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INFLUENCE OF N,N–BIS(3–AMINOPROPYLO)DODECYLOAMINE ON THE ULTRASTRUCTURE OF NUCLEI IN *ASPERGILLUS NIGER* MYCELIUM AND ON CELL PROLIFERATION AND MITOTIC DISTURBANCES IN *ALLIUM CEPA* L. ROOT MERISTEM

BARBARA GABARA[1*], JOANNA KALWINEK[1], ANNA KOZIRÓG[2], ZOFIA ŻAKOWSKA[2], AND BOGUMIŁ BRYCKI[3]

[1]*Department of Plant Cytology and Cytochemistry, University of Łódź, ul. Banacha12/16, 90-227 Łódź, Poland*
[2]*Institute of Fermentation Technology and Microbiology, Technical University, ul. Wólczańska 171/173, 90-530 Łódź, Poland*
[3]*Department of Chemistry, Adam Mickiewicz University, ul. Grunwaldzka 6, 60-780 Poznań, Poland*

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Hyphae growing for 1 h in the presence of 0.05% and 0.1% N,N-bis(3-aminopropylo)dodecyloamine (APDA) showed malformations in the nuclear structure, including changes in shape, budding, progressive disappearance of the nucleolus and chromatin, and damage of the nuclear envelope. The Allium test revealed that at both concentrations used (0.005%, 0.01%) the tested substance lowered the frequency of dividing cells and induced mitotic abnormalities such as c-metaphases, anaphase-telophase bridges and sticky chromosomes, lagging chromosomes, micronuclei, binucleate cells, budding nuclei and partial extrusion of the nucleolus from the nucleus. These data indicate that APDA has cytotoxic, mitodepressive and turbogenic effects, especially at the higher dose. The results showed that APDA may be safely used as a microbicidal agent to destroy microorganisms developing on experimental material.

Key words: *Aspergillus niger*, *Allium cepa*, nuclear ultrastructure, mycelium, root meristem, mitotic index, disturbances in mitosis, N,N–bis(3–aminopropylo)dodecyloamine.

INTRODUCTION

Disinfectants that quickly and efficiently kill mold fungi have been sought by researchers for years (Denyer and Stewart, 1998; Strzelczyk, 2001). N,N-bis(3-aminopropylo)dodecyloamine (APDA), a derivative of fatty amines with two amino groups and a 12-carbon aliphatic chain, is a triamine that seems to meet this need; at 0.05% and 0.03% concentrations it completely blocked germination of *Aspergillus niger* conidia, and at 0.01% it diminished the number of germinating conidia and retarded this process by 3 h (author's, unpubl. data). The reaction of 48-h-old *A. niger* mycelium to APDA was slightly different. Despite degeneration of some hyphae after 1 h and significant changes in the ultrastructure of numerous cells following 0.05% APDA treatment for 2 and 4 h, the mycelium kept on growing and after 24 h it resembled the control. On the other hand, in the presence of 0.1% APDA, after 24 h only multilamellar bodies were visible in most of the *A. niger* cells as a result of progressive destruction of hyphae (author's unpubl. data). The organelles most resistant in such hyphae were mitochondria, which had partly preserved inner membranes.

In the present study we investigated how APDA affected the ultrastructure of nuclei in *A. niger* mycelium. To check whether APDA induced mutagenic effects, we also applied the Allium test, commonly employed for studies of physical and chemical agents (Fiskesjö, 1985; Dash et al., 1988; Wierzbicka, 1988; Kristen, 1997; Ateeq et al., 2002).

*e-mail: bargab@ biol.uni.lodz.pl

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MATERIALS AND METHODS

MATERIALS

The experiments used *Aspergillus niger* strain Ł0 439 from the Culture Collection of the Institute of Fermentation Technology and Microbiology, Technical University of Łódź, registered as LOCK 105 in the World Collection, as well as adventitious roots of *Allium cepa* var. *Polanowska* (Polan, Cracow, Poland).

