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Cytotoxicity and Genotoxicity in *Allium cepa* L. Root Meristem Cells Exposed to the Herbicide Penoxsulam

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Abstract

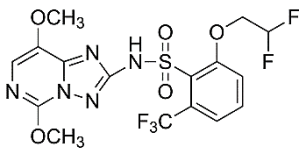
Penoxsulam is a new sulfonamide herbicide used on rice crops for annual grasses, broadleaf weeds, and sedges control, either grown through transplanting or direct dry or direct seeding methods of planting. In this study, *Allium cepa* ana-telophase and comet assay were used to examine the cyto-genotoxic effects of herbicide penoxsulam on *A. cepa* roots. *A. cepa* bulbs were exposed to ½xEC50 (12.5 µg/L), EC50 (25 µg/L) and 2xEC50 (50 µg/L) concentrations of penoxsulam for 24, 48, 72 and 96 h. Distilled water and 10 ppm of methyl methanesulfonate (MMS) were used as negative and positive control groups, respectively. Penoxsulam showed a cytotoxic effect by reducing root growth and mitotic index (MI), a genotoxic effect because it statistically increased chromosome aberrations (CAs, anaphase bridge, chromosomal laggards, polyploidy, disturbed ana-telophase and stickiness) and DNA damage as compared to control. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was also used for quantitative analysis of penoxsulam in *A. cepa* root meristem cells. Further molecular toxicological evaluations associated in the cyto-genotoxicity of penoxsulam on plants are needed to confirm these results.

Keywords: *Allium cepa* Ana-Telophase Test, Chromosome Aberrations, Comet Assay, Mass Spectrometry, Penoxsulam, Herbicide.

1. Introduction

Fluorinated class of triazolopyrimidine sulfonamide herbicides (flumetsulam, florasulam, penoxsulam and pyroxsulam) are one of the Acetolactate Synthase (ALS) inhibitors [1]. ALS enzyme is necessary for the biosynthesis of branched chain amino acids isoleucine, leucine and valine, which is only found in bacteria, plant and fungi but not in mammals. Therefore, they prevent plant growth by preventing cell division. Penoxsulam, registered in 2005, is a new sulfonamide herbicide broadly used on rice crops for annual grasses, broadleaf weeds, and sedges control, either grown through transplanting or direct dry or direct seeding methods of planting [2-4]. So, it's cytotoxic and genotoxic effects on plants should be explored. Some properties about penoxsulam are given Table 1.

Table 1. Some features of penoxsulam.

Chemical name	IUPAC Name	Chemical structure	Molecular Weight	Water solubility (mg/L at 20 °C)
Penoxsulam	2-(2,2-difluoroethoxy)-N-(5,8-dimethoxy-[1,2,4]triazolo[1,5-c]pyrimidin-2-yl)-6-(trifluoromethyl)benzenesulfonamide		483.37 g/mol	5.7 mg/L at pH 5 408 mg/L at pH 7 1460 mg/L at pH 9

Allium test has been frequently used for assessing the toxic effects of pesticides due to its high sensitivity, low cost, availability throughout the year, easy and rapid performance along with small number and large chromosomes. Different parameters (root growth, MI, CAs like c-mitosis, stickiness, bridges, micronucleus etc.) can be assessed easily. It is also validated by International institutions such as UN Environmental Program, US EPA, WHO and International Program on Plant Bioassay as test organism [5]. DNA damage can be also studied in *A. cepa* root meristematic cells independent than mitosis through the comet assay which is reproducible, sensitive, inexpensive and well-established test for the evaluation of genotoxic effects of pesticides [6].

The aim of the study was to investigate the potential cyto-genotoxic effects of penoxsulam on onion roots by *A. cepa* ana-telophase and comet tests. LC-MS/MS analysis was also performed to determine the amount of penoxsulam in onion root tips.

2. Materials and Methods

2.1. Materials

Equal-sized *A. cepa* L. bulbs (25–30 mm in diameter) were bought from market without any treatment (Uşak, Turkey). Penoxsulam (CAS Number 219714-96-2) and other chemicals were bought from Sigma Aldrich (Munich, Germany).

