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TRIFLURALIN-INDUCED DISORGANIZATION OF MICROTUBULAR CYTOSKELETON ALTERS THE DEVELOPMENT OF ROOTS IN *HORDEUM VULGARE* L.

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The extensive use of herbicides in agriculture becomes an important factor in environmental pollution, especially in case of slowly degradable compounds. Some agents act on plants during a long period of time, even if a very low concentration of the herbicide remains in the soil. Here, we investigated the toxicological effect of a low concentration of dinitroaniline herbicide, trifluralin, on growing seedlings of *Hordeum vulgare* L. Trifluralin in concentration of 1 μg/ml inhibited root growth. The mitotic activity of meristematic cells was suppressed due to the retardation of metaphase progression – alteration that can be caused by cytoskeleton disorder. Using antibodies to α-tubulin, we investigated the distribution of microtubules in root meristem cells. During all stages of mitosis, the highly regular system of microtubular cytoskeleton observed in control cells was slightly disorganized. An examination of root structure using light and electron microscopy demonstrated that the cell walls did not form normally during cell division that led to the appearance of large multinucleated cells. Also, the premature (pathological) cell differentiation was induced by trifluralin. A part of differentiating cells showed intracellular structural changes that are consistent with programmed cell death. It seems that the development of alterations in trifluralin-treated roots was due to the microtubular cytoskeleton disorganization.

Keywords: Herbicides – trifluralin – roots – microtubules – differentiation – programmed cell death

INTRODUCTION

An extensive use of herbicides significantly contributes to environmental pollution. However, the toxicological action of most herbicides on plant and animal cells is poorly understood. Some agents act on plants during a long period of time, even if a very low concentration of the herbicide remains in the soil. It has been shown that grasses are more sensitive to dinitroanilines then dicotyledonous species [3]. These

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data indicate that diverse plant species have different sensitivity to herbicides. Therefore the residual amounts of certain herbicides can be toxic for some plants and relatively harmless to more resistant species. Low concentration of herbicides may alter plant cell structure and development in a different mode compared to high concentrations, used for inhibition of weed growth. Importantly, low concentration of chemicals may cause the development of drug resistance, and this unavoidably brings about the application of higher concentrations of herbicides and use of more potent compounds. A lot of known agents are slow degrading compounds, and after application they might remain in the soil for extended period, thus affecting plant growth and development. Here, we investigate the effects on plant cells of trifluralin, a dinitroaniline herbicide that has high persistence in soil [14, 22]. Trifluralin is extensively used for the control of weed growth in areas under sunflower, onion, rape, etc. The residuals of the herbicide in soil can damage the seedlings of cereals (barley, oats, wheat), that cultivated on the same areas on the next year after trifluralin treatment.

Trifluralin is a well-known agent disrupting microtubular cytoskeleton in plant cells [1, 8]. Microtubules (MTs) are an integral part of the cytoskeleton of plant cells. They are composed of dimers of α- and β-tubulins, and MT associated proteins. Some classes of herbicides, such as dinitroanilines, phosphoric amides and N-phenylcarbamates, are MT depolymerizing compounds [1, 4, 15, 23, 24]. The mechanism of their action on plants has extensively been studied. Dinitroaniline herbicides, such as orysalin, interact directly with α,β-dimers of tubulin [7, 16]. The incorporation of dimer-herbicide complexes into growing tubulin protofilaments leads to MT disruption.

Here, we investigated the effects of trifluralin used in low concentration (1 μg/ml) on root growth, root cell structure, and organization of MT arrays in *Hordeum vulgare L*. The concentrations used (0.05–1 μg/ml) were significantly lower than that used in the study of Ovidi et al. [18] (1.53 mg/ml, the concentration similar to that normally used in agriculture). This allows us to investigate the possible mechanism of action of trifluralin residuals using simple *in vitro* system. We demonstrated that this herbicide slightly modifies the organization of MT cytoskeleton, and these changes lead to significant alterations of the cell structure and plant development.

MATERIALS AND METHODS

Plant material and growth conditions

Barley roots (*Hordeum vulgare L.*) were used as a subject of investigation. The seeds were germinated in 100 mm Petri dishes filled with filtered distilled water or trifluralin solution (0.05–1 μg/ml) at 24 °C in the dark. Treflan 24EC (a commercial formulation of trifluralin, lot NA21150121) was purchased from Dow AgroSciences.

Measurements of root growth

The root elongation rates of control and trifluralin-treated plants were determined on digital pictures of Petri dishes containing barley seeds. Measurements of the root length were performed with the public domain ImageJ program (available free on Internet at http://rsb.info.nih.gov/ij/). Three independent experiments did not show significant differences in length measurements.

