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Electron Microscopic Investigation of Nitrobenzene Distribution and Effect on Plant Root Tip Cells Ultrastructure

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Electron microscopic radioautography demonstrated the penetration of [1-6^{14}C]nitrobenzene in maize and soybean root tip cells: radioactive label was detected in cell wall, plasmalemma, nuclei, and cytoplasm. Among cytoplasmic organelles, the highest label was found in mitochondria and plastids. [1-6^{14}C]nitrobenzene and/or products of its transformation accumulated in vacuoles. Study of the action of different concentrations of nitrobenzene on cell ultrastructural organization revealed the following picture. Nitrobenzene concentration up to 0.015 mM was harmless for plant cells. Increase of nitrobenzene concentration from 0.015 to 1.5 mM induced several pathological changes, up to the complete destruction of cells. The most damaged organelles were nuclei, mitochondria, and plastids. In the presence of 0.15 mM nitrobenzene the intensification of contacts among cell organelles, especially between endoplasmic reticulum and mitochondria/plastids, was observed. The data indicate some coordination between detoxication activity and energy metabolism during cell reaction to xenobiotic toxicity.

Key Words: **[1-6^{14}C]nitrobenzene; cell ultrastructure; electron microscopic radioautography; maize; soybean; root tips.**

INTRODUCTION

A large part of environmental pollutants is constituted by hydrocarbons, many of which are carcinogens. The amount of these chemicals is continuously increasing, because of the construction and operation of oil and gas pipelines, and of chemical and oil processing factories.

Absorption of hydrocarbons by plant cells cause various types of ultrastructural disorganization, depending on the structure and concentration of the molecules (Buadze and Kvesitadze, 1997; Korte *et al*., 2000). They can inhibit DNA synthesis (Buadze *et al*., 1998) and disturb calcium homeostasis (Zaalishvili *et al*., 2000a, b), leading to complete cell destruction (Buadze *et al*., 1998; Korte *et al*., 2000).

Plants are able to detoxify limited amounts of xenobiotics, mainly via oxidative degradation. The pathways of absorption and transformation of different aliphatic (Durmishidze and Ugrekhelidze, 1968; Ugrekhelidze and Kvesitadze, 1997), aromatic (Durmishidze *et al*., 1974a,b; Ugrekhelidze *et al*., 1997), and polycyclic aromatic hydrocarbons (Devdariani, 1988) by plants have been studied.

On the basis of the author's data (Khatisashvili *et al*., 1993, 1997; Gordeziani *et al*., 1999) and those of others (Durst, 1991; Sandermann 1992, 1994), one can conclude that there is no universal mechanism of oxidation. Consequently, xenobiotic transformation could be carried out with the participation of different oxidative systems, located in different cell organelles.

With the aim of investigating possible mechanisms of xenobiotic oxidation, nitrobenzene, a highly toxic environmental pollutant characterized by its stability, its oxidative degradation via hydroxylation, and its lack of autooxidation, was selected.

The purpose was to examine the toxicity of nitrobenzene on plant cells and to identify the subcellular organelles involved (important for the study of oxidative degradation).

Examined in root tip cells were (i) the effects of different concentrations of nitrobenzene on the ultrastructural organization and (ii) the intracellular distribution of [1-6^{14}C]benzene (by electron microscopic radioautography).

MATERIALS AND METHODS

Materials

Maize (*Zea mays*), variety Adjametis tetri, and soybean (*Glycine max*), variety Kartuli 7, plants, representatives of different taxonomic groups with different types of metabolism, were selected for investigations. Plant seedlings were

