Prior microscope with Kodak C813 digital camera.

After the leaf and root lengths were measured at the end of the application, the leaves of the harvested plants were placed in 1 mL cold acetone and kept in dark until the tissues lose all color. The total chlorophyll content in the solution obtained from the leaf tissues was determined using the Lichtenthaler's method [16]. Lipid peroxidation was calculated by determining the amount of malondialdehyde (MDA) in 1g fresh leaf tissue according to Lutts' et al. [17]. The concentration of MDA was calculated from the absorbance at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecific turbidity) by using extinction coefficient of 155 $mM^{-1}.cm^{-1}$

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

RESULTS AND DISCUSSION

Mitotic index (MI)

Mitotic index (MI) in the root tips of *A. cepa* exposed to TBM are given in Table 1. The cytotoxic effects of TBM were determined based on Mitotic index (MI). Mitotic index of control sets (distilled water) was 8,51± 0,961 in 2h, 4.51± 0,582 in 48h, 4,04 ± 0,387 in 72h. MI was reduced significantly as treatment time and concentration of herbicide increased. All of the treatment periods and concentrations significantly affected mitotic phases. No cell division was observed at 300 mg/L concentrations for 48 and 72 hours.

Chromosome aberrations

Chromosome aberration types and percentage of mitotic abnormalities in the root tips of *A. cepa* exposed to TBM are given in Table 1. The genotoxic effects of TBM were determined based on chromosomal aberrations. Compared to control groups, the frequency of CA increased dose-dependent at all concentrations and durations of treatment. Complete inhibition of cell division was observed in the treated roots with the highest herbicide concentration (300 mg/L) used for 48 and 72 hours.

Figure 1 shows the different deviation types induced by TBM at the root tips of the *A.cepa*. In all treatments in different mitotic phases five types of chromosomal aberrations were recorded. These CAs were C-metaphase (Fig.1a), chromosomal stickiness (Fig.1b), anaphase bridge (Fig.1c), disturbed anaphase (Fig.1 d-e) and micronucleus (Fig.1f). In the present study, C-mitosis was discerned as the most common type of abnormality (42.68%) and the frequency of sticky metaphase was 24.43%. Disturbed anaphase and

anaphase bridges were monitored as 22,13% and 6,52% respectively. Micronuclei are also observed in the interphase cells as 4,24 %.

Effects on leaf and root length

The effects of TBM on leaf and root length depends on concentration and duration of treatment. Our results show that TBM treatment significantly decreased the leaf and root length (Figure 2 A and B). As a result of TBM treatment, the average decrease in leaf and root length was determined as 58%, 65% and 75% at the end of the 24, 48 and 72 hours, respectively. The highest leaf and root length were determined in the control group at the end of 72 hours. The least leaf and root length were observed in the sample which was treated with 300 mg/L of TBM. The differences between treatment groups were statistically significant ($P<0.05$).

Effects on malondialdehyde contents

Lipid peroxidation is often measured as MDA which is an oxidized product of membrane lipids, and commonly considered as a good marker of lipid peroxidation as well as stress level [2, 3, 18].

The changes in MDA contents are given in Figure 2 C. When compared to the controls, TBM caused significant ($P<0.05$) increase in MDA contents at 24, 48 and 72 hours of all treatments except 37,5 mg/L. The increase in MDA content was determined as 52%, 90% and 97% at the end of the 24, 48 and 72 hours, respectively.

Effects on photosynthetic pigments contents

Results of pigment analysis showed that the chlorophyll pigments of the *A. cepa* leaves were significantly reduced with increasing TBM concentrations represented in Figure 2(D). When compared to the control group, the least pigment contents were observed in the group which was treated with 300 mg/L of TBM especially at 72 hours. The carotenoid contents decreased with TBM treatments, but the reduction rate of carotenoids was slower than chlorophyll contents.

Table 1. *Mitotic index (MI), type and percentage of CAs in the root tips of A. cepa exposed to Tribenuron methyl*