2.1.1. Growth inhibition test

The growth inhibition test was performed with some modifications as described previously [6] to determine the doses to be utilize for the study of cyto-genotoxic effects of penoxsulam on *A. cepa* root meristem cells. The onions were removed from some brownish bottom plates and yellow-brownish outer surface scales without destroying the root primordia. Concentrations of penoxsulam at 6.25, 12.5, 25, 50, 100, 250, 500 and 1000 µg/L and distilled water (control group) were continuously employed the onions at room temperature (21 ± 4 °C) for 96 h (Fig. 1.) In the end, whole 50 root bundle from each bulb (n = 5 onion bulbs per exposure) were measured for single control groups and concentration as described by Fiskesjö [7]. The amount of concentration that reduces the root tips growth by 50% by correlating with negative control group gives EC_{50} value.

2.1.2. *A. cepa* ana-telophase test

A. cepa ana-telophase test was performed as described by Rank and Nielsen [8] with some modifications. ½xEC50 (12.5 µg/L), EC50 (25 µg/L) and 2xEC50 (50µg/L) concentrations of penoxsulam, 10 ppm of MMS (positive control) and distilled water (negative control) were treated with bulbs (2–3 cm long roots after 2 days) at room temperature (21 ± 4 °C) for 24, 48, 72 and 96 h. Total number of three onions were utilized to carry out each application. 15-20 root tips (~1 cm) from each bulb were settled in Carnoy's fixative (glacial acetic acid/absolute ethanol, 1:3) and stocked at 4 °C for 24 h. After this, the onions were washed and cleaned by pure water and then reserved in 70% alcohol at the refrigerator until analyses. The roots were incubated in 1 N HCl at 60 °C for 8-10 min for hydrolysis. The root tips were cleaned with distilled water thrice and then they were stained with Feulgen method at the room temperature for 25-30 minutes. At the end of the period, 1-2 mm long dark-stained root tips were cut with razor blade and lysed with a drop of 45% acetic acid. Semi-permanent preparations were prepared by covering finger nail polish. For each application, 1000-1200 cells were counted in five different roots. MI values (expressed as a percentage) were determined by dividing the cells that had undergone mitosis by dividing into the total cells. In order to detect the CAs (Anaphase bridge, chromosome laggards, polyploidy, stickiness and disturbed anaphase-telophase), 500 anaphase-telophase cells (100 cells per slide) were counted in each application group with concentration and shown in the form of percentage [9].

2.1.3. Comet assay

This assay was performed according to Tice et al. [10] with some alterations. Seven root tips were cut about 1 cm long for each experiment and chopped immediately with 600 µL precooled Tris-$MgCl_2$ buffer (0.2 M Tris, 4 mM $MgCl_2.6H_2O$, 0.5% w/v Triton X-100, pH 7.5) to separate root nuclei in buffer. The sampled obtained were then centrifuged at 1200 rpm at 4 ºC for 7 min. The supernatant was wielded for quantitative analyses, while the pellet was utilized in the comet assay. 50 µL of 1.5% low melting point agarose at 37 °C was blended with 50 µL of the nuclear suspension. The mixture put on the slide with coated 1% normal melting point agarose and covered with cover slip. Slides were kept on an ice-cooled tray for 5 min to solidify and then gently cover slip was removed. The samples were moved to electrophoresis tank and dipped in the electrophoresis buffer solution (1 mM EDTA and 300 mM NaOH, pH> 13) for 20 minutes at 4 °C before the electrophoresis. The electrophoresis was operated at 300 mA at 25 V for 20 min. After performing the electrophoresis, the slides were cleaned with distilled water and with neutralization buffer (0.4 M Tris pH 7.5) washed thrice for just 5 min. The staining was done with 70 µL EtBr solution (20 µg/mL) for about 5 min. Randomly selected 50 comets per slide (three slides per treatment) to determine the degree of DNA damage between class 0 to 4 (0-no visible damage, 1-low level damage, 2-moderate damage, 3-high level damage and 4-maximal damage) were counted using fluorescence microscope. The arbitrary unit (AU) for DNA damage was evaluated by performing calculation [11].