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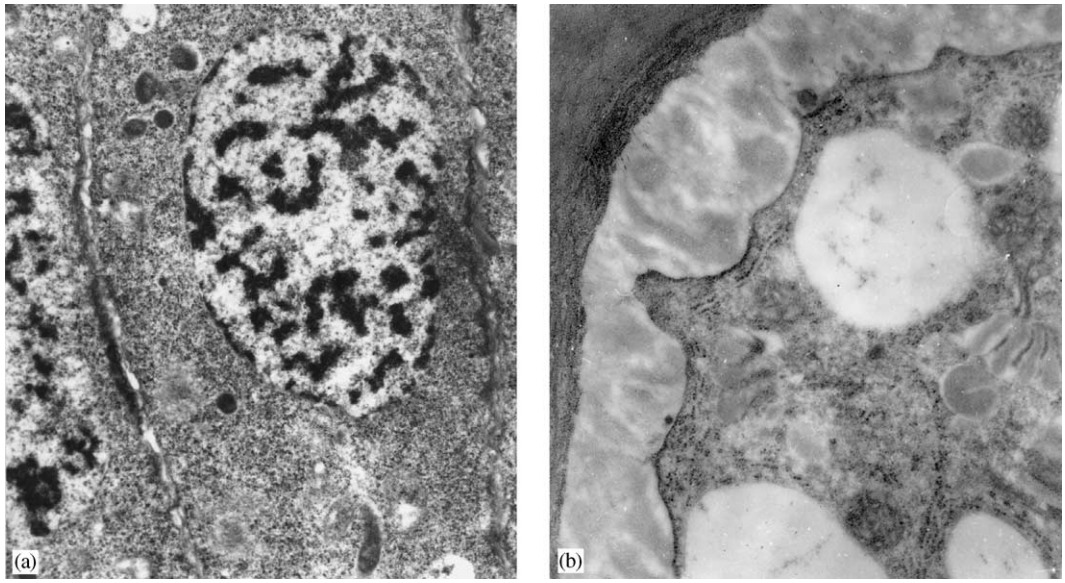

FIG. 1. Root tip cells of maize seedlings treated with 0.015 mM nitrobenzene. (a) Cells of the meristematic zone (× 15,000). (b) Secretory cells (× 25,000).

grown in the dark under sterile conditions on tap water at 20–22°C.

Nitrobenzene Treatments

Seven-day-old seedlings were exposed for 24 h to aqueous solutions of nitrobenzene of three different concentrations: 0.0154, 0.154, and 1.54 mM. Some nitrobenzene evaporated during the treatments, so that the concentration of the solutions decreased by 8%. The nitrobenzene concentrations were thus assumed to be 0.015, 0.15 and 1.5 mM (average values between initial and final concentrations). Control plants were left on water.

Electron Microscopy

Cell ultrastructural organization was studied by the generally accepted method (Buadze and Kvesitadze, 1997; Buadze *et al*., 1998). Root tips were excised and 1-mm^3 samples were fixed in a 2.5% solution of glutaraldehyde with postfixation in 1% osmium tetroxide. After dehydration in a graded series of ethanol solutions, the samples were embedded in Epon–Araldite resin (1.5:1.0) and poured into gelatine capsules. Thin serial sections were made using Reichert and LKB III ultramicrotomes, stained with uranyl acetate and/or lead citrate, and examined in a Telsa BS 500 electron microscope.

Nitrobenzene Distribution in Root Cells

Seven-day-old plant seedlings were transferred to 0.015 and 0.15 mM aqueous solutions of [1-6^{14}C]nitrobenzene. Control plants were transferred to a nonradioactive nitrobenzene solution of the same concentration. The length of treatments was 24 h.

Synthesis of [1-6^{14}C]Nitrobenzene

The synthesis started from 3 mCi of a commercial preparation of [1-6^{14}C]benzene made in Russia. It was diluted in 10 ml of nonradioactive benzene. Synthesis was conducted using a nitriding mixture (a mixture of concentrated nitric and sulfuric acids) by a standard method (Vasil'eva *et al*., 1986). The reaction temperature did not exceed 60°C, to minimize the formation of the by-product *m*-dinitrophenol. The product, [1-6^{14}C]nitrobenzene, was purified by two distillations at 210°C. Its purity was checked by UV spectroscopy and thin-layer chromatography (Dünnschicht Cromatografie, 1962).

Electron Microscopic Radioautography

Intracellular distribution of [1-6^{14}C]nitrobenzene was studied by a modified method of radioautography, based on the use of heavy metals (Buadze *et al*., 1985a,b). Ultrathin sections of golden color were incubated in a 10^{-4} M solution of nickel (II) chloride for 30 s, twice. Development was then carried out in a 2% solution of hydroquinone in water for 40 s, twice.

