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Effect of "External" Superoxide Anion on Apoptosis in Coleoptiles of Wheat Seedlings

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Abstract—A derivative of phthalic acid, dibutylphthalate (DBP), which has gametocidal effect at the concentration of ~10^{-4} M, increased apoptosis in coleoptiles of wheat seedlings. This was associated with activation of chromatin margination and generation of mitochondria-containing vesicles. At the same concentration, DBP activated the release by the coleoptiles of superoxide anion into the environment. Lower (10^{-5} M) and higher (10^{-3} M) concentrations of DBP virtually had no effect on either process. A probable mechanism of effect of the "external" superoxide anion on apoptosis within the plant cell is discussed.

Key words: wheat seedlings, apoptosis, superoxide anion, mitochondrial ultrastructure

Production of reactive oxygen species (ROS) and especially of superoxide anion ($O_2^{\cdot-}$) is usually an indispensable stage of apoptosis in animal and plant cells [1]. The generation of ROS within the cell is associated with mitochondria, microsomes, and other cell structures. NADPH-oxidases play a special role among the ROS-generating systems. These enzymes are usually located in the plasma membrane. Utilizing the intracellular NADPH, they reduce oxygen on the external side of the membrane and thus generate superoxide anion $O_2^{\cdot-}$ extracellularly [2, 3]. The probable effect of this "external" $O_2^{\cdot-}$ on apoptosis has not been discussed to date.

In the present work we have attempted to show the association between generation of "external" $O_2^{\cdot-}$ and apoptosis in plants. As an apoptotic tissue, we took coleoptile of etiolated wheat seedlings, the model already used in our studies on apoptosis in plants [4]. Apoptosis is known to begin in coleoptile after emergence of seedlings from the ground, and its relative activity increases with time and results in the coleoptile die-off. The rate of $O_2^{\cdot-}$ generation in coleoptiles of etiolated wheat seedlings can be increased by treatment with gametocides, which we studied earlier [5].

The special purpose of the present work was to demonstrate the acceleration of apoptosis concurrently with acceleration of production of the external $O_2^{\cdot-}$. As an external factor activating the $O_2^{\cdot-}$ generation, we used the gametocide dibutylphthalate (DBP) that we described earlier.

The same concentration of DBP increased both the rate of production by coleoptile of "external" $O_2^{\cdot-}$ and intensity of formation of mitochondria-containing vesicles, which is a specific sign of apoptosis in coleoptile [4]. This correlation is in agreement with the hypothesis about the probable involvement of generation of "external" $O_2^{\cdot-}$ in the cascade of apoptosis-associated biochemical reactions.

MATERIALS AND METHODS

Wheat seeds (*Triticum aestivum* L.) Priokskaya cultivar were grown in the dark on wet filter paper in a plastic cuvette at 26°C; cuvettes with the experimental plants contained aqueous solutions of DBP in concentrations from 10^{-7} to 10^{-3} M. To determine generations of superoxide anion and hydrogen peroxide and for isolation and fixation of mitochondria, we used 4- and 5-day-old seedlings.

To determine the rate of $O_2^{\cdot-}$ generation, two seedlings prewashed in distilled water were placed into 4 ml of incubation medium totally or turned over, with the corn and roots remaining in the air. These two variants of the plant immersion into the incubation medium gave

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qualitatively similar results. The medium contained 10 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 0.1% Triton X-100 (Fluka, Switzerland), and 0.6 mM nitro blue tetrazolium (NBT) (Sigma, USA) as a "trapper" for $O_2^{\cdot-}$. Incubation was performed in the dark at 26°C for 1 h. Then the seedlings were taken out from the incubation medium, and the quantity of formazan was determined in it (superoxide anion released by the seedling in the aqueous medium reduced NBT to formazan) at the wavelength of 530 nm. The molar extinction coefficient for formazan was taken to be 15,000 $M^{-1}\cdot cm^{-1}$ [6]. To exclude the contribution of nonspecific reduction of NBT unbound to $O_2^{\cdot-}$, in each experiment two parallel samples were used, one of which was supplemented with superoxide dismutase (50 activity units per ml of the medium). According to [7], superoxide dismutase fully converted the generated $O_2^{\cdot-}$ into hydrogen peroxide. The amount of the generated $O_2^{\cdot-}$ was determined by the difference of formazan absorptions in two parallel samples. All determinations were performed with a Hitachi 557 spectrophotometer (Japan).

