Polish J. of Environ. Stud. Vol. 19, No. 1 (2010), 193-199

Original Research

Mitodepressive and Cytotoxic Effects of Short-Term Exposure to Relatively Small Doses of Pendimethalin Evaluated by *Allium* Test

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Received: 4 July 2008
Accepted: 17 August 2009

Abstract

Pendimethalin is an extensively used dinitroaniline herbicide. The half-life of pendimethalin in soil can be long, and accumulation of this herbicide in the environment is probable. Therefore, we investigated the influence of pendimethalin (0.033, 0.066, 0.099, 0.132 and 0.264 g/L) on cell division during short-term treatment (24 and 48 hours) using *Allium* test. We observed inhibition of root elongation after 48 hours of incubation with pendimethalin. This effect was caused by the inhibition of mitoses varying from 1/3 to 1/2 in the case of 0.033, 0.066 and 0.099 g/L of pendimethalin, and almost complete restriction of mitoses under higher concentrations. Pendimethalin caused mitotic disturbances (c-metaphases, anaphasal and telophasal chromosome bridges, multipolar anaphases) and interphase abnormalities (micronuclei, multinuclear cells). This effect was irreversible during a 48-hour postincubation in water. Mitotic disturbances were caused by abnormalities in the organization of the tubulin cytoskeleton. Results suggest that even small amounts of pendimethalin can be a danger for dividing cells and embryos.

Keywords: mitotic index, mitotic aberrations, tubulin cytoskeleton, root anatomy

Introduction

The release of many organic and inorganic pollutants into all environmental elements (water, soil, and air) may be traumatic for living organisms. Their residues have caused strong cytotoxic effects [1-4]. Many kinds of pesticides are widely used in agriculture and therefore the residues of these chemicals pose a risk for local and global biocenoses. Plants (including transgenic ones [5]), owing to their settled style of life and constant exposure to pollution, are the universal models for toxicology screening studies. The *Allium* test is an efficient cytological model for chromosome aberration and mitotic activity assay of different environmental pollutants [1, 4, 6, 7-11].

One widely used component of herbicide for the control of annual grasses and certain broadleaf weeds in commercial crops is pendimethalin (in the USA, 2 to 4 million pounds in 1997 and 3 to 5 million pounds in 1999 [12]). One example of a pendimethalin-containing herbicide is STOMP 330 EC (330 g/l of pendimethalin). The herbicide is applied to the soil or on leaves (doses range from 3.5-6 l/ha). Pendimethalin has a similar chemical structure to nitro compounds such as dinitrobenzene. The U.S. Environmental Protection Agency classifies pendimethalin as a slightly toxic compound, class III, and a possible human carcinogen, group C (US Environmental Protection Agency [13]). There are some data stating that there is no

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Table 1. Increase of root length (cm±SE) in comparison to control after 24 hours of incubation in pendimethalin and 24 and 48 hours of postincubation in water.

	Control (incubation in H_2O)	Incubation in pendimethalin (g/L)				
		0.033	0.066	0.099	0.132	0.264
24 hours incubation	0.43 ±0.05	0.58 ±0.06	0.43 ±0.1	0.21* ±0.06	–	–
24 hours postincubation in H_2O	1.42 ±0.2	1.14 ±0.14	0.83* ±0.13	0.51* ±0.14	–	–
48 hours postincubation in H_2O	2.09 0.25	1.79 0.15	1.32* 0.16	0.87* 0.14	–	–

* differences between control and pendimethalin treated root length statistically significant (t-Student test $p<0.05$);
– no increase of root length observed.

correlation between pendimethalin exposure and the incidence of cancer. However, some other data indicate that occupational exposure to pesticides (including pendimethalin) may have serious consequences to both human health [14-15] and non-target live components of the environment [16-18].