RESULTS

Effect of Different Concentrations of Nitrobenzene on the Ultrastructure of Root Tip Cells

Maize: *treatment by* 0.015 *mM nitrobenzene.* The cells had some distinctive features: small vacuoles with

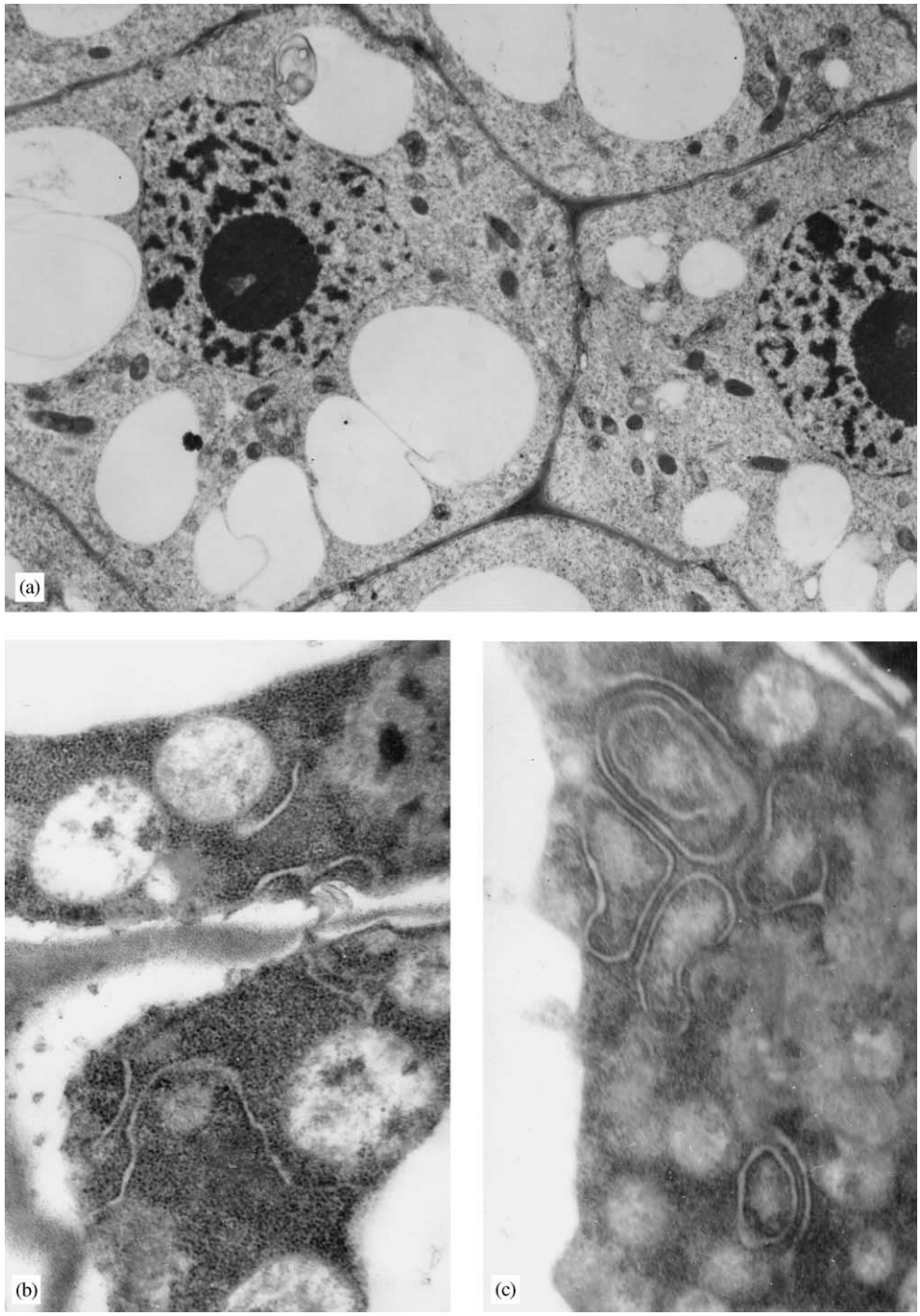

FIG. 2. Root tip cells of maize seedlings treated with 0.15 mM nitrobenzene. (a) Cells of the merisrematic zone with large vacuoles and electron-dense cell wall (× 8000). (b) "Dark" cells of the cap zone. Contacts of endoplasmic reticulum with vacuoles, mitochondria, plasmalemma, and plasmodesm (× 40,000). (c) Mitochondria surrounded by endoplasmic reticulum (× 40,000).

osmiophilic inclusions, and some plastids and mitochondria with electron-dense matrices. Periplasmic space was enlarged, and cell walls were darker than those of controls and were distinguished by a higher electron density (Fig. 1a). In secretory cap cells mucus secretion was slightly disturbed. The content of Golgi vesicules and the structure of insertions in the periplasmic space were similar to those of the controls, and vesicle translocation and their fusion with plasmalemma resulted in the release of their content into the periplasm (Fig. 1b). The slight decrease in secretion could be