2.1.4. Quantitative analysis of penoxsulam in *A. cepa* root meristem cells by LC-MS/MS

The quantitative analysis of penoxsulam was performed with LC-MS/MS by using supernatants (described above). An UPLC (Agilent 1200 series) is equipped with cooling system, binary pump, auto-sampler and column oven was used. The 6460 Triple Quadrupole LC–MS/MS system was used for detection of the Mass spectrometry. The mass spectrometer has triple quadrupole was furnished with system of electrospray jet steam ionizer. The ion source is ionization mode in positive electrospray. The MS/MS system was conducted in the multiple reaction monitoring mode. Zorbax Eclipse plus C18 (2.1×50 mm, 2.7 µ) column was applied for chromatographic separation. The mobile phase constitutes of acetonitrile 50% (mobile phase B) and 0.1% formic acid in water 50% (mobile phase A), having column temperature of 40°C and 1.2 mL/min flow rate. The 2.966-min isocratic elution was achieved using chromatographic separation. The MRM transition was

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detected: m/z 484.1 → 194, 484.1 → 164 and 484.1→124.

2.1.5. Statistical analysis

One-Way Analysis of Variance (ANOVA) with Duncan multiple range tests (P <0.05) were used to for the comparison of the results (mean ± standard deviation) using the IBM SPSS version 23. The dose-response and time-response relationship were determined by Pearson correlation test.

3. Results and Discussion

The cytotoxic and genotoxic of penoxsulam detection was carried by Allium Test. As a result of growth inhibition test, the EC_{50} of penoxsulam was found as approximately 25 µg/L (49.25%) as shown in Table 2 and Figure 1. Penoxsulam exposure statistically decreased average root length and dose dependently (r=-0.982 p=0.01). The average length of Allium root control was 4.67±0.08 cm, while the root length decreased up to 0.58±0.07 cm at 1000 µg/L of penoxsulam. The root length on *Oryza sativa* was decreased with increasing rates of penoxsulam [12]. Penoxsulam+cyhalofop butyl showed inhibitor effect on the radial mycelial growth of *Rhizoctonia solani* [13]. EC_{50} for penoxsulam after 30 days exposure was found 0.72 µg/L for *Mytilus galloprovincialis* [4]. EC_{50} for penoxsulam after 66 days was found 12, 64, 630, 51 and 23 µg/L for *Scirpus validus* Vahl., *Paspalidium geminatum*, *Panicum hemitomon*, *Pontederia cordata* L. and *Sagittaria lancifolia* L., respectively [2].

Table 3 illustrates the effect of penoxsulam on MI and mitotic phases in the *A. cepa* root tips. The value of MI was changed between 61.58±0.75 and 48.62±0.95 by penoxsulam applications, and statistically these decreasing values were compared with control group. The decreased MI for penoxsulam was found statistically significant not only dose dependently for 24 h (r= -0.943 p= 0.01), for 48 h (r= -0.97 p= 0.01), for 72 h (r= -0.913 p= 0.01) and for 96 h (r= -0.914 p= 0.01) but also time dependently for 12. 5 µg/L (r= -0.957 p= 0.01), for 25 µg/L (r= -0.961 p= 0.01) and for 50 µg/L (r= -0.94 p= 0.01). The MI in 25 and 50 µg/L at 48, 72 h, and 96 h was lower than MMS. Unlike other result, Scorpion® (flumetsulam as active ingredient, one of the triazolopyrimidine sulfonamide herbicides) induced MI in *A. cepa* [14].

Penoxsulam treatment statistically decreased prophase index (except at 50 µg/L in 48h) and simultaneous statistically increased telophase index compared to control group. The prophase index reduction (except at 50 µg/L in 48h) may initiate mitosis by halting of interphase as a result of penoxsulam [15] while delay in mitotic cycle accomplishment is due to telophase index increase [16].

Table 2. *Allium* root growth inhibition test results of penoxsulam.