Determination of the trifluralin effect on the mitotic indices (MIs)

The roots (five for each experiment) were placed in a solution of ethanol (99%) and glacial acetic acid (3 : 1) for 24 h, washed with distilled water three times, and then stained using acetic orcein (1% solution in 45% acetic acid). The stained root tips were squashed in 45% acetic acid and observed under the light microscope. 200 cells for each root tip were analyzed.

Immunostaining

The immunolabeling was done as described elsewhere [13]. Briefly, the root tips were fixed in fresh 4% paraformaldehyde prepared in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$) for 1.5–2 h and then incubated for 15–20 min in 0.4 M mannitol solution containing 1% cellulase and 5 mM EDTA. After maceration, cell suspension was dried overnight at 4 °C. Then preparations were incubated for 30 min in 0.5% Triton X-100 prepared in PHEM buffer supplemented with 5% DMSO. The mouse monoclonal antibodies against α-tubulin (clone DM1A) (Sigma) were used as primary antibodies. After several washes in Tris-HCl with 10 mM $MgCl_2$ and 0.1% BSA, cells were incubated with AlexaFluor-488-conjugated antibodies (Molecular Probes) for 45 min at 37 °C. Cells were embedded in Vectashield (Vector Laboratories) containing DAPI. The preparations were analyzed using a microscope (Axiovert 200M, Carl Zeiss) equipped with Neofluar 100×/1.3 oil immersion objective. Images were recorded with CCD-camera Axiocam HRm (Carl Zeiss).

Microscopy

For electron microscopy, cells were fixed for 2 h in 2.5% glutaraldehyde (Sigma) prepared on 0.1 M Sorensen buffer (pH 7.4), postfixed with 1% OsO_4 for 2 h, dehydrated (70% ethanol contained 2% uranyl acetate) and embedded in Epon 812 (Fluka). Epon blocks were used for analysis of general organizations of root tips. The samples were investigated and photographed with a stereomicroscope (Leica) equipped with digital camera. Semithin sections (1 μm) were obtained from the Epon

blocks using LKB Ultratome-III. The sections were attached to the microscope slides, stained with toluidine blue (Sigma) and examined in Axiovert 200M microscope (Carl Zeiss). Ultrathin sections were cut with a LKB Ultratome-III, stained with lead citrate and examined in HU-11B transmission electron microscope (Hitachi).

RESULTS

The effect of trifluralin on root growth and morphology

We have analyzed the effect of different concentrations of trifluralin (0.05–1 μg/ml) on the germination of seeds. The number of germinated seed at low doses (0.05–0.25 μg/ml) of trifluralin was slightly higher than in control (Fig. 1A). At 0.5–1 μg/ml of trifluralin, the number of germinated seeds decreased, yet the difference was not sig-

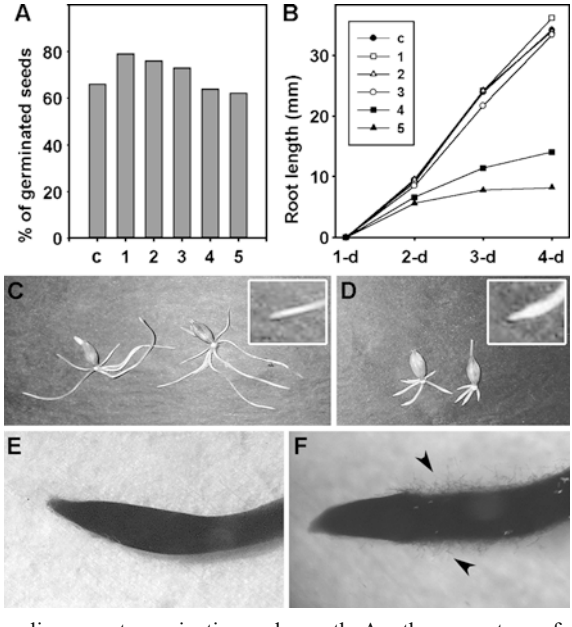

Fig. 1. Effect of trifluralin on root germination and growth. A – the percentage of germinated 2-day-old seedlings in control (c) and in the presence of different concentrations of trifluralin (1 – 0.05 μg/ml, 2 – 0.1 μg/ml, 3 – 0.25 μg/ml, 4 – 0.5 μg/ml, 5 – 1 μg/ml). B – root length in seedlings germinated in distillate water (c – control) and different concentrations of trifluralin (concentrations are the same as in A). The graphs A and B are representative examples of three identical experiments with similar outcome. C, D – morphology of germinated 3-day-old seedlings. The roots of control plants (C) are longer and thinner than the roots of trifluralin-treated (1 μg/ml) plants (D). The inserts illustrate the enlarged root tips. E, F – root tips morphology of samples fixed with glutaraldehyde and osmium tetroxide. The numerous root hairs (arrowheads) surround roots of trifluralin-treated seedlings (F), but are never observed near root tips of control plants (E)

nificant (66% of germinated seeds in control versus 62% in the presence of trifluralin).