In the face of contradictory data about pendimethalin's effects on living organisms, and taking into consideration the possible accumulation of pendimethalin residues in the environment [17, 19-21], we have tried to establish the action of this herbicide at the cellular level. This paper presents the results of the cytotoxic effects of pendimethalin, an active factor of the herbicide STOMP 330EC, on dividing cells. In our investigation we used the *Allium* test as an efficient procedure for evaluating the influence of relatively small doses and short-term periods of action of pendimethalin on mitotic activity and tubulin cytoskeleton of dividing onion cells.

Material and Methods

Meristematic cells of *Allium cepa* roots were the study material. The *Allium* test procedure was performed according to Fiskejö [6]. The effect of pendimethalin (STOMP 330 EC concern 330 g/L of pendimethalin) on mitosis was studied. The experimental concentrations of pendimethalin were 0.033, 0.066, 0.099, 0.132 and 0.264 g/L. These concentrations are relatively small (agriculture effective concentrations range between 0.115 and 0.2 g/m^2). The onion bulbs were cultured on distilled water in the dark and at room temperature for 2-3 days until the roots achieved 1-2 cm length. Then three bulbs were transferred to each pendimethalin concentration. Two different conditions of incubation were used: the first – 24 h of incubation in specific pendimethalin solutions, rinse in water and 48 h of postincubation in distilled water; the second – 48 h of incubation, rinse in water, and postincubation according to the above procedure. During the research we measured the increase in root length in control and the pendimethalin-treated bulbs.

After each incubation and postincubation period nine root tips (three from each of three bulbs) were cut off, fixed for two hours in Carnoy's fixer and stained in 2% acetoorceine. After staining, each root tip was squashed on a microscope slide for analysis of the mitotic index scored from 2,000 cells and aberration index scored from 500 mitoses [6].

Three root tips (one from each of three bulbs), after fixation in Carnoy's fixer, were dehydrated in ethanol, embedded in paraffin and sectioned into 7.5 μm sections. Microscopic slides with sections were stained with 0.1% alcianate blue after the Feulgen reaction.

Another three root tips (one from each bulb) were digested in an enzymatic solution (pH 5.0) containing 0.5% cellulase R-10 and 0.25% pectolyaseY-23 for 30 min. at 37ºC, and washed in 0.5% triton X-100 in MSB. Subsequently, the root tip was transferred to PBS (pH 7.0) and incubated with mouse monoclonal anti-α-tubulin antibody diluted 1:100 (PBS) for 12 h at 4ºC, washed in PBS, and incubated with monoclonal IgG rabbit anti-mouse antibody labelled with FITC (1:200 PBS) for 2-3 h at 37ºC. After labelling, material was washed in PBS (according to [22]).

Acetoorceine- and Feulgen-alcianate-blue-stained slides were studied with Olympus BX41 microscope. For observation of the tubulin cytoskeleton we used Olympus filter V-MNB2 (excitation wave length 470 nm). The results are shown in tables and figures.

For statistical evaluation of the collected data we performed Shapiro-Wilk W-test to check normal distribution and Levene L-test to check the homoscedastic of variances in particular experimental groups of results. For comparison between control and experimental groups Student t-test was performed. Tables 1 and 2 present data as average increase in length of roots ± standard error. Figs. 1 and 2 show results calculated as percent of dividing cells or percent of aberrations in dividing cells.

Results

Obtained results indicate that doses of 0.099 g/L, 0.132 g/L and 0.264 g/L of pendimethalin cause strong inhibition of root growth during 24-hour incubation, which was irre-

Table 2. Increase of root length (cm ± SE) in comparison to control after 48 hours of incubation in pendimethalin and 24 and 48 hours of postincubation in water.