Doses (µg/L)	Average length (cm) ± SD*	Growth (%)	Decrease in growth (%)
Control	4.67± 0.08a	100.00	0
6.25	2.83±0.07b	59.53	40.47
12.5	2.49±0.06c	52.25	47.75
25	2.35±0.07d	49.25	50.75
50	1.83±0.1e	38.54	61.46
100	1.2±0.09f	25.27	74.73
250	1.02±0.05g	21.41	78.59
500	0.74±0.06h	15.85	84.15
1000	0.58±0.07i	12.63	87.37

* Means with the same letter do not differ statistically at the level of 0.05.SD: Standard deviation

Figure 1. *A. cepa* roots exposed to penoxsulam after 96 h. Doses from left to right 0, 6.25, 12.5, 25, 50, 100, 250, 500 and 1000 µg/L.

Table 3. Changes of mitotic and phase index of *A. cepa* root meristematic cells treated with penoxsulam.

Concentration (µg/L)	CCN	MI±SD*	Phase index (%)±SD*			
			Prophase	Metaphase	Anaphase	Telophase
Control-24 h	5080	71.69±0.61a	89.35±0.79a	1.81±0.19a	2.45±0.28a	6.39±0.6a
MMS-10 ppm	5134	56.51±0.53b	88.03±0.9b	2.83±0.34b	2.52±0.41a	6.62±0.36a
12.5	5113	61.58±0.75c	85.68±0.98c	2.51±0.31b	2.73±0.6a	9.08±0.52c
25	5093	58.53±0.67d	84.61±0.37d	2.45±0.28b	2.61±0.24a	10.33±0.35d
50	5032	57.07±0.25b	87.46±0.45b	2.51±0.2b	2.58±0.45a	7.45±0.38e
Control-48 h	5080	70.29±0.8a	88.88±0.29ab	2.41±0.2a	2.8±0.22a	5.91±0.33a
MMS-10 ppm	5072	55.45±0.93b	88.44±0.62a	2.24±0.14ab	2.7±0.32a	6.62±0.49b
12.5	5040	58±0.76c	86.15±0.88c	2.16±0.3ab	2.74±0.28a	8.96±0.67c
25	5090	55.19±0.6b	87.54±0.41d	1.99±0.2b	2.49±0.18a	7.98±0.5d
50	5080	51.97±0.66d	89.71±0.87b	1.63±0.19c	1.97±0.2b	6.7±0.62b
Control-72 h	5117	70.32±0.53a	89.47±0.63a	2.05±0.25a	2.59±0.38a	5.89±0.46a
MMS-10 ppm	5091	54.18±0.84b	88.83±0.54ab	2.1±0.5a	2.65±0.42a	6.42±0.42a
12.5	5153	55.05±0.99b	88.66±0.21c	2.19±0.29a	2.36±0.28a	7.79±0.36b
25	5122	51.62±0.57c	88.13±0.97bc	2.08±0.3a	2.35±0.42a	7.45±0.54b
50	5117	50.48±0.46d	88.11±0.78bc	2.05±0.35a	2.36±0.37a	7.47±0.68b
Control-96 h	5113	69.88±0.7a	89.48±0.46a	2.07±0.3a	2.71±0.22ab	5.74±0.43a
MMS-10	5151	53.42±0.23b	88.38±0.45b	2.07±0.37a	2.76±0.24b	6.79±0.39b
12.5	5128	53.53±0.86b	88.16±0.84b	2.19±0.4a	2.26±0.41a	7.39±0.62b
25	5142	50.43±0.98c	88.01±0.74b	2.04±0.28a	2.51±0.46ab	7.44±0.62b
50	5190	48.62±0.95d	88.19±0.42b	2.02±0.2a	2.46±0.29ab	7.33±0.48b

*Means with the same letter in the same column for each application time do not differ statistically at the level of 0.05. CCN: Counting Cell Numbers. SD: Standard Deviation.

Different types of CAs were induced by penoxsulam in *A. cepa* root ana-telophase cells are shown in Table 4 and Figure 2. Total CA frequency was increased significantly and dose dependently for 24 h (r= 0.686 p= 0.01), for 48 h (r= 0.641 p= 0.05), for 72 h (r= 0.796 p= 0.01) and for 96 h (r= 0.526 p= 0.05) and also time dependently for 12.5 µg/L (r= 0.788 p= 0.01), for 25 µg/L (r= 0.749 p= 0.01) and for 50 µg/L (r= 0.821 p= 0.01). But these CAs were lower than MMS. While the most visible CAs of penoxsulam was a stickiness (4% at 12.5 µg/L for 96 h), the least visible CAs of penoxsulam was an anaphase bridge (0.4% at 50 µg/L for 24 h). Unlike other result, penoxsulam did not show any genotoxic potential in Chinese hamster ovary cells, *Escherichia coli*, *Salmonella typhimurium*, rat lymphocytes and mouse bone marrow cells [17].

