THE ASSESSMENT OF PENDIMETHALIN CYTOTOXITY BY *ALLIUM* ASSAY

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Abstract. *The widespread use of herbicides, despite their beneficial effects on agricultural production, may constitute a threat to the environment. The accumulation of active substances in soil, depending on their half-time life, can affect not only the target species but also non-target ones. Pendimethalin is an extensively used pre-emergent herbicide in vegetable crops, including for onion or garlic. The aim of the study is the assessment of the potential cytotoxic effect using the Allium assay. Allium sp. are very sensitive to different potential harmful elements for the environment. As biologic material were used 10 healthy Allium cepa L. same size bulbs (4.4 ± 0.3 g) for each experimental variant. For 4 days all bulbs were grown in tap water in tanks (10/20/10 cm). After this period the bulbs were placed in 5 experimental variants: Control – tap water; E1-E4 - increasing concentrations of the herbicide solution (1.365 – 4.550 g l^{-1} pendimethalin). The solutions in tanks were renewed at each 24 h. After 48, when the length of the root was 15- 20 mm were harvested 3 roots from each plant for cytological investigations. The roots were placed in Carnoy fixing solution for 24 hours, in the refrigerator. The hydrolysis was performed with HCl 1N for 7 minutes at 60°C. The staining was performed with Carr solution. The cytotoxicity was evaluated by the mitotic index (MI), as well as by aberrant divisions counting. All data were processed statistically with STATISTICA 10 software (Basic statistics, 2 way -ANOVA, Duncan test, Correlations). The results show that pendimethalin induces a mitodepressive effect depending on dose correlated with a significant percent of genomic mutations. The frequency of aberrant cells increased significantly with the herbicide concentration. The accumulation of pesticides in the soil can represent an environmental hazard.*

Keywords*: cytotoxicity, Pendimethalin, Allium test*

INTRODUCTION

Pesticides have made huge contributions to mankind by reducing the number of diseases and pests typical of each crop, as well as weeds and increasing production. They have many advantages, both economically and productively, but improper use can be dangerous. The accumulation of active substances in soil, depending on their part-time lifetime, may affect not only target species but also non-target species. The use of pesticides for a longer period has been reported to induce toxicity. These chemicals or their derivatives accumulated in organisms may cause the risk of mutagenicity, carcinogenicity or teratogenicity (DULIO and VON DER OHE, 2013). Bio-tests are currently used to detect the effects of pesticides. Researchers use different pesticides for the evaluation of cytotoxicity and genotoxicity: pendimethalin (VERMA and SRIVASTAVA, 2018), fenaminosulf (LIMAN et al., 2010) and metolcarb (LIMAN et al., 2011), atrazine (FELISBINO et al., 2018), diclofop methyl, lindane (MESI and KOPLIKU., 2013), mancozeb and chlorpyrifos (FATMA et al., 2018a)

Pendimethalin is part of the dinitroaniline class and is a herbicide that is used in prime-emergence and post-emergence applications to control annual grass and certain weeds and has the molecular formula (fig 1) C13H19N3O4. It is lipophilic, with a 5,18 log kow (WSSA, 2002), with strong uptake in soil. This chemical property is linked to the strong persistence of organic soil and the low leachability which greatly improves the potential risk of polluting the environment. Pendimethalin has a down effect on the mitosis in the root's meristem of *Allium cepa* L. and it disrupts the distribution of chromosomes. The effects of the

pesticide also occur in the inhibition of root growth and the thickening of the meristematic area. (BEURET, 1980).

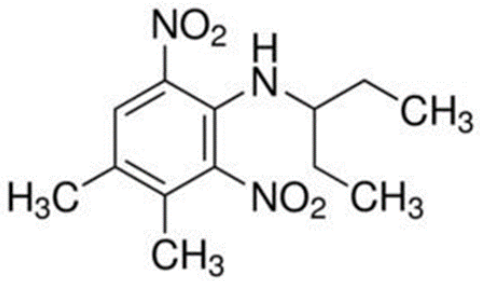

Figure 1. The chemical structure of pendimethalin

Certain plant species are recognized as excellent genetic models for the detection of changes due to their sensitivity to certain toxic substances. One of these is *Allium cepa*, which was used as test plant due to its long chromosomes, as well as low chromosome number (2n = 16) (BARBERIO et al., 2011; FIRBAS, P., and AMON, T., 2014; FATMA et al., 2018b; KARAISMALOGLU et al., 2015, 2016, 2017; BONCIU et al., 2018)).