The effect of trifluralin on the growth of roots was dose-dependent (Fig. 1B). Low concentrations of trifluralin (0.05–0.25 μg/ml) did not alter the root growth, but at a concentration 0.5–1 μg/ml the growth was inhibited. Figures 1C and 1D show the effect of trifluralin treatment (1 μg/ml) on seedling morphology. Besides the reduced root length, the enhanced thickness of roots is clearly visible (inserts in Fig. 1C and D). The root tips were investigated using stereomicroscope (Fig. 1E and F). The trifluralin-treated roots were slightly thicker than control and were surrounded above the zone of meristem by a dense 'halo' of root hairs (arrowheads in Fig. 1F), which were never visible in this region of control plants. Thus, the enhanced thickness of trifluralin-treated roots was partially due to development of numerous root hairs.

Effect of trifluralin on the mitosis progression

The root growth is a complex process that depends on the rate of cell proliferation in meristem region. Therefore, the mitotic indices (MIs) were studied in control and in

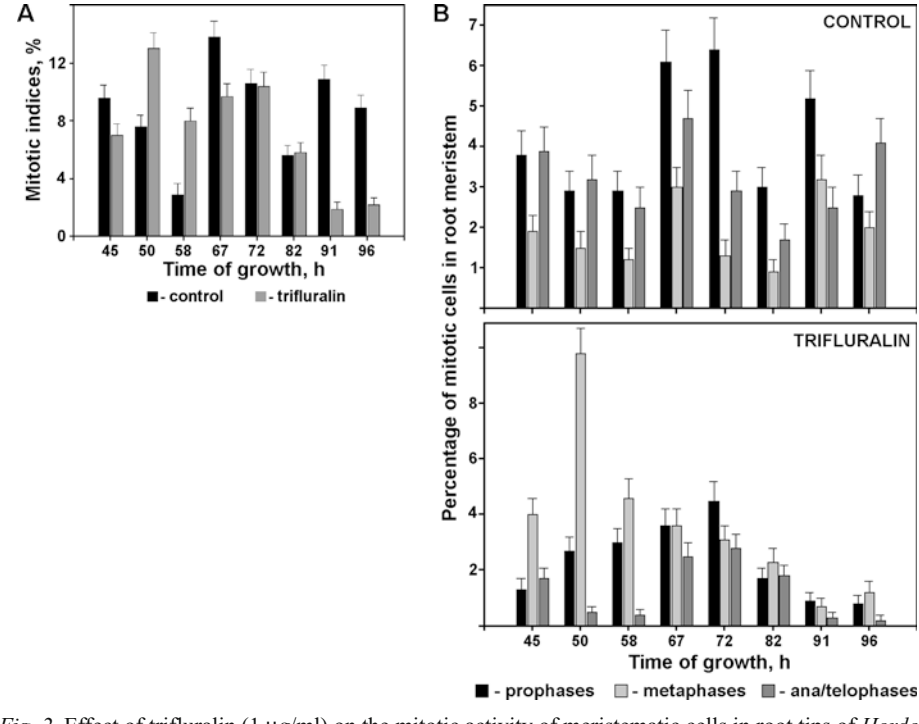

Fig. 2. Effect of trifluralin (1 μg/ml) on the mitotic activity of meristematic cells in root tips of *Hordeum vulgare* L. (A) Mitotic indices for control and trifluralin-treated seedlings. (B) Mitosis progression in root meristem of *Hordeum vulgare* L.

trifluralin-treated (1.0 µg/ml) root tips (Fig. 2A). The MIs had no constant value, but were rather altered during root growth, and these alterations had "wavy" character. The peaks of the fluctuations were slightly shifted among trifluralin-treated seedlings if compared to non-treated ones (50 h and 72 h versus 67 h and 91 h). On the other hand, the decline of MI was evident in 4-day-old (91 h and 96 h) trifluralin-treated seedlings (Fig. 2A). However, it is not clear what is the mechanism of growth inhi-