CULTURE CONDITIONS

Conidia of *Aspergillus niger* were inoculated to contain 1 ml/10^8 cells of mineral medium composed of 3% glucose, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.5% $MgSO_4 \times 7H_2O$, and 0.5% yeast extract, pH 6.2 (before sterilization). Conidia were germinated in 250 ml Erlenmeyer flasks in static conditions at 30°C. When 48-h-old mycelia appeared, N,N–bis (3–aminopropylo)dodecyloamine (APDA) (IODEX, Poznań, Poland) was added to the mineral medium at concentrations of 0.05% and 0.1% (v:v; each) and the fungi were grown for a further 1 h.

Root tips from *Allium cepa* bulbs 1.5 cm long, grown on Hoagland's medium, were transferred to 0.005% and 0.01% (v:v, each) APDA dissolved in distilled water as recommended by IODEX, for 1, 2 and 24 h. The APDA concentration had to be reduced because the roots became slimy and completely destroyed at the concentrations applied to *A. niger*. To check whether APDA effects can be reversed, some roots were post-incubated in Hoagland's medium for 24 h after their 1 or 2 h treatment. Roots kept only in distilled water and others subsequently transferred to nutrient medium were the control.

LIGHT MICROSCOPY

Root meristems were fixed in freshly prepared acetic ethanol (1:3, v/v) for 24 h and stained by the Feulgen method. Squashed preparations were made using the dry ice method. The mitotic index and the frequency of disturbances in mitosis (c-metaphases, anaphase-telophase bridges, lagging chromosomes, micronuclei, binucleate cells, budding nuclei, cells with partial extrusion of the nucleolus from the nucleus) were calculated in 10,000 cortex cells of 10 onion root meristems in each experimental series. Slides were coded and randomized before scoring. The statistical significance of differences was checked by Student's t test.

TRANSMISSION ELECTRON MICROSCOPY

Mycelia 48-h-old after 1 h growth in APDA at 0.05% and 0.1% (v:v) concentrations were examined by electron microscopy. Material cultivated on mineral medium was the control. The specimens were fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 6.2, prepared with distilled water and mineral medium (2:1 v:v), for 3 h at 0–4°C, washed in the buffer, then postfixed in 1% $0s0_4$ for 2 h, dehydrated in an ethanol-propylene oxide series, and infiltrated in Spurr-Epon 812 mixture according to the routine procedure for electron microscopy. Ultrathin sections cut on a Reichert ultratome were analyzed with a Jeol 1010 transmission electron microscope at 80 kV after staining with 2% (w/v) uranyl acetate in 50% ethanol and lead citrate (Reynolds, 1963).

RESULTS

ULTRASTRUCTURE OF *A. NIGER* NUCLEI

In the control material, the nuclei, spherical in shape, had a large nucleolus tightly pressed to nuclear envelope (Fig. 1a). After 1 h treatment with 0.05% APDA, many nuclei became irregular in shape (Fig. 1b,c), and sometimes resembled budding nuclei (Fig. 1b). In such deformed organelles, the nucleolus was frequently reduced in size.

Drastic changes in nuclear ultrastructure appeared after the 0.1% APDA treatment. In many nuclei, often heart-shaped (Fig. 1d), a decrease in nucleoplasm density was accompanied by progressive disappearance of nucleoli. In most of those altered nuclei, chromatin aggregates and local damage of the nuclear envelope became evident (Fig. 1e,f).

ALLIUM TEST

Mitotic index

In the control roots of *A. cepa* growing on Hoagland's medium and transferred to distilled water, the mitotic index dropped drastically from 10.9 ± 0.1 to 3.02 ± 0.01 after 1 h, and after 2 h culture in distilled water it increased to 8.87 ± 0.62 (Tab. 1). Prolongation of culture in distilled water to 24 h did not cause further changes in the frequency of dividing cells in these roots. After post-incubation in Hoagland's medium for 24 h, however, the mitotic index increased in roots growing in distilled water for 1 h but not in those growing in distilled water for 2 h (Tab. 1).