The purpose of the study is to assess the potential cytotoxic effect of pendimethalin using the *Allium* assay. The *Allium cepa* test has been used since 1940 and is characterized as low-cost and is more practical compared to other short-term tests. The mitotic index together with chromosomal aberrations is used for the evaluation of cytotoxicity and micronucleus analysis for the verification of different mutations (LEME et al., 2009). This test provides information on the evaluation of an agent about its effects on the genetic material (clastogenic and aneugenic effects) (LEME et al., 2009).

MATERIAL AND METHODS

As biologic material were used 10 healthy *Allium cepa* L. same size bulbs (4.4 ± 0.3 g) for each experimental variant. For 4 days all bulbs were grown in tap water, in tanks (10/20/10 cm). After this period the bulbs were placed in 5 experimental variants: Control – tap water; E1-E4 - increasing concentrations of the commercial herbicide (pendimethalin) solution (g/l, considered concentration to a volume of 300 l/ha), as follows: E1 – 1.365 g l^{-1}; E2 -2.275 g l^{-1}; E3 – 3.185 g l^{-1}; E4 – 4.550 g l^{-1}). The solutions in tanks were renewed every 24 hours. After 48 hours, when the length of the roots was 15- 20 mm were harvested 3 roots from each plant for cytological investigations. The roots were placed in Carnoy fixing solution for 24 hours, in the refrigerator. The hydrolysis was performed with HCl 1N for 7 minutes at 60°C. The staining was performed with Carr solution, 24 hours in the refrigerator. The cytological observations were made on squash slides. The cytotoxicity was evaluated by mitotic index (MI) and the genotoxicity by aberrant cells percent and micronuclei/1000 cells counting. For each variant were observed more than 5000 cels (5 slides, around 1000 cells each). All data were processed statistically with STATISTICA 10 software (Basic statistics, 2 way -Anova, Duncan' Multiple Range Test [DMRT], correlations).

RESULTS AND DISCUSSIONS

Cytotoxicity assessment in mitotic division cycle. The Mitotic Index (MI), characterized by the total number of dividing cells, reported to the total number of observed

cells is a very important parameter. The mitotic cycle can be disturbed by different toxic chemicals, that can affect chromatin condensation degree, the kinetochore activation, mitotic spindle, phragmoplast or can produce different chromosomal aberrations. Analysis of variance showed that the concentration of pendimethalin effect is significant on MI and very significant on different mitotic phases percentages (Table 1).

MI is significantly decreased in E3 and E4 samples (over the concentration of 3g l^{-1} pendimethalin), from 11.49% in Control to 6.94% in E3 and 2.87% in E4. At the highest concentration of pendimethalin (4.550 g l^{-1} the cells are arrested in prophase, no other mitotic division phases being observed (Table 2). These results show that high concentrations of pendimethalin induced inhibition of histonic and non-histonic proteins, that are involved in DNA condensation and spiralization, as well as mitotic spindle formation. Mitodepressive effects of herbicides were reported by other researchers too, not only in plant bioassay (KARAISMALOGLU, 2015; KUMAR and SRIVASTAVA, 2015) but also in animal ones (PRASATH et al., 2016).

Table 1.

The effect of pendimethalin on mitotic division

Analysis of Variance Marked effects (bold) are significant at p <0.05000		
Experimental variant	F	P
Mitotic Index %	5.356	0.014393
Prophase %	229.497	0.000000
Metaphase %	393.583	0.000000
Anaphase %	224.857	0.000000
Telophase %	105.794	0.000000

Table 2.