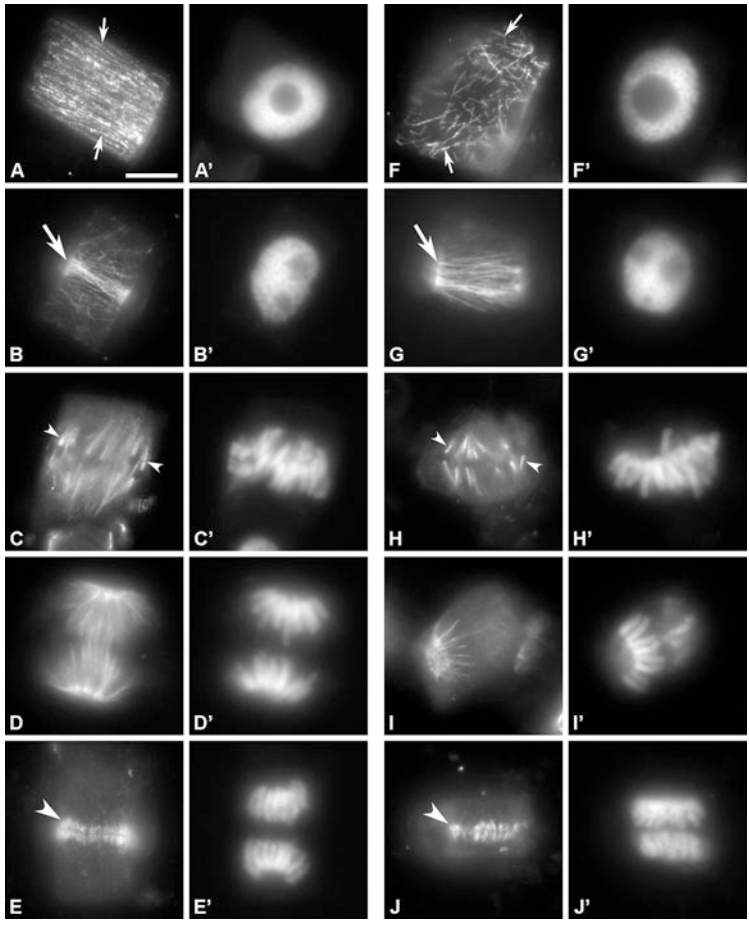

Fig. 3. Immunofluorescent images of root meristem cells of control (A–E) and trifluralin-treated seedlings (F–J). MTs are revealed using antibodies against α-tubulin. Images of nuclei and chromosomes stained with DAPI are marked by corresponding letters with apostrophes. A – interphase cell contains a regular system of cortical MT arrays. F – irregular network of cortical MT arrays in trifluralin-treated interphase cell. MT arrays are indicated by small arrows. Preprophase band (large arrow) in cortical cytoplasm of control (B) and in herbicide-treated prophase cells (G). The spindle organization in metaphase (C and H) in anaphase cells (D and I). Small arrowheads indicate the MT arrays forming mitotic spindles (K fibers). Phragmoplasts in telophase cells (E and J) are indicated by large arrowheads. Scale bars = 10 µm

bition – the block of cell division or an elongation of the cell cycle. Therefore, we determined the percentages of divided cells in different mitotic stages (Fig. 2B). The percentage of metaphase cells among mitotic cells was significantly higher if compared to control ones. These data indicate that the time of metaphase progression increased, but trifluralin (1.0 μg/ml) did not induce total block of metaphase progression. This delay in metaphase progression may be due to alterations in cytoskeleton organization.

Effects of trifluralin on the MT cytoskeleton in root meristem cells

The effect of trifluralin (1.0 μg/ml) on MT cytoskeleton was studied by immunofluorescent labeling of MTs with antibodies against α-tubulin. MTs formed several distinct arrays during the cell cycle in root meristem cells. Interphase cells displayed cortical MT arrays (Fig. 3), prophase cells had preprophase band of MTs and prophase spindle (Fig. 3B), metaphase and anaphase cells (Fig. 3C, D) formed the mitotic spindle, and cells in telophase developed phragmoplast (Fig. 3E).

After trifluralin treatment, cortical MT arrays of interphase cells were replaced by irregular web-like network (Fig. 3F). The network arrays were shorter and thicker than cortical MT bundles of untreated cells. In prophase cells, the appearance of preprophase band was not significantly altered (Fig. 3G). Vast majority of cells during metaphase had some distinctive features, if compared to untreated cells. Their mitotic spindles were composed of short and densely packed K-fibers (Fig. 3H). About 10% of metaphase cells had residual MT bundles emanating form the centromere regions of the chromosomes scattered in the cytoplasm (data not shown). These cells were arrested in C-metaphase. The chromosome segregation during anaphase was not disturbed, but MT bundles were altered similar to that observed in metaphase cells (Fig. 3I). In telophase cells, the distribution of MTs in the phragmoplast was not as uniform as in untreated cells, and the packing of MTs within phragmoplast seemed to be more compact than in untreated cells (Fig. 3J). These data show that drug treatment resulted in selective disorganization and shortening of MT arrays, indicating that MTs were affected by the trifluralin.