					Chromosomal aberrations (%)					
Treatment time (hours)	Concentration (mg/L)	Num. of observed cells	Number of dividing cell	MI (%± SE)	c-metaphase	Anaphase -bridge	Sticky metaphase	Micronucleus	Disturbed anaphase	Total aberration
24	Control	3197	272	8,51±0,961	0	0	0	0	0	(% ±SE)
	37,5	3179	99	3,11*±0,875	10	3	6	2	2	23,23
	75	3542	31	2,57*±0,538	13	3	3	0	6	80,65
	150	3339	84	2,52*±0,70	8	4	16	0	10	45,23
	300	3827	77	2,01*±0,621	3	0	12	1	7	29,87
48	Control	3435	155	4,51±0,582	0	0	0	0	0	0
	37,5	2612	67	2,57*±1,182	8	0	2	5	8	34,33
	75	4337	101	2,33*±1,165	7	0	1	0	5	12,87
	150	3840	40	1,04*±0,06	4	1	7	0	1	32,5
	300	0	0	0,00*±0	0	0	0	0	0	0
72	Control	3345	135	4,04±0,387	0	0	0	0	0	0
	37,5	4177	67	1,58*±0,192	4	1	7	0	1	19,4
	75	5048	52	1,15*±0,099	7	1	8	2	1	36,54
	150	1810	18	0,99*±0,369	6	0	0	0	2	44,44
	300	0	0	0,00*±0	0	0	0	0	0	0
Frequency of abnormalities (%)					42,68	6,52	24,43	4,24	22,13	

*Means significantly different at the $p<0.05$ level compared with compared with control for MI (LSD % 5 = 1,12).

Extreme and uncontrolled usage of herbicides cause problems on biological processes such as mitotic division, root and leaf growth, photosynthesis, lipid peroxidation [2, 3, 15, 18-22].

In this study cytotoxic and genotoxic effects of TBM were determined based on chromosomal aberrations and Mitotic index (MI). It was observed that increased duration and concentration of TBM has decreased the MI significantly. This results revealed cytotoxic effect of TBM. In previous studies, it has been reported that the herbicides reduce mitotic activity [23-27]. Mitotic activity inhibition is often used to monitor cytotoxicity of the test substances. Reducing the MI level points out that the test substance has a mitodepressive effect that causes cells to reach mitosis and it is related to the effect of herbicides on DNA synthesis [28]. Kim and Bendixen stated that mitotic activity depression is caused by inhibition of protein synthesis [29]. Depression of mitotic activity is attributed to suppression of protein synthesis. Pesticides in a cell may have some fundamental effects on enzyme production, induction, function, suppression and may cause a low mitotic index [21, 22].

Disturbed anaphase and C-mitosis were aneugenic; bridges and fragments were clastogenic chromosome aberrations. The appearance of C-mitosis indicates that TBM have similar effect to that of colchicine which caused the inhibition of spindle formation. Kovalchuk et al. stated that chromosome stickiness and C-mitosis spindle-related abnormalities are evidence of cytotoxicity [30]. Leme and Marin-Morales represented that chromosome bridges and breaks indicate clastogenicity and C-metaphases result from aneugenic effects [31]. C-metaphases are presumably the result of disarrangement of tubulin cytoskeleton organization, and disturbing mitotic spindles which may cause c-metaphases [32].

Chromosome stickiness is responsible for the separation of chromosome segments and free inversion [24]. Sticky chromosomes have an irreversible toxic effect which may lead to cell death. Chromosome stickiness may be result of protein adhesion [33]. This result suggests that TBM induced cytological changes and mitotic abnormalities in the root tip cells of onion. In mitotic phases clastogenic and nonclastogenic chromosome abnormalities were investigated. Stickiness and chromosome bridges are major clastogenic aberrations [34].

It was reported that chromosome bridges were also induced by other herbicides. Meanwhile, the formation of bridges and chromosome breaks caused by the effect of TBM may be determinative of the mutagenic potential of the herbicide. Chromosome bridges can be the result of chromosomal adhesion, so bridges may possibly be formed by breakage and fusion of chromosomes and chromatids. Failure of anaphase separation can be attributed to the inversion of chromosome segments and unequal translocation. Bridges can be caused by DNA breaks and may cause the occurrence of interphase anomalies like cells with micronuclei. Micronucleus formation was also observed in this study. This type of formation shows loss of genetic material; they might be the consequences of lagging chromosomes or acentric fragments. Micronuclei were the result of the removal of the acentric chromosome fragment outside of the nuclear envelope when the cell division was completed. Chromosomal aberrations work as an important factor for assessing the genotoxicity capacity of pesticides. All these CAs may be related with the mutagenic potential of the TBM.