Mitotic index (MI) in *Allium cepa* L., depending on pendimethalin concentration (significant differences [DMRT] in comparison with Control are marked in bold)

Variant	Total analyzed cells	Mitotic index %		Division phases %							
				Prophase		Metaphase		Anaphase		Telophase	
		Average	SE	Average	SE	Average	SE	Average	SE	Average	SE
C	5395	11.49	0.48	54.47	1.88	9.53	0.19	11.23	0.72	27.10	1.59
E1	5197	9.62	0.29	73.43***	1.45	10.10	0.58	1.44^{000}	0.12	16.69^{000}	0.96
E2	5205	7.50	3.06	89.90***	0.29	0.00^{000}	0.00	0.00^{000}	0.00	10.10^{000}	0.29
E3	5330	6.94^{0}	0.35	87.80***	1.04	0.00^{000}	0.00	0.00^{000}	0.00	12.20^{000}	1.04
E4	5434	2.87^{00}	0.19	100.00***	0.0	0.00^{000}	0.00	0.00^{000}	0.00	0.00^{000}	0.00

The obtained results are also sustained by the calculated correlations between herbicide concentration and MI, as well as with different mitotic phases. Between pendimethalin concentration and MI was registered a very significant negative correlation (r = -0.8025) and a very significant positive correlation with arrested cells in prophase, while with the other mitotic phases the correlations are negative ones (Table 3). This phenomenon is due to the mitotic spindle inhibition or some metabolic disorders in G2, which induce delays in the chromatin fiber's condensation.

Genotoxicity evaluation in the mitotic division cycle. Most of the pesticide compounds have mutagen potential (VERMA and SRIVASTAVA, 2018; LIMAN et al., 2010; FELISBINO et al., 2018; MESI and KOPLIKU, 2013). They can act at genes level by the inhibition of proteins synthesis and induce the metabolic disorders, or at chromosomes level producing chromosomal aberrations. They can affect the entire genome, inducing polyploidy or

aneuploidy by the mitotic spindle or kinetochore inactivation or by inhibition of phragmoplast formation. Chromosomal aberrations (CA) are produced due to free radicals action and can be highlighted especially in anaphase and telophase: chromosome breakage and reunion of broken ends (ring chromosomes, dicentric chromosomes – bridges, arches), changes in chromosome structure or loss of genetic material (acentric fragments, that in the next division can be observed as micronuclei -MN). Chromosomal bridges are associated with clastogenic and aneugenic effects of the herbicide (FELISBINO et al., 2018). These aberrations were produced in the first 24 hours of herbicide solution action and after 48 hours chromosomal aberrations percent decreased (Fig. 2, D – H), while MN and cells with two nuclei linked by a chromatin bridge could be observed in a significant number (Fig. 2, I – J).

In the samples treated for 48 hours with pendimethalin in the lowest concentration (1.365 g l^{-1}) were observed aspects similar to the ones obtained after colchicine treatment: C-metaphase, C-anaphase, as well as aberrant cells in interphase: binucleated cells and micronuclei (Fig 2, J –N). Lobulated nuclei, with different shapes, were observed frequently. The percent of binucleated cells and micronuclei/1000 cells increased with herbicide concentration, the differences in comparison with Control being very significant. In these samples also the mitotic index was at the lowest values. The behavior of pendimethalin like colchicine, affecting tubulin polymerization was also reported by UPADHAYAYA and NOODEN (1977) and TYLICKI et al. (2010). VERMA and SRIVASTAVA (2018) considered that the C-metaphases are produced by the disorders in the organization of the tubulin cytoskeleton started in the early stages of its origination.

Table 3.

Correlation matrix between the pendimethalin concentration, MI and division phases
(r marked in bold is significant for p<0.0500)

	MI %	Prophase%	Metaphase %	Anaphase %	Telophase %
Herbicide concentration (g/l)	-0.8025^{000}	+0.9388***	-0.8544^{000}	-0.7668^{00}	-0.9286^{000}
	p=0.000	p=0.000	p=0.000	p=0.001	p=0.000
MI %		-0.7718^{00}	+0.6547**	+0.5964*	+0.7936***
		p=0.001	p=0.008	p=0.019	p=0.000
Prophase%			-0.8693^{000}	-0.8954^{000}	-0.9544^{000}
			p=0.000	p=0.000	p=0.000
Metaphase %				+0.6761**	+0.7702**
				p=0.006	p=0.001
Anaphase %					+0.8100***
					p=0.000

Table 4.