Morphology of trifluralin-treated roots

For detection of sensitive to trifluralin treatment cell types, we have analyzed the semithin sections of roots (Fig. 4). In control roots, the meristem and zone of active division were clearly visible (Fig. 4A). The formation of longitudinal cell walls was typical for proliferating cells in the zone of active division, so these cells were expanded transversely (Fig. 4C). The distinct zones of root were also visible in trifluralin-treated seedling (Fig. 4B). However, the region of apical meristem was much smaller if compared to the untreated roots. It appears that the zone above meristem corresponded to the zone of active division in control roots. However, the size of this

zone was decreased if compared to the untreated roots. In this region, we have found multinucleated cells with abnormal cells walls (Fig. 4D). The analysis of orcein-stained preparations showed that the percent of multinucleated cells in trifluralin-treated roots reached 36.9%. The zone above the region, in which we revealed numerous multinucleated cells with damaged cell walls, may be considered as an analogue of a zone of elongation and differentiation. In particular, root hairs were seen on the root periphery (Fig. 4B, E). In normal conditions, root hairs grow in the

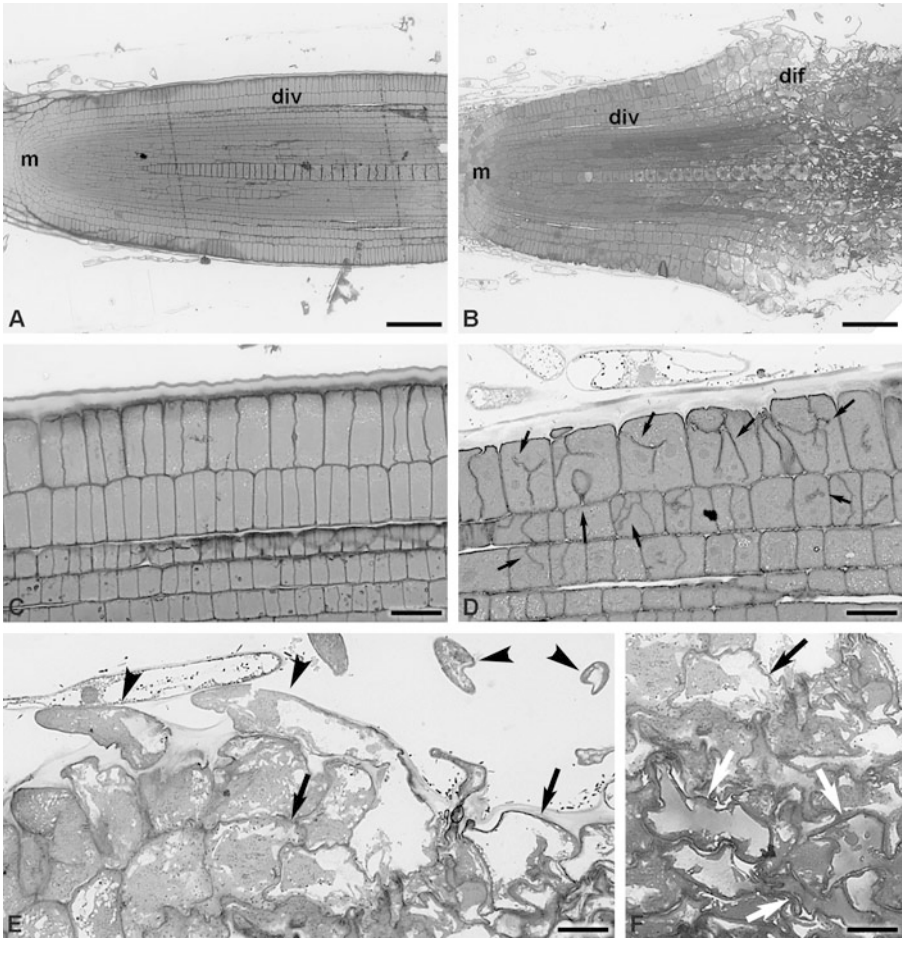

Fig. 4. Morphology of control and trifluralin-treated roots (semithing sections stained with toluidine blue). Root tip organization in control (A) and in trifluralin-treated plants (B). m – meristem, div – division zone, dif – differentiation zone. Large magnification of division zone in control (C) and in trifluralin-treated plants (D). Irregular cell walls inside multinuclear cells are indicated by small arrows. E, F – differentiation zone in trifluralin-treated root. Root hairs marked by arrowheads, cells with deformed cell walls – by large black arrows. Cells with highly basophilic cytoplasm and basophilic vacuoles are indicated by white arrows. Scale bars: A, B = 100 μm; C–F = 20 μm