	Control (incubation in H_2O)	Incubation in pendimethalin (g/L)				
		0.033	0.066	0.099	0.132	0.264
48 hours incubation	1.43 ±0.12	1.07* ±0.10	1.04* ±0.11	0.58* ±0.09	–	–
24 hours postincubation in H_2O	2.12 ±0.14	1.63* ±0.15	1.60* ±0.13	0.73* ±0.10	–	–
48 hours postincubation in H_2O	2.76 ±0.13	2.12 ±0.16	2.21 ±0.16	0.91* ±0.21	–	–

* statistically significant differences between control and pendimethalin-treated root length (t-Student test $p<0.05$);
– no increase of root length observed.

versible during postincubation. In the case of a concentration of 0.066 g/l, we observed inhibition of root growth just after postincubation. The lowest concentration of pendimethalin (0.033 g/L) did not cause inhibition of root growth (Table 1). After 48 hours of incubation in pendimethalin, we observed a reduction of root growth even at the lowest concentration of pendimethalin (Table 2).

Our results indicate that relatively small doses of pendimethalin caused a strong mitodepressive effect on the meristematic cells of root tips of *Allium cepa*. The mitodepressive action resulted in significantly reduced mitotic indexes (Fig. 1A and 1B). The highest concentrations of pendimethalin (0.132 g/L and 0.264 g/L) almost completely inhibited cell division. The effects of these concentrations

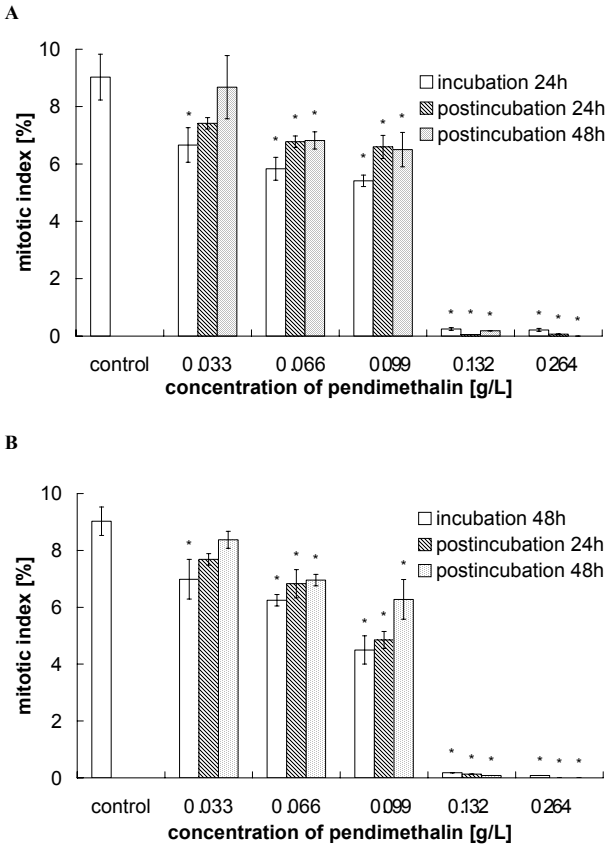

Fig. 1. Changes in the mitotic index of onion root tip cells after 24 hours of incubation in pendimethalin and 48 hours of postincubation in water (A), and 48 hours of incubation in pendimethalin and 48 hours of postincubation in water (B).
* – statistically significant differences between control and experimental groups (Student t-test $p<0.05$).