APDA at both tested concentrations reduced the mitotic index in *A. cepa* roots (Tab. 1) throughout the experiment. As in the control material, the frequency of mitoses, lowest after 1 h of APDA treatment, increased after 2 h, especially in the presence of the lower dose of APDA, and remained unchanged until the end of treatment (24 h).

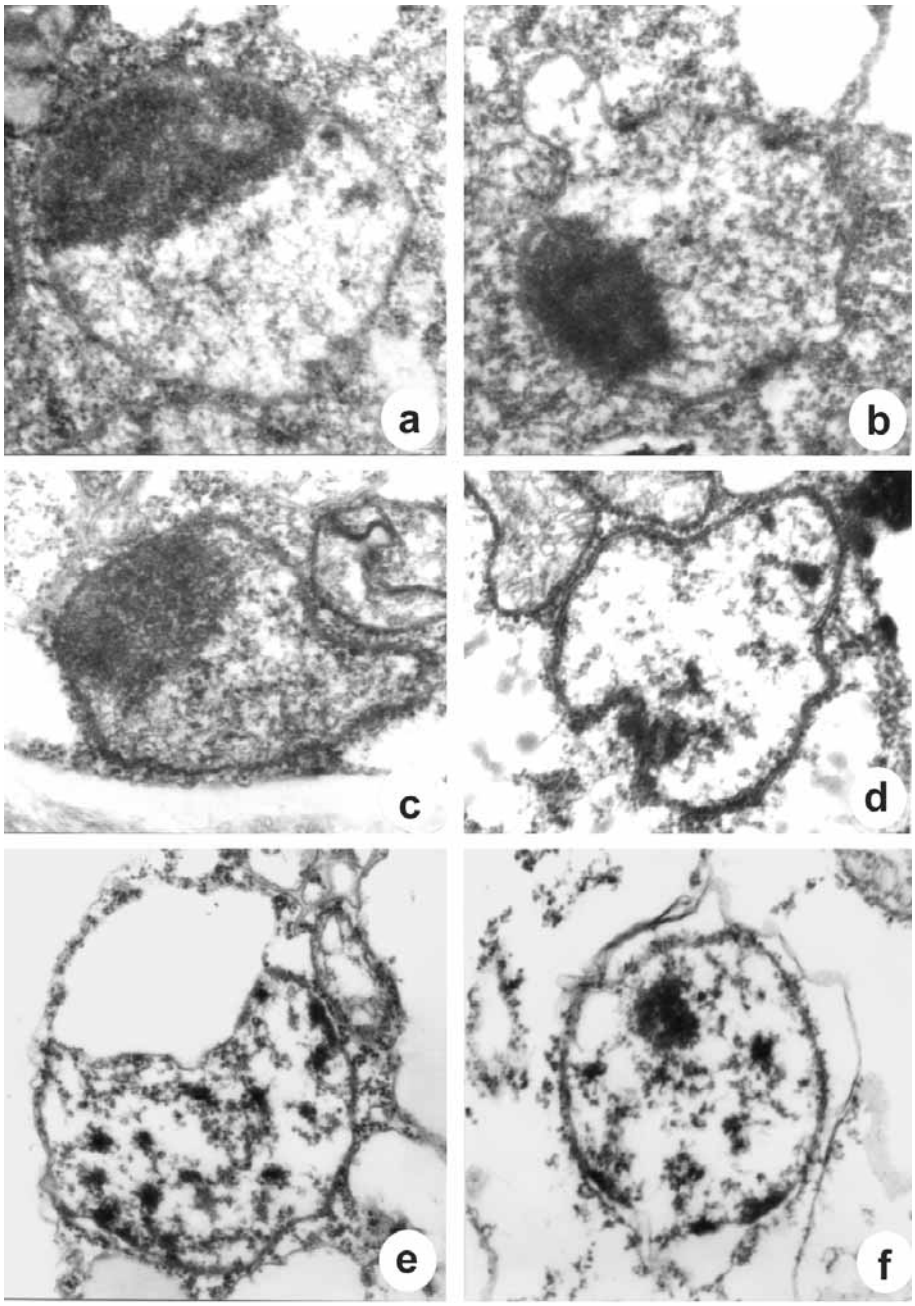

Fig. 1. Ultrastructure of nuclei in *Aspergillus niger* hyphae. (**a**) 48-h-old hypha growing on mineral medium. Nucleus with single large nucleolus, (**b**) Budding nucleus in material treated for 1 h with 0.05% APDA, (**c**) Irregularly shaped nucleus from mycelium treated for 1 h with 0.05% APDA with nucleolus still present, (**d**) Heart-shaped nucleus from hyphae treated for 1 h with 0.1% APDA, with small aggregates dispersed in chromatin in the minute nucleolus, which is more electron-transparent than in earlier figures, (**e**) Nucleus with many aggregates of chromatin and the partly separated and damaged outer membrane of the nuclear envelope in material treated for 1 h with 0.1% APDA. (**f**) Partial damage and separation of the outer membrane of the nuclear envelope, and remnants of nucleolus in hypha treated for 1 h with 0.1% APDA. × 29,000.

After 24 h post-incubation in Hoagland's medium, the frequency of dividing cells was higher, especially in roots treated for 2 h with 0.005% APDA, where it reached a level slightly lower than in the control material (Tab. 1). Only a slight diminution of the mitodepressive effect was noted in the roots treated for 1 h with 0.01% APDA followed by transfer to Hoagland's medium (Tab. 1).