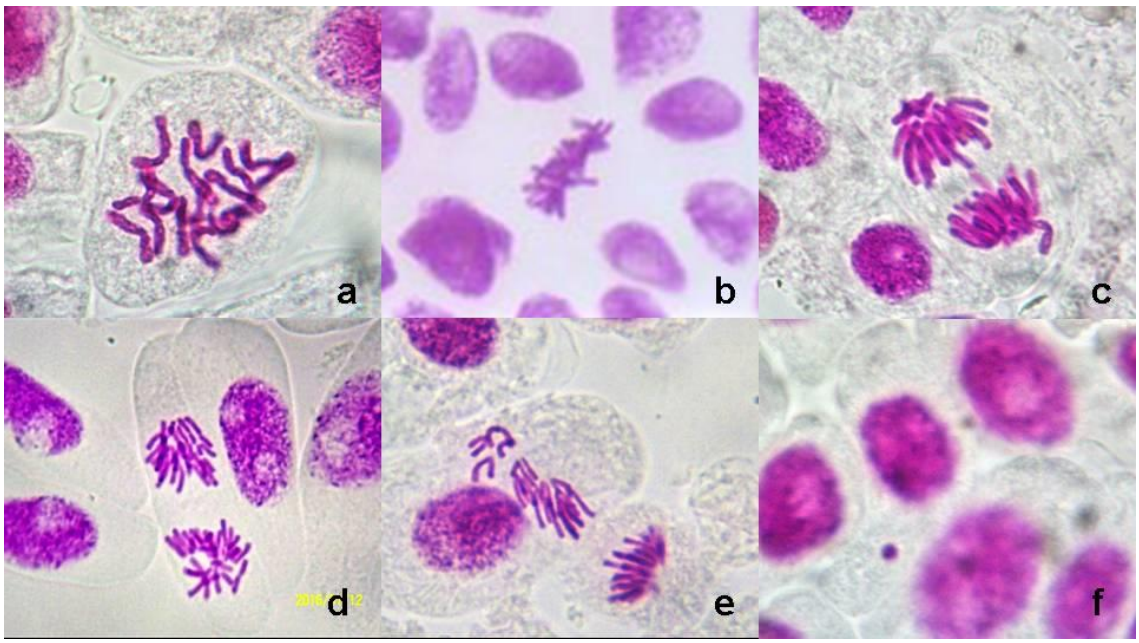


Fig. 1. *CAs induced by TBM in A. cepa root tips: a: C-metaphase, b: Sticky metaphase, c: Anaphase bridge, d-e: Disturbed anaphase, f: Micronucleus*

The safety of herbicides recommended for use in plants might be demonstrated by the analysis of agronomic properties (height, leaf area, leaf color, plant growth speed, biomass, yield, etc.) and physiological changes (lipid peroxidation, protective enzyme activities, etc.) [2, 3, 15, 19, 20, 35, 36]. Previous studies have shown that several pesticides inhibit growth at higher doses. Growth indicators such as dry weight and height are important parameters in assessing the physiological effects of toxic chemicals on

plants. This result has also been reported in several studies with the herbicide TBM [3, 5, 6, 15, 19, 20]. Our results show that the cytotoxic effect of TBM was accompanied by a significant decrease in plant growth measured as leaf and root length (Figure 2 A and B). This data show that TBM treatment significantly decreased the leaf and root length. Similarly, Enayati et al. reported that increasing the dose of herbicide (10g/ha, 15g/ha, 20g/ha TBM) would lead to a decline in the weed's (*Galium aparin*) dry weight and its height [15]. Our results are similar to those of Ning et al. [19] in which application of TBM inhibited the growth of foxtail millet, which was reflected by reduced root and shoot biomass. Previous studies reported that TBM reduced weed growth by disrupting the synthesis of branched amino acids and disorders the cell metabolism, which in turn reduces the number of cell divisions and causes a low height and dry weight in weeds [5, 6, 15]. Several studies have shown that herbicides cause lipid peroxidation and subsequent oxidative stress [36-38]. Duman et al. [3] investigated that the influence of TBM on a non-target aquatic plant (*Nasturtium officinale*). Their results indicated that TBM caused an increase in MDA contents. They also reported that the herbicide concentration and its exposure period had significant effects on the biological responses against TBM stress. Our results are similar to those of Agostinetto et al. [2] in which application of different herbicides increased MDA contents in wild radish control. Similarly, Garkova et al [14] reported that MDA contents increased, especially at the highest herbicide concentration, in cereal plants treated with herbicide Granstar (an active ingredient is TBM).