The effect of pendimethalin on aberrant divisions

Analysis of Variance Marked effects (bold) are significant at p <0.05000		
Characters	F	P
Total aberrant cells (%)	73.323	0.000000
Ab. cells interphase (%)	201.728	0.000000
Binucleated cells (%)	88.615	0.000000
MN/1000 cells	467.262	0.000000
C -mitosis (%)	4749.000	0.000000

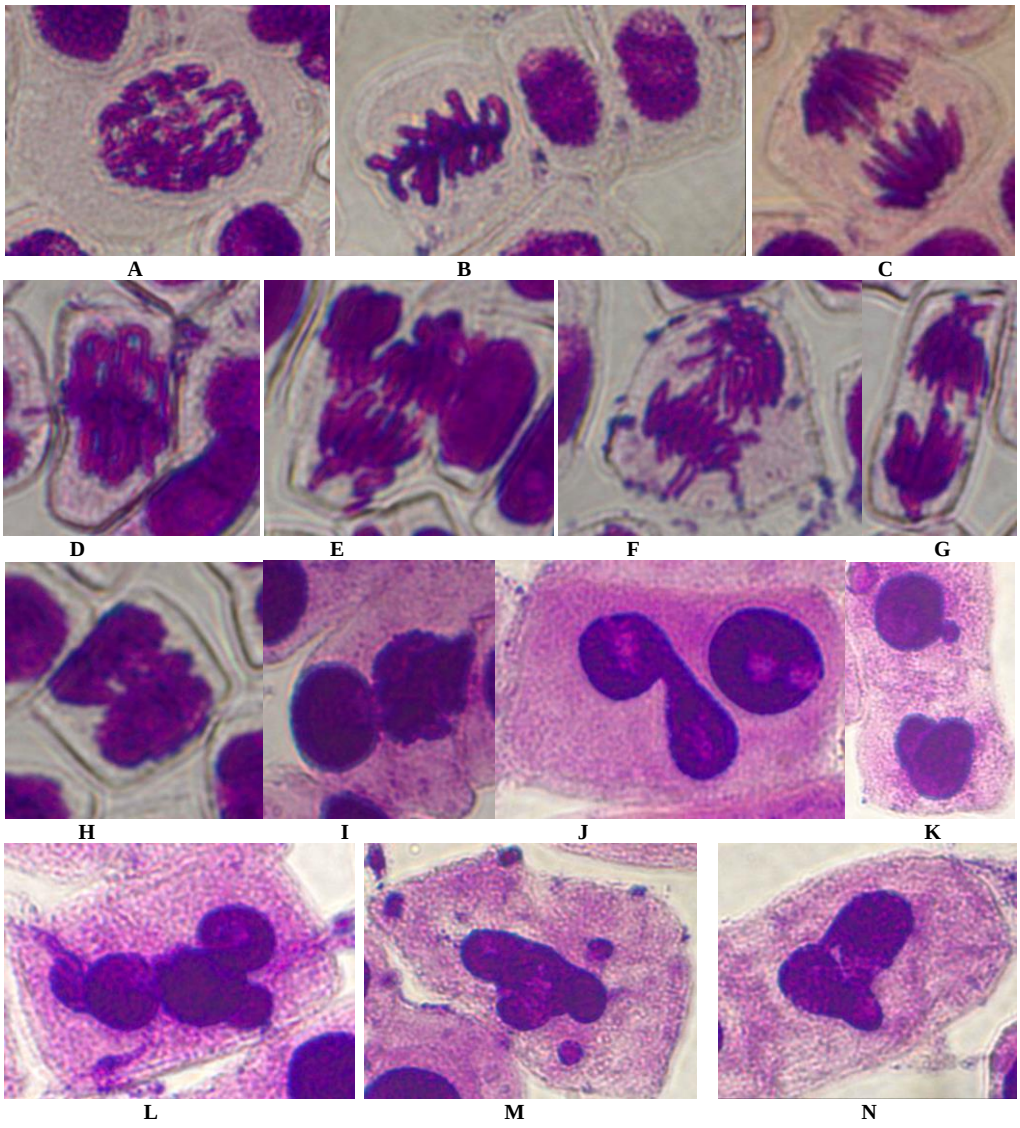

Figure 2. Normal and aberrant aspects of mitosis induced by pendimethalin in *Allium cepa* L.: (A) normal prophase; (B) normal metaphase (left) and telophase (right); (C) normal anaphase; (D) metaphase DCC (delay in chromatin condensation) [E1]; (E) anaphase with multipolar spindle [E1]; (F) disorganized anaphase [E2]; (G) bridge in anaphase [E2]; (H) multiple bridge in telophase [E2]; (I) binucleated cell (the two nuclei are linked by a chromatin bridge and present asynchrony in their activity [E3];); (J) binucleated cell, one of them is lobulated [E3]; (K) binucleated cell, one of them lobulated and the other one with bud [E3]; (L) binucleated cell, both lobulated nuclei [E4]; (M) lobulated nucleus and 4 MN [E4]; (N) lobulated nucleus [E4].

Analysis of variance shows that pendimethalin had a very significant effect inducing aberrant divisions (Table 4). The number of cells with MN increased with the concentration of pendimethalin in solution, the differences in comparison with Control being very significant (Table 5), as well as the calculated correlations (Table 6).

Table 5.

The percent of aberrant divisions and micronuclei in *Allium cepa* L., depending on pendimethalin concentration (significant differences [DMRT] are marked in bold)

Exp. Var.	Total aberrant cells (%)		Interphase (%)				Mitosis (%)			
			Binucleated cells (%)		Micronuclei/ 1000 cells		C-metaphase		C-anaphase	
	Average	SE	Average	SE	Average	SE	Average	SE	Average	SE
C	0.10	0.01	0.00		0.00		0.50	0.06	0.00	0.00
E1	2.40***	0.06	0.90***	0.06	1.30	0.06	5.80***	0.06	0.60***	0.06