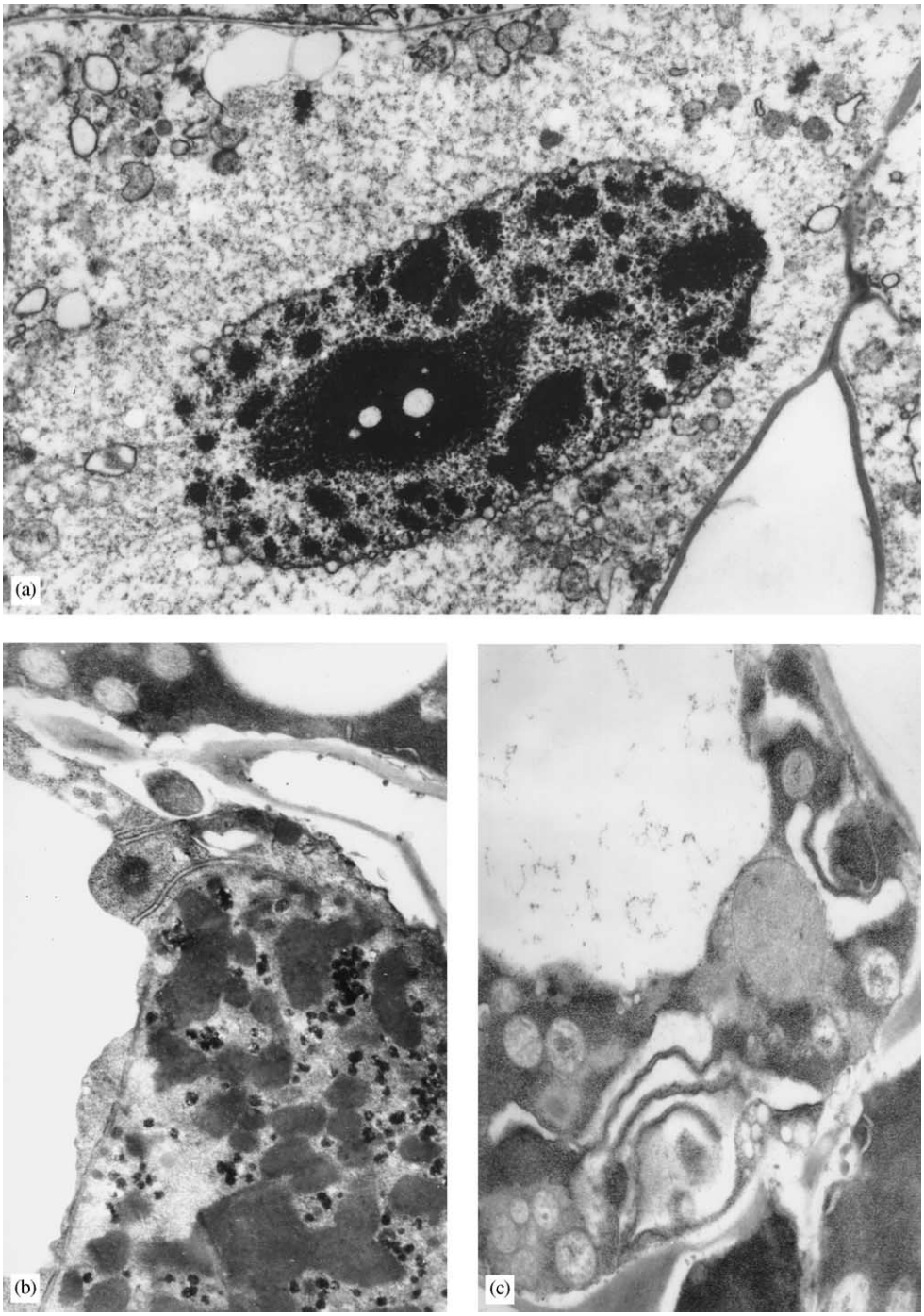

FIG. 3. Root tip cells of maize seedlings treated with 1.5 mM nitrobenzene (a) Damaged cells of the epidermal zone (× 12,000). (b) Cells of the cap meristematic zone. Dark cells on the left. Granular inclusions in the nucleoplasm. Contacts of endoplasmic reticulum with vacuoles and plasmalemma (× 28,000). (c) Dark cells; widened cysterns of endoplasmic reticulum, and their contacts with plasmalemma, vacuoles, and mitochondria (× 28,000).

explained by the existence of vacuole-like vesicules as well as by a tight disposition of mature Golgi vesicles.