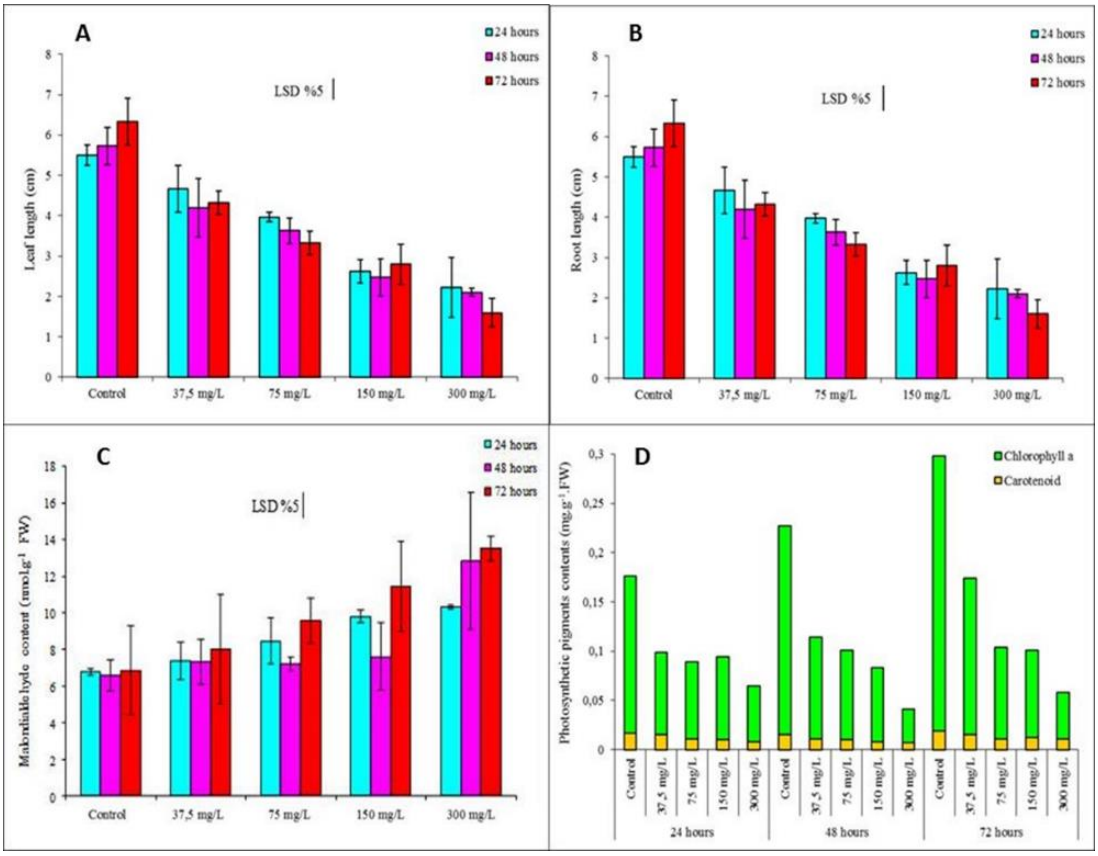


Fig. 2. *The effects of TBM on leaf (A), root (B) length, MDA (C) and photosynthetic pigments (D) contents of A. cepa. Values were the means of three replicates and vertical bars represent the standard errors*

Results of pigment analysis showed that the chlorophyll pigments of the *A. cepa* leaves were significantly reduced with increasing TBM concentrations represented in Figure 2(D). Similar studies pointed out chlorophyll content decreased in response to increasing stress conditions [3, 35, 39]. In this study, the contents of carotenoid decreased with TBM treatment, but the reduction rate was lower than the Chl contents. It has been shown in many studies that the reduction of chlorophyll content as a result of stress may be a good indicator for monitoring the damage in the growth and development of the plant [36, 38]. Guo et al. (2018) indicated that, increased herbicide concentrations decreased plant height and chlorophyll accumulation [36]. Also, they reported that differences in chlorophyll reduction might be due to the diversity of herbicide tolerance. Similarly, Agostinetto et al. [2] reported that herbicides belonging to sulfonylureas cause chlorosis and necrosis and thus growth retardation.

The conclusions to be made from this study are as follows: increasing TBM concentrations; (i) have a significant effect on cytological process of *A. cepa* (ii) inhibits the growth parameters like leaf and root length, and also photosynthetic pigments; (iii) increased MDA contents. Our results indicated that TBM should be regarded as a cytotoxic agent for plants. Hence, the use of this herbicide should be under control at these concentrations. The use of herbicides not only controls weeds development but also ensures the safety of the crops. The inhibition of MI due to concentration and exposure time as well as the emergence of mitotic abnormalities indicate the cytotoxic potential of the herbicide TBM in *A.cepa*. Consequently, these results indicated that tribenuron-methyl should be regarded as a cytotoxic agent for plants. Therefore, the use of this herbicide should be kept under control at these concentrations because of the ecological risk assessment of pesticides and their impact on organisms in the context of future use of pesticides in agriculture.

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