E2	3.00***	0.06	0.70***	0.03	28.20***	1.15	0.00	0.00	0.00	0.00
E3	4.40***	0.12	0.90***	0.06	36.10***	1.15	0.00	0.00	0.00	0.00
E4	6.20***	0.58	1.10***	0.06	53.40***	1.73	0.00	0.00	0.00	0.00

Table 6.

Correlation matrix between the pendimethalin concentration, MI and division phases (r marked in bold is significant for p<0.0500)

	Total aberrant cells (%)	Ab. cells interphase (%)	Binucleated cells (%)	MN/1000 cells	C -mitosis (%)
Herbicide concentration (g/l)	+0.9699	+0.9204	+0.7829	+0.9700	-0.5025
	p=0.000	p=0.000	p=0.001	p=0.000	p=0.056
Total aberrant cells (%)		+0.9163	+0.8593	+0.9186	-0.3516
		p=0.000	p=0.000	p=0.000	p=0.199
Ab. cells interphase (%)			+0.8010	+0.8670	-0.2908
			p=0.000	p=0.000	p=0.293
Binucleated cells (%)				+0.6303	0.1195
				p=0.012	p=0.671
MN/1000 cells					-0.6742
					p=0.006

KARAISMAILOĞLU (2017) in his study on fipronil action on mitotic division observed that micronucleus formation augmented with increasing concentration of insecticide as compared to control. All these genotoxic effects were mentioned in the last years in many research papers on *the Allium* test and are in accord with our findings (FISKESJÖ, 1993; LEME and MARIN-MORALES, 2009; BONCIU et al., 2018; VERMA and SRIVASTAVA, 2018).

CONCLUSIONS

Mitodepressive effect of pendimethalin is a very significant positive correlated with herbicide concentration, high doses inducing the cells arrest in prophase.

Pendimethalin had a very significant effect inducing aberrant divisions. Pendimethalin behaves like colchicine affects tubulin polymerization in the early stages of protein synthesis. Binucleated cells, lobulated nuclei, micronuclei and C-mitosis were the most frequently observed mutations in the analyzed samples.

The frequency of aberrant cells increased significantly with the herbicide concentration.

The accumulation of pesticides in the soil can represent an environmental hazard.

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