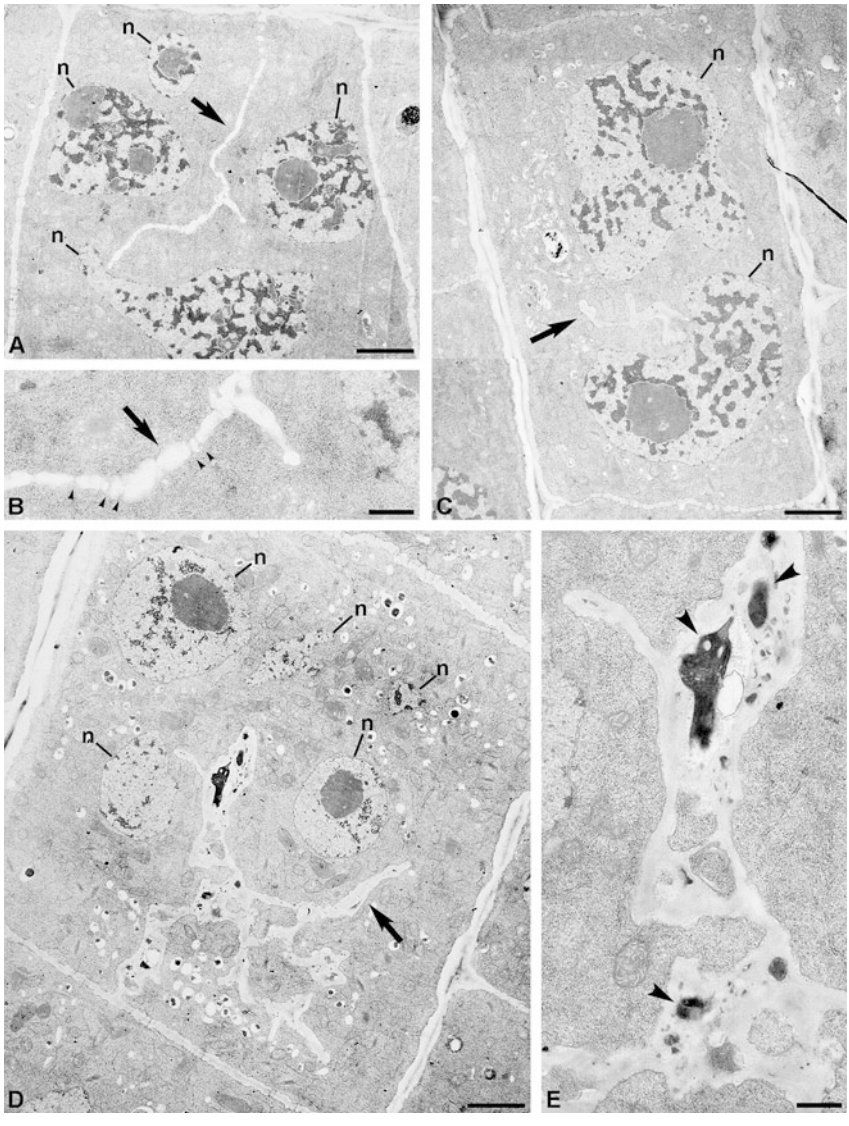

Fig. 5. Ultrastructural organization of multinucleated cells in division zone of trifluralin-treated roots: organization and formation of abnormal cell walls. A – multinuclear cell (n – nuclei), B – fragment of the cell wall (arrow). Plasmodesmata are indicated by small arrowheads. C – binuclear cell with plate-like cell wall (arrow). D – multinuclear cell with branched cell wall (arrow) having extremely thickening central segment (E) with electron dense inclusions (large arrowheads). Scale bars: A, C, D = 5 μm; B, E = 1 μm