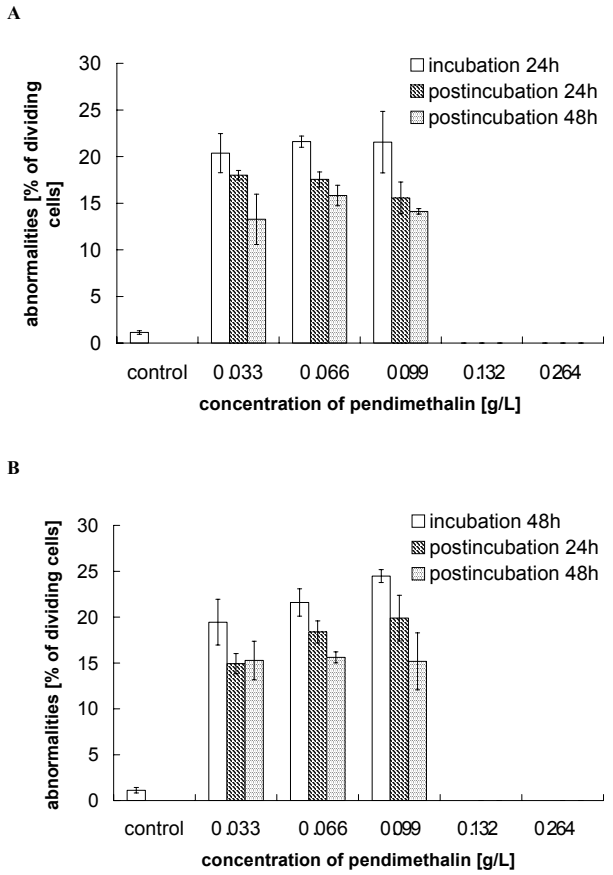

Fig. 2. Total number of mitotic irregularities in onion root tip cells after 24 hours of incubation in pendimethalin and 48 hours of postincubation in water (A), and 48 hours of incubation in pendimethalin and 48 hours of postincubation in water (B). In all cases of incubation and postincubation, Student t-test $p<0.05$ in comparison to control.

were irreversible during postincubation (Fig. 1A and 1B). Our data shows that the mitodepressive effect caused by 0.033 g/L, 0.066 g/L and 0.099g/L of pendimethalin was slowly reversible during postincubation. However, the rate of mitotic aberrations after 48h of postincubation was still much higher than in the control (Fig. 2A and 2B). In the case of the highest pendimethalin concentrations (0.132 g/L and 0.264 g/L), we could not estimate the rate of mitotic aberrations because the mitotic index in these cases was too low (about 0.05%, Fig. 1A and 1B). We noted the highest number of mitotic aberrations in the case of 48h of incubation in 0.099 g/L pendimethalin (about 25% of the dividing cells, fig. 2B). Cells that were incubated for 24h in concentrations of 0.033 g/L, 0.066 g/L and 0.099 g/L had a lower number of aberrations (about 20% of the dividing cells, Fig. 2A). Mitotic disturbances after treatment with pendimethalin concerned different stages: the metaphase (Fig. 3A and 3B), telophase (Fig. 3C), and anaphase (Fig. 3D and 3E). We noted chromosome aberrations, like anaphasal and telophasal chromosome bridges, caused by DNA breaks. This kind of aberration may cause the occurrence of interphasal anomalies like multinuclear cells and cells with micronuclei (Fig. 3F and 3G). Observed c-metaphases, multipolar anaphases, and delayed or eliminated chromosomes were probably caused by disorders in organization of the tubulin cytoskeleton. Tubulin cytoskeleton analyses indicate abnormalities in preprophase band organization (Fig. 4A, 4B, 4C), which could effect the abnormal direction of division; disturb the mitotic spindle, which could cause c-metaphases (Fig. 4D) and multipolar anaphases