TABLE 1. Effect of APDA on mitotic index [%] in root meristem of *Allium cepa* L.

APDA concentration [%]	Time of treatment [h]		
	1	2	24
0	3.02±0.10	8.87±0.62	8.43±0.79
	[a]13.21±3.22*	[a]9.50±1.41	
0.005	0.62±0.16*	3.91±0.67*	4.83±0.67*
	[a]2.32±1.10*	[a]6.86±0.93*	
0.01	0.32±0.07*	1.12±0.71*	1.15±0.28*
	[a]0.96±0.12*	[a]1.00±0.30*	

[a] material post-incubated in Hoagland's medium for 24 h;
* statistically significant difference from control at $p < 0.05$.

Prophases and metaphases dominated in *A. cepa* roots growing in distilled water and also in those post-incubated in Hoagland's medium for 24 h; there were fewer prophases, more metaphases, and sometimes more telophases in those treated with APDA (Fig. 2). This effect was more pronounced at the lower concentration of the tested disinfectant, except that metaphases were more frequent at 0.01% APDA.

Post-incubation for 24 h in the nutrient medium also changed the percentage shares of mitotic phases in *A. cepa* roots treated with APDA. As shown in Figure 2, the higher frequency of metaphases and telophases was accompanied by reduction of prophases in these roots. Post-incubation in Hoagland's medium also caused a slight increase of the frequency of anaphases in roots treated for 2 h with 0.01% APDA (Fig. 2).