Maize: 0.15 *mM nitrobenzene*. An intensive vacuolization was observed in the meristematic cells of the root tip. Large vacuoles were probably the result of the fusion of smaller ones, as suggested by the presence of numerous membrane fragments and osmiophilic insertions (Fig. 2a). Mitochondria were characterized by swollen crysts and transparent matrixes. Cell walls were electron-dense; some

"dark" cells appeared in the root cap zone. Large numbers of mitochondria, mostly with an abnormal matrix, were found in these cells. There were numerous contacts of widened cysterns of endoplasmic reticulum with mitochondria, plasmalemma, and vacuoles (Fig. 2b). Mitochondria were sometimes surrounded by endoplasmic reticulum (Fig. 2c). Some extension of periplasmic space was noticeable (Figs. 2b and 2c). Vacuoles of different sizes were found in the central zone of epidermal cells, and they tended to merge into bigger structures. The periplasmic space of these cells was markedly restricted. These data indicate that under the influence of nitrobenzene, the vectorial translocation of secretory vesicules from the center to the periphery of the cell is disturbed, and that consequently the process of exocytosic secretion is inhibited.

Maize: 1.5 *mM nitrobenzene*. In comparison with control preparations, cells treated with nitrobenzene presented a general expansion of intracellular structures. The electron density of the cell wall was strongly increased. In addition, a marked destruction of epidermal cells was observed. In these cells, the cytoplasm was devoid of most organelles. Only altered nuclei and lysed mitochondria accumulated at the cell periphery, and fragments of endoplasmic reticulum could be recognized. The inner, hydrophobic layers of membranes, especially of nuclear membranes, were swollen into strings of vesicles. The presence of similar structures around the nuclei suggested that these vesicles could be expelled from the membranes (Fig. 3a).

In the meristematic and the differentiated cell zones, nuclear membranes were less altered, but their hydrophobic layer was widened, and in addition, the membrane pores were enlarged (Fig. 3b). The presence and the morphology of granular inclusions in the nucleoplasm indicated the presence of salt deposits, probably resulting from the release of Ca^{2+}, Mg^{2+}, and phosphate groups after the destruction of chromatin. Some cells appeared very dark, and the electron density of their cytosol was related to a high concentration of ribosomes (Fig. 3b). The cells of this zone were also characterized by huge vacuoles. Endoplasmic reticulum with large cysterns made extensive contacts with vacuoles, plasmalemma, plasmodesmata, and mitochondria, and connected vacuoles to plasmalemma (Fig. 3c). Vesicles of smaller sizes also made frequent contacts with plasmalemma. All these connections between various structures indicated exocytosic processes involved in the detoxication of xenobiotics and/or their metabolites. A marked extension of periplasmic spaces, which is not typical for this type of cells, was probably related to the active secretion process (Figs. 3a–3c).

Soybean: 0.015 *mM nitrobenzene*. In the cells meristematic zone, the following ultrastructural changes were observed: along with some normal mitochondria, there were others with widened crysts and electron-dense matrices. The matrices of the plastids were osmiophilic and the thylakoids were swollen. Reserve protein conglomerates in vacuoles were relatively few (Fig. 4) in comparison with controls;

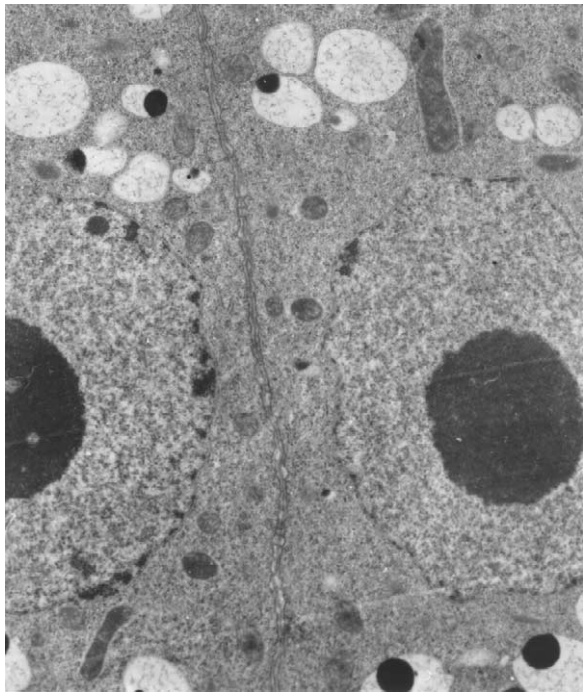

FIG. 4. Root tip meristematic cells of soybean seedlings treated with 0.015 mM nitrobenzene (× 5000).