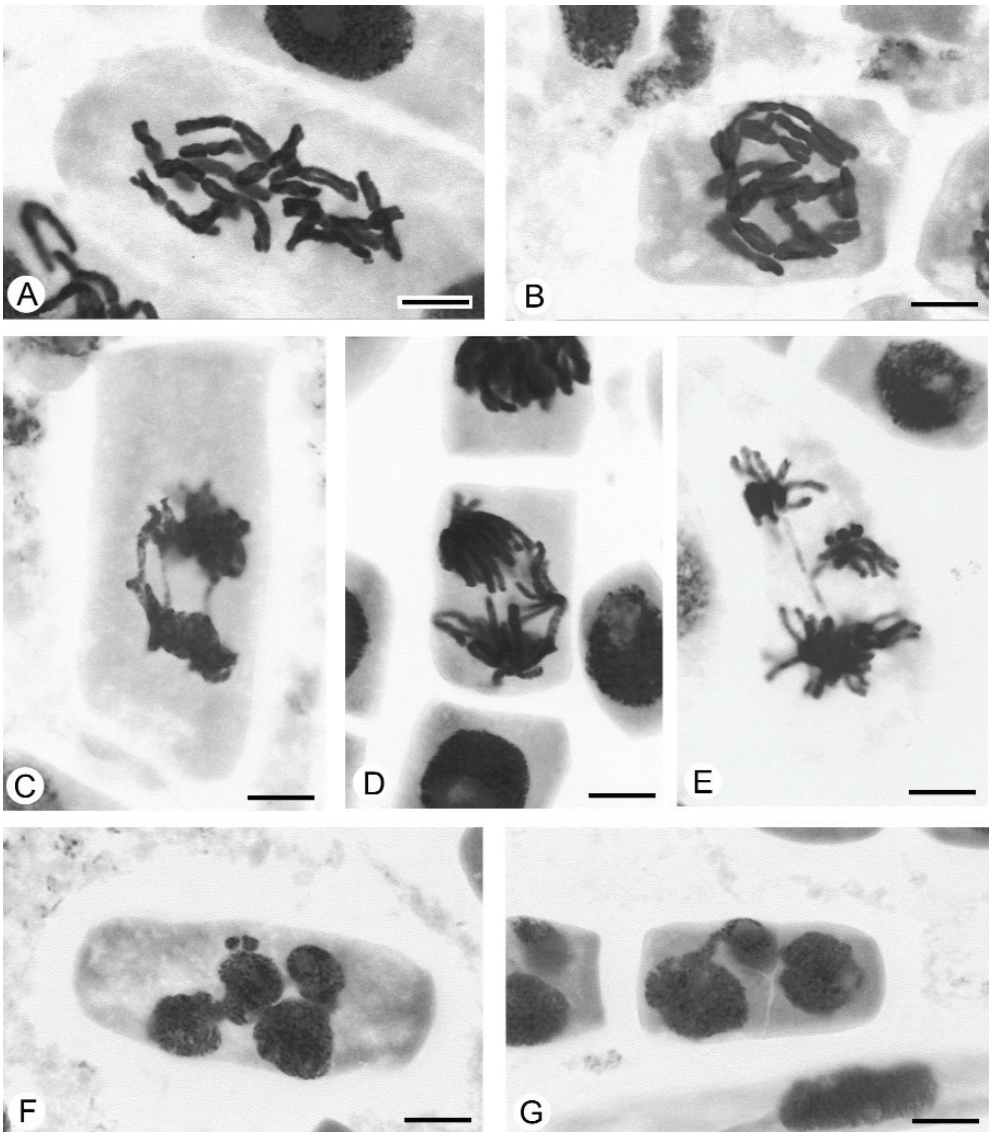

Fig. 3. Examples of most commonly observed mitotic and interphase abnormalities during treatment of onion roots with pendimethalin (bar = 5μm).
C-metaphases observed in root cells after 24 hours (A) and 48 hours (B) of incubation in 0.066 g/L of pendimethalin. Telophasal bridges after 24 hours of incubation in 0.099 g/L of pendimethalin (C). Tripolar anaphases in cells after 24 hours of incubation in 0.099 g/L of pendimethalin and 24 hours of postincubation in water (D), and after 48 hours of incubation in the same concentration of pendimethalin and 24 hours of postincubation (E). Tetra-nuclear cell with two micronuclei after 48 hours of incubation in 0.033 g/L of pendimethalin (F). Tri-nuclear cell with abnormally formed cell wall after treatment for 24 hours with 0.033 g/L pendimethalin (G).

(Fig 4E); and chaotically distributed microtubules (Fig. 4F), which could cause improper elongation of cells.

As a result of the disorders observed after treatment of the roots with lower concentrations of pendimethaline (0.033 g/L and 0.066 g/L), we observed swelling in the elongation zone of roots, especially after postincubation (compare Fig. 5A, 5B, 5C). In the region of swelling, we observed oddly shaped cells (Fig. 5D), and cells that had no normal plane of division (Fig. 5E).