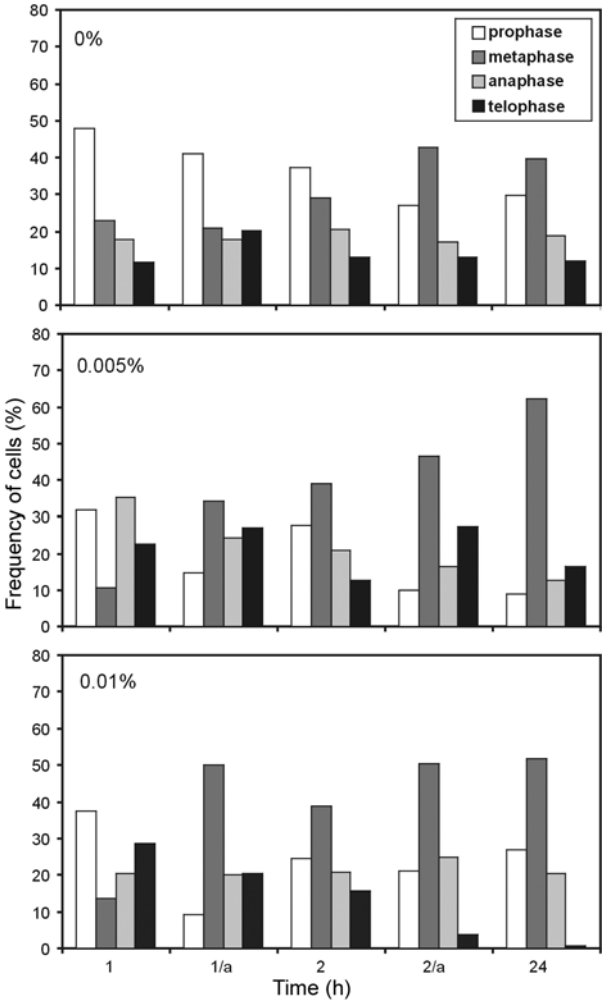

Fig. 2. Changes in the percentage share of mitotic phases in *A. cepa* roots treated with APDA. Top: material post-incubated in Hoagland's medium for 24 h.

Disturbances in mitosis

In *A. cepa* roots, besides typical phases of mitosis (Fig. 3a–d), abnormalities such as c-metaphases (Fig. 3e), lagging chromosomes (Fig. 3g, arrow), anaphase-telophase bridges and sticky chromosomes (Fig. 3h), budding nuclei (Fig. 3i), partial extrusion of the nucleolus from the nucleus (Fig. 3j), micronuclei (Fig. 3k) and binucleate cells appeared. Cells with abnormally oriented (Fig. 3f) and lost chromosomes (Fig. 3g) were also observed.

The total percentage of disturbances in mitosis rose with the duration of the 0.01% APDA treatment, but significantly decreased after 24 h treatment with the lower dose (Tab. 2). In roots post-incubated in Hoagland's medium and earlier treated with 0.01% APDA there were no changes in the total percentage of mitotic abnormalities but there were two more types of disturbances: micronuclei and binucleate cells. In contrast, roots treated with the lower dose of the disinfectant had lower frequencies of most of the described mitotic disturbances, and disturbances such as lagging chromosomes and partly binucleate cells disappeared after post-incubation (Tab. 2).

Of all the mentioned disturbances, c-metaphases and anaphase-telophase bridges dominated, and micronuclei were the least frequent (Tab. 2). After 24 h treatment with 0.01% APDA, the percentages of c-metaphases and bridges increased. On the other hand, in the presence of 0.005% APDA the frequencies of these abnormalities declined in spite of their significantly higher number after 2 h (Tab. 2).

These disturbances in mitosis, and the lagging chromosomes and binucleate cells observed mostly in the presence of 0.01% APDA, indicated that