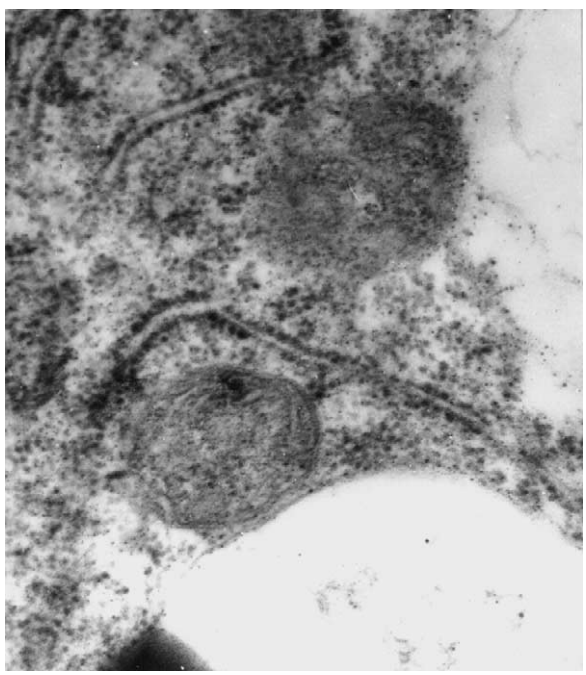

FIG. 5. Root tip cells of soybean seedlings treated with 0.15 mM nitrobenzene. Contacts between endoplasmic reticulum and mitochondria (× 55,000).

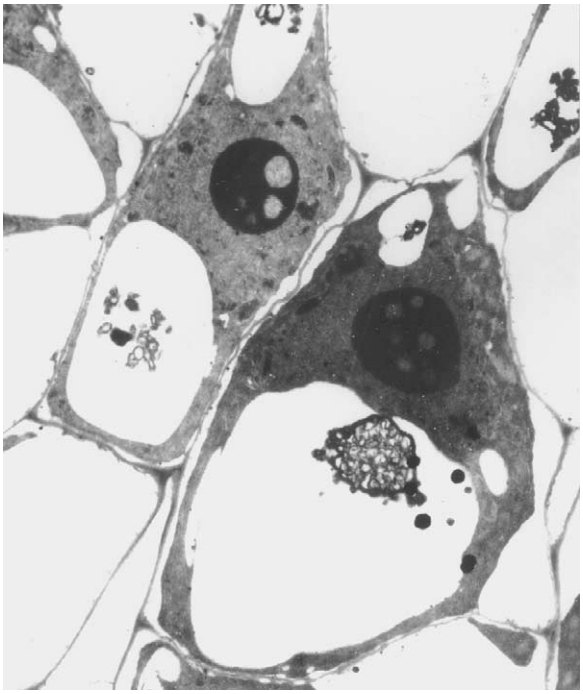

FIG. 6. Root tip meristematic cells of soybean seedlings treated with 1.5 mM nitrobenzene (× 5000).

protein secretion was minimized, and intercellular vesicles were enlarged.

Soybean: 0.15 *mM nitrobenzene*. Comparatively large vacuoles containing proteins were found in cells of the meristematic zone. The contacts between vacuoles and plasmalemma were limited. The nuclei were invaginated; some mitochondria had electron-dense matrices; plastids were osmiophilic with swollen thylakoids. Cell walls and glycocalyx zones were characterized by electron density. Frequent contacts were observed between mitochondria and endoplasmic reticulum and numerous osmiophilic insertions were found in intracellular vesicules (Fig. 5).

Soybean: 1.5 *mM nitrobenzene*. Considerable destruction of cap meristematic cells was indicated by reduction of hyaloplasm, enlargement of periplasmic space, huge vacuoles, invaginated nuclei, and perforated nucleoli. Mitochondria and plastids had electron-dense matrices and swollen crysts and thylakoids. Dark cells contained plastids surrounded by electron-transparent zones (Fig. 6) and granular osmiophilic insertions were often found in vacuoles. The inner hydrophobic layers of nuclear membranes, as well as cysterns of endoplasmic reticulum, were relatively widened. There were frequent contacts between endoplasmic reticulum and mitochondria. Compared with previous concentrations, fewer protein conglomerates were observed in vacuoles.