Table 5 presents the results of the comet assay obtained in *A. cepa* root meristamic cells after exposure penoxsulam. Penoxsulam induced DNA damage dose dependently for 24 h (r= 0.933 p= 0.01), for 48 h (r= 0.959 p= 0.01), for 72 h (r= 0.968 p= 0.01) and for 96 h (r= 0.927 p= 0.01) and also time dependently for 12.5 µg/L (r= 0.919 p= 0.01), for 25 µg/L (r= 0.908 p= 0.01) and for 50 µg/L (r= 0.842 p= 0.01). The highest DNA damage (141±2.65) was obtained from 50 µg/L of penoxsulam for 96 h and the lowest one (63±2.65) at 12.5 µg/L of penoxsulam for 24 h. DNA damage treated with 50 µg/L of penoxsulam (except 24 h) was higher than MMS. Penoxsulam caused induction of ROS production and DNA damage by comet assay in hemocytes of *Mytilus galloprovincialis* after 15 and 30 days [4]. Costa et al. [18] showed that penoxsulam induced DNA damage to the *Procambarus clarkii*. Penoxsulam also induced oxidative stress in *Oryza sativa* [19] and *Oreochromis niloticus* [20]. Penoxsulam exposure in the *A. cepa* might result in generation of ROS which could decrease root growth and MI and also increase CAs and DNA damage.

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Table 4. Genotoxic effects of penoxsulam on *A. cepa* root cells.

Concentration (µg/L)	CCN	Anaphase-Telophase Anomalies %					
		DAT	CL	S	P	AB	TA± SD*
Control-24 h	500	1.2	0.8	0.4	0.8	0.2	**3.4±0.55a**
MMS-10 ppm	500	2.2	3.2	3.2	3.8	1.6	**14±.1.00b**
12.5	500	2.4	3.6	3.4	0.8	-	**10.2±0.45c**
25	500	2.4	2.8	2.6	1.8	0.8	**10.6±0.89c**
50	500	1.8	3.4	3.6	2.4	0.4	**11.6±0.55d**
Control-48 h	500	0.2	1	0.6	0.8	-	**2.6±0.55a**
MMS-10 ppm	500	3	3.2	3.2	3.4	2.2	**14.8±0.45b**
12.5	500	2.2	2.6	3.2	1.8	0.6	**10.4±0.89c**
25	500	2	2.8	2.6	3.	1.4	**11.8±0.84d**
50	500	2	3	2.6	3.2	1.2	**12±0.71d**
Control-72 h	500	0.8	1	0.6	0.8	-	**3.2±0.84a**
MMS-10 ppm	500	2.6	3.4	3.4	3.2	2.4	**15±0.71b**
12.5	500	2.2	3.2	3.2	2.2	0.8	**11.8±0.45c**
25	500	2.6	2.8	2.8	3.2	1.2	**12.6±0.55cd**
50	500	2.8	3	2.8	3	1.4	**13.2±0.45d**
Control-96 h	500	0.6	1	0.6	1.2	0.2	**3.6±0.89a**
MMS-10 ppm	500	2.6	3.2	3.6	3.4	2.4	**15.2±0.84b**
12.5	500	2	3.2	4	2	1.4	**12.6±1.14c**
25	500	2	3.2	3	3.4	1.2	**12.8±0.84cd**
50	500	2.8	3.6	3.4	3.2	1	**14±1bd**

*Means with the same letter in the same column for each application time do not differ statistically at the level of 0.05. SD: Standard Deviation. CCN: Counting Cell Numbers. DAT: Disturbed Anaphase-Telophase. CL: Chromosome Laggards. S: Stickiness. P: Polyploidy. AB: Anaphase Bridge. TA: Total Anomalies

Table 5. DNA damage in *A. cepa* bulbs treated with penoxsulam.