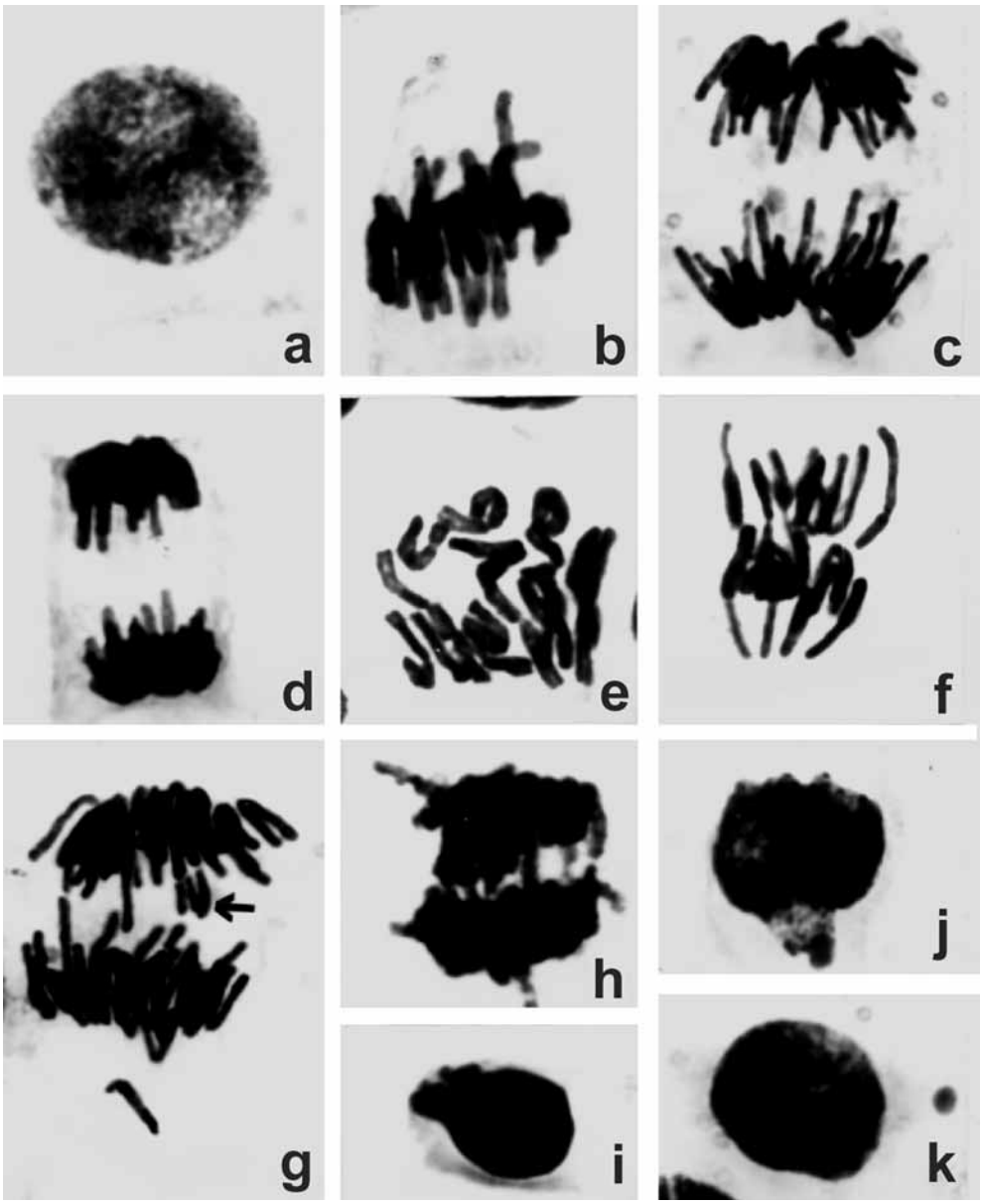

Fig. 3. Mitoses of *Allium cepa* roots. (**a**) Interphase, (**b**) Metaphase, (**c**) Anaphase, (**d**) Telophase, (**e**) c-metaphase, (**f**) Anaphase with chromosomes in atypical position, (**g**) Lagging (arrow) and lost chromosomes, (**h**) Anaphase bridge and sticky chromosomes, (**i**) Budding nucleus, (**j**) Extrusion of nucleolus into cytoplasm, (**k**) Micronucleus near the nucleus. × 1200.

APDA caused turbogenic effects in addition to the cytotoxic and mitodepressive effects. In *A. cepa* roots, cells with nucleoli partly extruded from the nuclei, as well as budding nuclei similar to those described in *A. niger* mycelium, were noticed in APDA-treated hyphae throughout the experiment, especially at the higher concentration.

After post-incubation in Hoagland's medium following APDA treatment, the frequency of most of the mentioned disturbances decreased. This effect was more pronounced under the lower disinfectant concentration; the disappearance of lagging chromosomes and binucleate cells (especially after 1 h) confirms this trend (Tab. 2). On the other hand, 24 h post-incubation did not diminish the frequency of partly extruded nucleoli, lagging chromosomes and budding nuclei; on the contrary, partly extruded nucleoli and budding nuclei became more frequent in APDA-treated roots at both concentrations used (Tab. 2).