Nitrobenzene Distribution in Cells

After treatment of maize and soybean seedlings for 24 h with 0.015 and 0.15 mM [1-6^{14}C]nitrobenzene, the label was detected in almost all cells of the root tip zone. In maize root cells the radioactive label of 0.15 mM [1-6^{14}C]nitrobenzene was detected in cell wall, plasmalemma, and

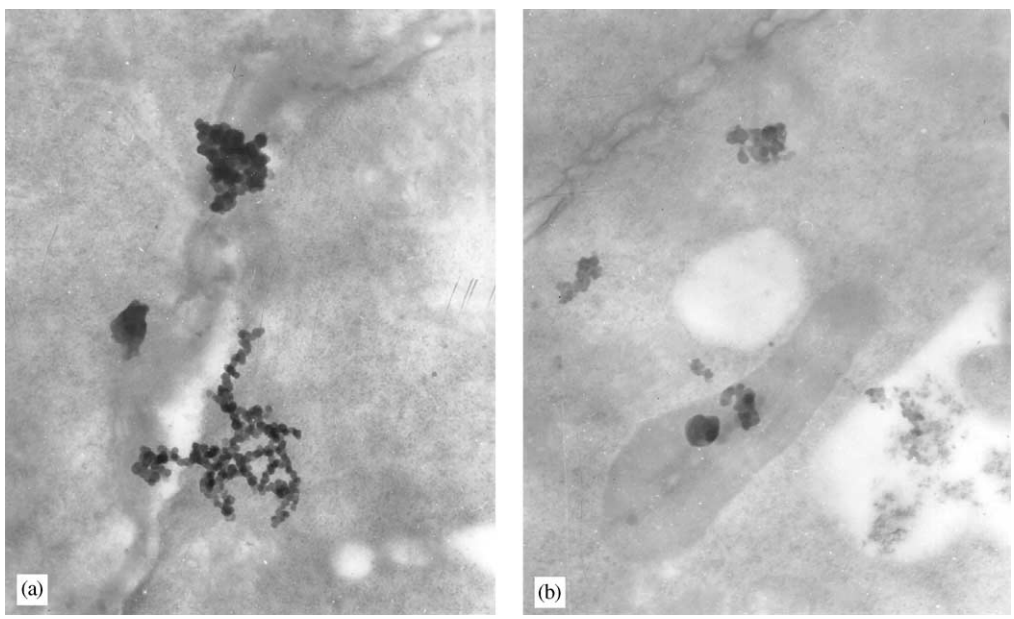

FIG. 7. Radioautograph of root tip cap cells of maize seedlings treated with 0.15 mM [1-6^{14}C]nitrobenzene.(a) Radioactive label in cell wall, plasmalemma, and peryiherial cytoplasm (× 28,000). (b) Radioactive label in mitochondrion, plastid, vacuole, and peripheral cytoplasm (× 28,000).

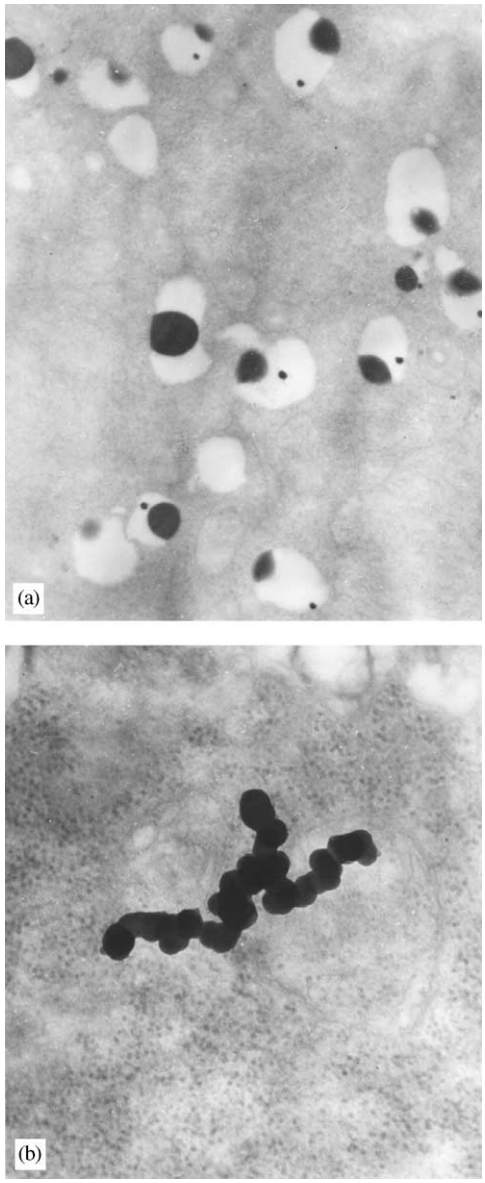

FIG. 8. Radioautograph of root tip cap cells of soybean seedlings treated with 0.15 mM [1-6^{14}C]nitrobenzene. (a) Radioactive label in vacuoles (× 12,000). (b) Radioactive label in mitochondrion and cytoplasm (× 48,000).