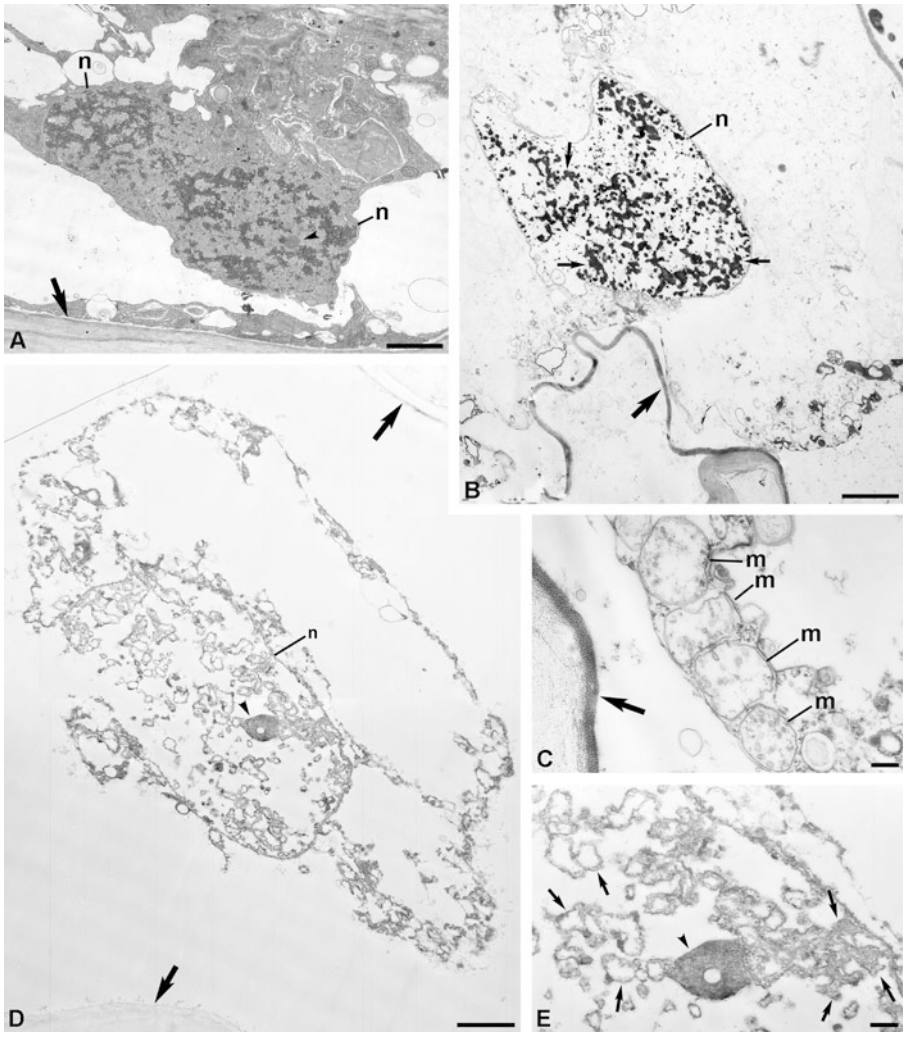

Fig. 6. Morphology of cells in the differentiation zone of trifluralin-treated roots: structural changes that are consistent with PCD. A – a cell with condensed cytoplasm. n – nuclei, nucleolus is indicated by small arrowhead. B – a cell with ruptured tonoplast. Small arrows indicate electron dense complexes of condensed chromatin (so-called chromonemata). C – enlarged fragment of this cell. The cytoplasm with swollen mitochondria (m) is separated from cell wall (large arrows). D – shrinkage of protoplast. E – enlarged fragment of this cell. The chromonemata are either filled with chromatin residuals (small arrows in the right part of the figure), or these residuals are digested, and the only peripheral shell of these chromatin complexes is not destructed (small arrows in the left part of the figure). Scale bars: A, B, D = 5 μm; C, E = 1 μm

region of differentiation. Thus, trifluralin induced the atypical development of these highly specialized structures. We also observed large vacuolated cells of irregular shape (black arrows in Fig. 4E, F). Some vacuoles contained basophilic material, which was never observed in untreated roots (white arrows in Fig. 4F). These cells probably appeared due to premature cell differentiation.

Ultrastuctural analysis

The organization of irregular cell walls inside large multinucleated cells was studied using high-resolution ultrastructural analysis. The morphology of the cell wall correlated with the number of nuclei: we detected small plate-like cell walls in binucleated cells (Fig. 5C) and branched cell walls in multinucleated cells (Fig. 5A). Numerous plasmodesmata were visible inside these cell walls (Fig. 5B). However, we also observed cells with very thick cell walls containing fewer plasmodesmata (Fig. 5D). Such cell walls contained dense inclusions of various size and morphology (arrowheads in Fig. 5E), and were typical only for very large multinuclear cells.

The cells of the region of differentiation in trifluralin-treated roots had irregular shape, but the cytoplasmic organelles and chromatin complexes were not changed (data not shown). However, some cells with cytoplasm of enhanced electron density were also observed (Fig. 6A). The nucleoli were small and dense, indicating that transcription in these cells was inhibited. The condensation of cytoplasm is the typical feature of the programmed cell death (PCD) [19]. Also, DNA fragmentation and activation of protease activity, typical features of programmed cell death, were observed (data not shown). The percent of cells with condensed cytoplasm was significantly higher in the upper region of differentiation and, especially, on the 5th day of trifluralin treatment. The dead cells appeared in 4- and 5-day-old plants treated with trifluralin. The cell presented in Fig. 6B had swollen cytoplasm. The tonoplast was ruptured, indicating that the swelling was due to the degradation of cytoplasmic organelles (Fig. 6C). The nuclear structures were altered: the condensed chromatin was extremely dense, interchromatin spaces did not contain material, which usually occupied the regions between condensed chromatin complexes in control nuclei. The protoplast in some region did not contact the cell wall. In some cells, the chromatin was partially digested (Fig. 6D, E), but the periphery of condensed chromatin was not damaged. Thus, three types of cells were detected – cells with condensed cytoplasm, cells with swollen cytoplasm and condensed chromatin, and cells with digested nuclei. These cell types may represent either different stages of PCD induced by trifluralin or different types of PCD.