Discussion

All observed abnormalities demonstrate that even short-term exposure to relatively small doses of pendimethalin significantly affects the mitotic index of root meristematic cells of onion and seriously disturbs mitotic spindle formation. The large number of c-metaphases suggests that mitotic spindle damage begins in the early stage of its origination. Chromosomal anaphase bridges affect normal caryokinesis and cytokinesis, as well as not allowing complete cell wall formation. The mitotic spindle is a dynamic molecular machine composed of tubulin, motor proteins, and other molecules. It has great importance for both division and differentiation of cells [23]. Observation of the action of dinitroaniline herbicides reveals that they can act like colchicine [24], affecting tubuline polymerization. Trifluralin and oryzalin bind specifically to tubulin, suggesting that tubulin is the primary sub cellular target for dinitroaniline herbicides. When the herbicide-tubulin complex is added to the growing microtubule, further growth of the microtubule is stopped [25-26]. Some results indicate that spindle microtubules are most sensitive, whereas cortical microtubules are the most resistant to oryzalin [25]. Our results indicate that the tubulin cytoskeleton is strongly affected also by pendimethalin, which disturbs both spindle and cortical microtubules. We consider that disturbance of the tubulin cytoskeleton to be responsible not only for mitotic abnormalities and cytostatic effects, but also for improper differentiation of cells.

Earlier investigations [27] demonstrate that depressive effect of pendimethalin on onion root tips cells starts between 10 and 1M, but functional disturbance can be observed at 0.1M. Author also have observed inhibition of root growth and thickening of the meristematic area of root. We observed similar effects, but at much lower concentrations of pendimethalin (about 0.1 – 1mM). Other results [28], contrary to ours, show that Stomp did not induce chromosomal aberration in the cells of *Crepis capillaris* (two hours treatment with concentration of 0.005 – 0.4%) but