peripheral cytoplasm (Fig. 7a), as well as in mitochondria, plastids, and vacuoles (Fig. 7b). The radioactive label of 0.15 mM [1-6^{14}C]nitrobenzene was also detected in soybean root tip cells cytoplasm, vacuoles (Fig. 8a), and mitochondria (Fig. 8b).

DISCUSSION

Incubation of maize and soybean seedlings for 24 h in [1-6^{14}C]nitrobenzene at different concentrations clearly indicated that the xenobiotic penetrates into root tip cells. The intracellular distribution of radioactivity demonstrated that nitrobenzene and/or its metabolites reach the cell wall, the plasmalemma, the nucleus, and the cytoplasm and its organelles: mitochondria, plastids, and vacuoles.

According to the above observations, the ultrastructural changes of maize and soybean root tip cells, as well as the rate of cell destruction, were directly dependent upon the toxicant concentration. Among cell organelles, the most sensitive were nuclei, mitochondria, and plastids, i.e., the cell genetic apparatus, the sites of energy formation, and all organelles determining cell and plant vitality. Structural changes of organelles at the lower concentrations of toxicant are balanced by compensatory mechanisms, suggesting that after removal of the xenobiotic action all organelles should recover their normal ultrastructure and function.

On the basis of the electron microscope data, the following speculations could be made concerning maize:

- The existence of dark cells suggests that cellular protein content increases with nitrobenzene exposure (the darkness is related to the increase in the density of ribosomes in the hyaloplasm). This increase in protein content could be related to the biosynthesis of enzymes and/or proteins capable of detoxifying and conjugating xenobiotics.
- Endoplasmic reticulum with widened cysterns is connected with vacuoles and plasmalemma. This indicates some excretion of the xenobiotic and its metabolites from the cells.
- Special attention must be paid to the behavior of endoplasmic reticulum membranes, because cytochrome P450-containing monooxygenases—the basic enzymatic system in xenobiotic oxidative degradation—are located on these membranes (Gordeziani *et al*., 1991). In parallel with the increase of nitrobenzene concentration, the frequency of contacts of endoplasmic reticulum membranes with mitochondria (Fig. 2b) intensifies. Recent data have revealed that when NADPH is lacking, the microsomal monooxygenase system can draw the electrons required for xenobiotic detoxication from mitochondria (Gordeziani *et al*., 1999). Moreover, the present observations point out frequent contacts between endoplasmic reticulum and vacuoles (Fig. 3b). This is probably indicative of some accumulation of nitrobenzene degradation products in vacuoles (Sandermann, 1992, 1994). The lowest nitrobenzene concentration—0.015 mM—is considered to be compatible with the normal cell metabolism, since its detoxication is accomplished without ultrastructural changes of the cells.

In soybean, contrary to maize in which an intensification of protein synthesis is observed during nitrobenzene detoxication, the consumption of storage protein, contained in great amounts in vacuole-like reservoirs in root cells, was observed. One observation that can be made for both plants is that the process of nitrobenzene detoxication is correlated with protein consumption.

Thus, two levels of nitrobenzene concentration can be distinguished: concentrations upto 0.015 mM are harmless for plant cells, as they do not cause noticeable changes in cell ultrastructure; at 0.15 mM and higher concentrations, several ultrastructural changes, leading to the complete destruction of the cell, occur.

CONCLUSIONS

The effect of increasing concentrations of nitrobenzene on maize and soybean root tip cells ultrastructure was correlated with the intensity of pathological changes. Maize appeared to be more sensitive to nitrobenzene. Among cell organelles, the most damaged were nuclei, mitochondria, and plastids, i.e., the cell genetic apparatus and the sites of energy formation, which determine cell and plant vitality. On the basis of ultrastructural investigations it could be concluded that at 0.015 mM concentration, nitrobenzene can be detoxicated by the cell. In cells treated with 0.15 mM nitrobenzene, an intensification of contacts among cell organelles, especially between endoplasmic reticulum and mitochondria/plastids, was observed. These data probably indicate a coordination between organelles responsible for energy metabolism and those responsible for xenobiotic degradation, in order to protect cells from xenobiotic toxicity.

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