DISCUSSION

Ovidi et al. [18] have found that 1.53 mg/ml of trifluralin (Treflan) led to complete depolymerization of MTs in generative cells of pollen tubes (this concentration was

similar to that normally used in agriculture). In order to investigate toxicological effects of trifluralin residuals on plants, we used lower concentrations of herbicide (0.05–1 μg/ml). It appeared that this concentration of the herbicide induced disorganization of interphase and mitotic MT arrays, yet all systems retained their structural entities. The effect of the drug on the different MT systems also varied. Thus, preprophase band was not affected notably, yet K-fibers of the spindle lost regular alignment and shortened. These results indicate that trifluralin induced selective depolymerization of MTs. The latter did not, however, cause mitotic arrest, but affected the growth of phragmoplast during anaphase-telophase. Alterations in phragmoplast development resulted in formation of multinuclear cells with aberrant cell walls. It should be stressed that recently it was described that trifluralin leads to a loss of control in the cellular division in *Allium cepa*, bringing about polyploid cells [5]. The exceeding genetical material of theses polyploidized cells tends to be eliminated from the nucleus in the form of micronucleus [5]. It seems that this phenomenon is different from the micronuclei formation due to telophase pathology described here.

Disorganization of cortical MT arrays in trifluralin-treated rootlets may contribute to the changes in shape and size of growing cells and overall root development. We believe that the disorganization of the interphase MT cytoskeleton results in the inhibition of directional cellular elongation. Elongating cells lose the ability to regulate the directional growth, expand not only longitudinally but also transversely, and this leads to the formation of large cells with irregular shape.

Our results showed that early stages of root growth are less sensitive to trifluralin than later stages of differentiation. Trifluralin also does not influence the process of seed germination. Morphologically, the roots of seedlings germinated in the presence of herbicide are thicker than the roots of control plants, and this may occur due to the inhibition of directional cellular growth induced by the loss of cortical MTs. The trifluralin-treated roots are surrounded by a 'halo' of root hairs, developing in the region of elongation, where they are never observed in control roots. Thus, the process of root hair differentiation is not influenced by trifluralin, yet their growth is shifted down the root length, toward the root tip.

The premature differentiation of cells in the region of elongation induced by trifluralin, suggests the disorganization of MT cytoskeleton may be the reason of atypical root development. Our study demonstrated the presence of cells with condensed cytoplasm in 4-day-old roots that is a typical feature of plant cell during PCD [19].

The PCD observed in trifluralin-treated roots could be induced by irreversible depolymerization of MTs. Although the direct relationship between MT dynamics and PCD remains to be elucidated, some authors suggested that disruption of MTs might be a specific and early sign of a PCD [11, 20, 21]. Binet et al. [2] demonstrated that cryptogein, a protein secreted by *Phytophthora cryptogea*, caused a rapid disruption of MT network and induced the death of cells. Both processes depended on calcium influx, but direct role of MT loss for induction of PCD was not demonstrated. There are no sufficient data available to conclude that disorganization of MT network by trifluralin is directly involved in induction of PCD [9]. Another possibility would be that trifluralin interferes in regular differentiation of root meristem cells,

followed by the pathological differentiation pattern. This eventually leads to the activation of PCD. The final stage of PCD in trifluralin-treated roots was accompanied by tonoplast rupture. According to the published data, a vacuole rupture promotes PCD during differentiation of tracheary elements in *Zinnia elegants* L. [6, 12, 17; for review see: 10]. One can assume that trifluralin activates the mechanism similar to that described for the differentiation of tracheary elements.

Summarizing the data discussed above, we conclude that trifluralin in a relatively low concentration induced the disorganization of MT network, followed by altered mitotic events, abnormal differentiation of cells, and finally led to death of differentiated cells. Thus, the roots of seedlings germinated *in vitro* are a convenient model for investigations of mechanisms of herbicides toxicity and screening of resistant variants. An attractive feature of the experimental approach described here is the possibility to investigate various features of herbicide action and to determine, at least roughly, which of them are induced by herbicide or the induced by primary defects in cell structure (primary and secondary effects, respectively). It seems that the screening of secondary effects is a more simple way to study herbicide toxicity than the analysis of MT skeleton. The results of the current paper suggest that even relatively low concentrations of herbicides may disturb plant development. This is important for the slowly degradable herbicides since low concentrations may persist for a long time in the soil. It seems that the toxicological action of residuals of highly persistent herbicides may occur through the mechanisms different from those described for the high concentrations of the same drugs.

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