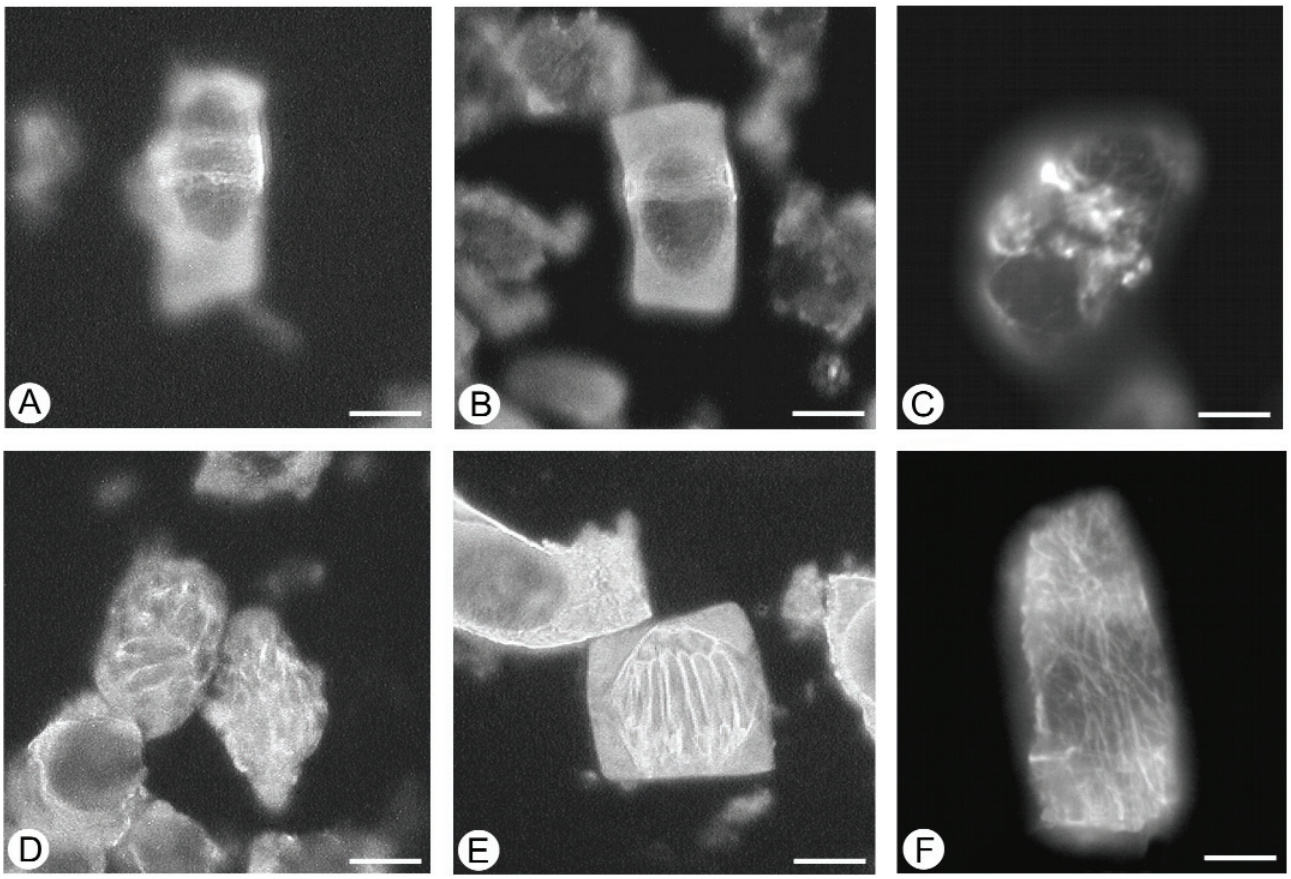

Fig. 4. Changes in organization of tubulin cytoskeleton in onion root tip cells caused by pendimethalin (bar = 7 μm).
Abnormalities in formation of preprophase band after 24 hours treatment with 0.066 g/L of pendimethalin: duplication of preprophase band (A), dislocation of preprophase band from the middle to pole of the cell (B), and disorganisation of preprophase band (C). Disorganisation of mitotic spindle microtubules, and image characteristic of c-metaphase (D). Widened pole of mitotic spindle during anaphase, which could cause multipolar anaphase (E). Chaotic distribution of cortical microtubules in a cell undergoing elongation (F).

increased their incidence in mouse cells (oral administration of 0.05 – 0.1 ml 15.5% Stomp solution). Authors of cited work suggest that the induction of aberrations in the case of mouse cells may be due to biosynthesis of genotoxic metabolites of pendimethaline. Cited results and our data indicate that different organisms show different responses to pendimethalin, depending on concentration. Among pendimethalin, its metabolites can be dangerous for living organisms.