TABLE 2. Mean number (%) of mitotic disturbances in root meristem of *Allium cepa* L. treated with ADPA

Culture time (h)	APDA conc. (%)	C-metaphases	Bridges	Lagging chromosomes	Micronuclei	Binucleate cells	Budding nuclei	Nucleolus partly outside nucleus	Total abnor. (%) (X ± S.E.)
1	0	1.62	1.46	-	-	-	-	-	3.08±0.52
1/a	0	3.37	1.59	-	-	0.22	-	-	5.43±0.96
2	0	3.45	1.98	-	-	-	-	-	7.18±0.94
2/a	0	3.67	1.44	-	-	0.56	-	-	5.67±0.68
24	0	5.24	2.45	0.94	-	0.49	-	-	11.12±0.53
1	0.005	9.79	4.67	0.11	-	-	1.33	0.29	16.19±2.00*
1/a	0.005	2.19	2.37	-	0.09	-	4.80	0.62	10.07±2.31*
2	0.005	12.14	8.21	0.45	-	-	3.29	0.86	24.95±1.45*
2/a	0.005	7.82	4.09	-	0.13	0.83	4.25	1.76	18.88±2.67*
24	0.005	6.17	5.27	1.11	0.21	1.59	2.11	4.54	21.08±1.40*
1	0.01	16.83	8.96	1.05	-	-	2.53	1.52	30.89±4.02*
1/a	0.01	8.45	7.15	1.01	0.34	1.54	6.67	3.38	28.54±1.90*
2	0.01	20.32	11.96	2.18	-	-	4.35	2.18	40.19±1.61*
2/a	0.01	11.12	7.47	2.13	0.38	1.25	8.81	6.19	37.35±1.96*
24	0.01	31.86	18.75	3.92	0.55	1.84	11.66	3.91	67.49±2.74*

a – material post-incubated in Hoagland's medium for 24 h; * statistically significant difference from control at $p < 0.05$.

DISCUSSION

It is well known that despite the significant differences in organization and metabolism between fungi and plants, plant reactions may be extrapolated to fungi (Grant, 1982a,b), and there is a high correlation between the mutagenic effects of chemicals in plant and animal cells (for references see: Osiecka, 1993; Grant, 1994). The ultrastructural changes in the nuclei of *Aspergillus niger* mycelium and the marked reduction in the percentage of dividing cells and high frequency of mitotic disturbances in *Allium cepa* roots under APDA treatment support the validity of such an extrapolation.

The higher dose of APDA induced drastic malformations of nuclear ultrastructure in *A. niger* mycelium, but only slight ones at the lower dose. Similarly, authors (data unpublished) observed that 0.1% APDA caused progressive damage to *A. niger* protoplasts, and after 24 h only mitochondria and multilamellar bodies were distinguished in the majority of cells; at half that dose there were disturbances in cell ultrastructure after 1–2 h treatment, but most of the mycelial cells growing for 24 h in the presence of 0.05% APDA resembled the control cells. Authors (data unpublished) suggested that such a reaction to 0.05% APDA indicates not only defense but also adaptation processes in these mold fungi. Changes in the activities of succinate (cytochrome c oxidoreductase), NADH (cytochrome c oxidoreductase and fumarase) in *A. niger* mycelium in the presence of APDA, indicating that its effects may be mediated by oxidative stress (Kuźniak et al., 2006) partly support that suggestion. It cannot be ruled out that adaptation to APDA also took place in *A. cepa* roots. Probably some meristematic cells regained the ability to divide, since the mitotic index, lower in the presence of 0.005% APDA, increased after post-incubation in Hoagland's medium, but this does not explain the higher frequency of abnormalities in mitosis such as budding nuclei and partly extruded nucleoli in roots treated with the lower APDA dose.