Compounds	Concentration (µg/L)	DNA Damage (Arbitrary Unit ±SD)*			
		24 h	48 h	72 h	96 h
Negative control	-	2±1a	3±1a	3.67±0.58a	5.33±0.58a
MMS	10 ppm	108±4.36b	119.33±3.21b	124.33±1.53b	126.33±1.53b
Penoxsulam	12.5	63±2.65c	77.67±2.52c	88.33±2.08c	90.67±2.31c
	25	70.33±2.31d	115.67±2.08b	121.33±2.52b	133±2d
	50	101±3.61e	129.33±3.79d	135±2d	141±2.65e

* Means with the same letter do not differ statistically at the level of 0.05. SD: Standard Deviation

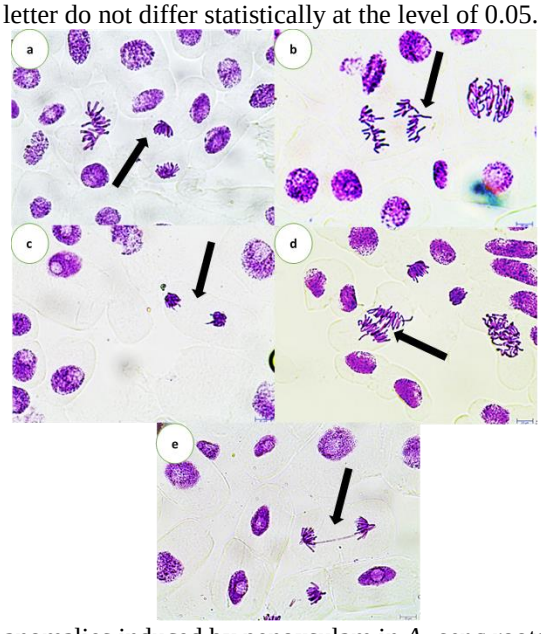

Figure 2. Anaphase-telophase anomalies induced by penoxsulam in *A. cepa* roots, a: Disturbed ana-telophase, b: Chromosome Laggards, c: Stickiness, d: Poliploidy e: Anaphase Bridge.

The *A. cepa* root cell quantitative analysis of penoxsulam by LC-MS/MS is shown Table 6. The penoxsulam amount observed was increased, both in terms of concentration (for 24 h r= 0.714 p= 0.01, for 48 h r= 0.693 p= 0.01, for 72 h r= 0.753 p= 0.01 and for 96 h r= 0.894 p= 0.01) and duration (for 25 µg/L r= 0.725 p= 0.01 and for 50 µg/L r= 0.792 p= 0.01). The highest value (0.0057±0.0012 ppb) was obtained from 50 µg/L of penoxsulam in 96 h, and the lowest one (0.0012±0.0004 ppb) was at 12.5 µg/L of penoxsulam in 24 h.

Table 6. Quantitative analysis of penoxsulam.

Compounds	Concentration (µg/L)	Amount (ppb ±SD)*			
		24 h	48 h	72 h	96 h
Negative control	-	-	-	-	-
MMS	10 ppm	-	-	-	-
Penoxsulam	12.5	0.0012±0.0004a	0.0013±0.0005a	0.0015±0.00005a	0.0017±0.0005a
	25	0.0015±0.0005a	0.0017±0.0005a	0.0018±0.0004a	0.0037±0.0008b
	50	0.0023±0.0005b	0.0025±0.0005b	0.0043±0.0015b	0.0057±0.0012c

* Means with the same letter in columns do not differ statistically at the level of 0.05. ppb: parts per billion. SD: Standard Deviation.

4. Conclusion

Results showed that penoxsulam induces cytotoxicity by reduction in root growth and MI and genotoxicity by increasing CAs and DNA damage to *A. cepa* roots. It should be considered when using because it poses a potential hazard to non-target organism. Further molecular toxicological evaluations involved in the cyto-genotoxicity of penoxsulam on plants are needed to confirm these results.

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Author's Contributions
Seçil Özkan: Drafted and wrote the manuscript, performed the experiment and result analysis.
Recep Liman: Assisted in analytical analysis on the structure, supervised the experiment's progress, result interpretation and helped in manuscript preparation.

Ethics
There are no ethical issues after the publication of this manuscript.

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