Pendimethalin is not neutral for non-target plants and soil organisms. Persistent levels of pendimethalin at field application levels (about 10 mg/kg of soil) will impact plant communities; however, invertebrate communities are more tolerant to this herbicide. Nevertheless, ten times higher concentrations result in a near complete disruption of invertebrate reproduction [18]. Other data indicates that pendimethalin application rate of 0.75 and 1.0 kg/ha reducing soil nematodes by 35-60%. After application of these doses, soil microbiota including *Rhisobium* were affected. In water, a 50% lethal concentration for *Daphnia* was 78 μg/l [17]. Pendimethalin also affect the specific growth rate, cell number, chlorophyll a level and dry weight of the green alga *Protosiphon botryoides* [16]. Pendimethalin contamination can accumulate in the environment. A degradation study indicates that pendimethalin could be considered a non-leaching compound in soil [19, 29]. Terrestrial studies show that over 20% of the herbicide evaporates during the first week after application. The half-life of pendimethalin residues in soil varies from a few days to over 200 days, but in conditions of reduced temperatures and drought its half-life is prolonged to over 2,000 days [17, 20, 30]. Investigation of the distribution of residues of pendimethalin in the household represented by farm, rural, and urban houses, shows that residues of the pesticide are present in all types of tested households. The maximum concentration of pendimethalin residues vary from less than 1 μg/m^2 on flat surfaces to over 5 μg/m^2 in carpets [21]. Confirmed concentrations of pendimethalin in the household are not very high but taking into consideration its relatively long half-life and the still growing up usage of this herbicide, there is a distinct possibility of its accumulation in the environment and food. Pendimethalin was found (0.16-0.21 μg/g) in garlic plant 28 days after application. However, two weeks later residues of it were undetectable [30]. Probably the herbicide was transformed by garlic cells in derivatives of pendimethalin. Residues of pendimethalin and its metabolites have been found in fruits and vegetables [31]. This fact can be dangerous even to the human population, especially children and embryos. In our opinion, if the toxic effects of a certain chemical are detected in the dividing cells of higher plants, it can also occur especially in the case of fast-dividing embryonic cells and the quickly differentiating cells of children. Pendimethalin poses a risk in the case of reproductive cells and blood. There is evidence [3] that some pesticides cause cancer (other authors suggest that there is no association of pendimethalin exposure with cancer incidence [32]) and affected murine embryos [14] as well as chicken embryos (especially together with cadmium and copper ions [33]).

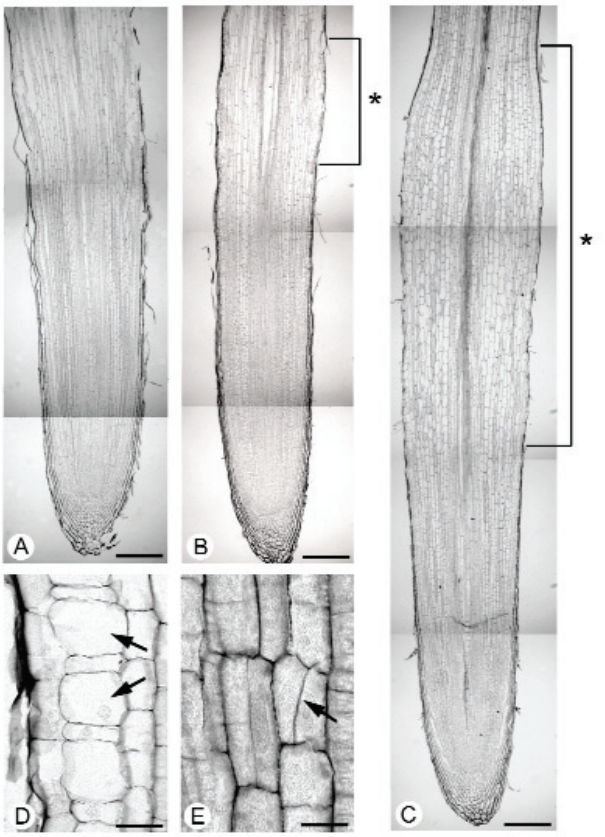

Fig. 5. Longitudinal sections of roots treated with pendimethalin. Longitudinal section of control root (A, bar = 100 μm), Section of root after 48 hours of incubation in 0.066 g/L of pendimethalin (B, bar = 100 μm) and after 48 hours of postincubation in water (C bar = 100 μm) area of swelling marked by asterisk. Not normal elongated cells (D, arrows) and atypical (diagonal) plane of division (E, arrow) in swelling area of root after 48 hours of incubation in 0.066 g/L of pendimethalin (bars = 10 μm).

Moreover *in vitro* experiments indicate that pendimethalin (0.1 μM) produces cytotoxicity and DNA damage in mammalian cells [34]. Based on our results and the results of other authors, we can conclude that pendimethalin is able to exert severe cytotoxic effects. Even small amounts of this compound can be dangerous, especially when it is extensively used in agriculture. Moreover, production as well as consumption of it is still increasing. Thus, only a relatively small safety margin exists, indicating that overapplication or point source contamination could impact all living organisms – including humans.

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