Degradation of nuclear material inside the nuclear envelope (karyorexis) in *A. niger* mycelium, and the decline of the mitotic index in *A. cepa* roots, revealed the strong cytotoxicity of APDA, especially at the higher dose. The numerous mitotic disturbances and the high frequency of c-metaphases and bridges in *A. cepa* roots indicated that APDA induced turbogenic as well as mitodepressive effects.

Mitodepressive action is suggested by the effect of APDA on the cell cycle, and turbogenic action by disturbances of the mitotic spindle. The high frequency of c-metaphases and anaphase-telophase bridges observed after 1 h treatment with APDA, and the low frequency of binucleate cells, also point to disturbances of the timing of the cell cycle. APDA probably affected the G_2 phase, since in this phase tubulin is synthesized in addition to proteins essential entry into mitosis and responsible for condensation of chromatin. The numerous shrinking nuclei observed in *A. cepa* roots after 1–2 h treatment with 0.01% APDA as well as the c-metaphases, bridges and lagging chromosomes support this idea. Hsu et al. (1986) suggested that the c-metaphases induced by mutagens result from blockage of tubulin polymerization or from aggregation of microtubules and tubulin to crystalline forms. It is difficult to say which of these processes was affected by APDA,

although no doubt this disinfectant diminished the frequency of proliferating cells and caused various malformations in mitosis.

Induction of even a small number of micronuclei by any substance indicates that it affected the timing of the cell cycle (Obe and Beek, 1982, cited after Stopper and Müller 1997). Micronuclei most arose from acentric fragments formed as a result of chromosome or chromatid breaks (Müller and Streffer, 1994). Lagging chromosomes or chromatids appearing as a consequence of kinetochore malformations or partial inactivation of a division spindle may also be responsible for micronuclei formation. Such chromosomes or chromosome fragments were not incorporated into daughter nuclei but remained in the cytoplasm where they formed micronuclei. The micronuclei observed in *A. cepa* roots treated with APDA, especially at the higher dose, perhaps do not represent typical micronuclei, but rather nuclear fragments formed as a result of apoptosis (cit. after Müller and Streffer, 1994). The budding nuclei may be a source of structures resembling micronuclei when buds separate from nuclei (Müller and Streffer, 1994). Budding nuclei such as were induced by APDA in *A. cepa* roots were also described in *Vicia faba* meristem treated with pesticides (DeKergommeaux et al., 1983) and with cyanobacterial extract (Kontek, 1999).

Besides the micronuclei, the lagging chromosomes also suggested that APDA had a slight clastogenic effect, resulting in rearrangement of chromosome pieces or in chromosome gain or loss within the genome (Panda and Panda, 2002). Reacting indirectly, clastogens become responsible for various cytotoxic effects (Scott, 1990) such as malformation of enzymes, structural proteins and endomembranes (for references see: Osiecka, 1993). In this light, it may be that the partial extrusion of nucleoli and budding of nuclei in onion roots treated with APDA were due to changes in membrane structure or composition. The intensified ergosterol synthesis in *A. niger* mycelium treated with APDA observed by authors (data unpublished), responsible for enhanced rigidity and in consequence lower permeability of endomembranes, supports that suggestion. Unlike in Pawiroharsono et al.'s (1987) work, however, such changes in endomembrane composition protected neither the hyphae nor roots from the action of APDA, as indicated by the higher frequency of mitotic abnormalities such as budding nuclei and partly extruded nucleoli in roots treated with the lower APDA dose.

The appearance of small droplets at the mycelial surface of *Aspergillus niger* observed by authors (data unpublished) relate to the mechanisms of action of APDA. Such droplets probably reduced the fungi's contact with the culture medium, which might induce cell necrosis. These droplets were not noted on *A. cepa* roots, but perhaps they contributed to the observed effects and then disappeared during slide preparation.

The present study showed that N,N–bis (3–aminopropylo)dodecyloamine may be safely used as a microbicidal agent to destroy microorganisms developing on experimental material, since it had strong mitodepressive and turbogenic effects, although a slight clastogenic effect apparently occurred at the higher concentration.

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