To determine the H_2O_2 production, two seedlings were washed in distilled water, dried with filter paper, and dipped into a solution which contained 3 mM Tris buffer (pH 8.75), $15\cdot10^{-5}$ M *p*-iodophenol, $15\cdot10^{-5}$ M luminol, and 0.1 nM horseradish peroxidase (the total volume of the solution was 930 µl). Amounts of H_2O_2 were determined at time intervals from 5 to 15 min. New seedlings were used for each time interval. Determinations were performed in four replicates. Intensity of chemiluminescence was recorded with a liquid scintillation counter for measurement of β-radiation. The quantity of H_2O_2 was determined from the calibration curve obtained for known concentrations of H_2O_2.

The wheat seedling coleoptiles for electron microscopy were fixed with 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) supplemented with 300 mg sucrose and 0.5 ml of 40% formalin per 20 ml of the buffer. Apices of the seedling coleoptiles were fixed and after vacuum treatment left in the fixer for 1.5-2 h at 4°C. Then the preparations were washed in fresh buffer for 30 min, fixed additionally with 1% OsO_4 in the same buffer for 1.5 h, and dewatered in ethanol solutions of enhancing concentration (70% ethanol contained the saturated solution (1.5%) of uranyl acetate). After dehydration, the preparations were placed in mixtures of acetone with Epon-812, with increasing concentration of the latter. Then the preparations were embedded in Epon-812. Ultrathin section were obtained with an LKB-III ultramicrotome (Sweden), stained with lead according to [8], and examined with an HU-11B electron microscope (Hitachi, Japan).

Mitochondria were isolated from coleoptiles of 5-day-old wheat seedlings. The coleoptiles were ground with a pestle in a mortar at 0°C in isolation medium that contained 0.4 M sucrose, 5 mM EDTA, 1 mM dithiothreitol, 20 mM Hepes-Tris (pH 7.5), and BSA (3 mg/ml), at the plant mass to medium ratio of 1 : 2 (g/ml). Remains of the plant tissue were removed by filtration of the homogenate through four layers of gauze, the filtrate was centrifuged at 28,000*g* for 5 min, and the precipitate was resuspended in isolation medium and centrifuged at 2500*g* for 20 min; the supernatant fluid was centrifuged at 28,000*g* for 40 min, the precipitate was resuspended in the BSA-free isolation medium and precipitated at 28,000*g* for 40 min, then the precipitate was suspended again in BSA-free isolation medium (final concentration of the mitochondrial protein was, on average, 50 mg/ml). Respiration of the mitochondria was determined with an LP7e polarograph (Czechoslovakia) using a Clark-type electrode in 1.5 ml of the incubation medium (0.4 M sucrose, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 20 mM Hepes-Tris, pH 8.0) at room temperature with constant stirring.

RESULTS

The rate of $O_2^{\cdot-}$ generation by wheat seedlings grown in the presence of different concentrations of DBP (10^{-7}-10^{-3} M) was studied. This series of experiments was replicated four times. A control sample (without DBP) was used in each experiment.

DBP significantly increased the rate of $O_2^{\cdot-}$ generation only when taken in the narrow range of concentrations (~10^{-4} M). Lower and higher concentrations of DBP had virtually no effect on the rate of $O_2^{\cdot-}$ generation by the seedlings (Fig. 1a).

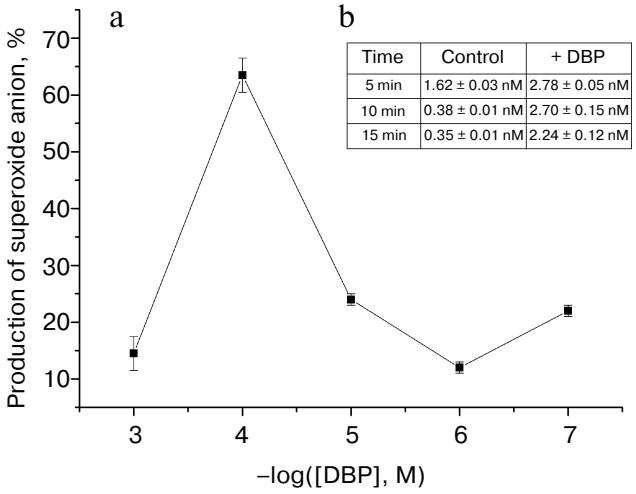


Fig. 1. Effect of dibutylphthalate on generation of $O_2^{\cdot-}$ and H_2O_2 by wheat seedlings. a) The curve shows the increase in the $O_2^{\cdot-}$ amount (in % versus control) released within 1 h by the seedlings grown in the presence of different concentrations of DBP (see "Materials and Methods"). b) The amount of H_2O_2 (nM) in the incubation medium after different time intervals from the experiment beginning (see "Materials and Methods"). Data for the control seedlings and the seedlings grown in the presence of 10^{-4} M DBP.

The effect of DBP on the release of H_2O_2 by the seedlings was also studied. The seedlings were grown in the presence of 10^{-4} M DBP. Contents of H_2O_2 in the incubation medium of the seedlings grown in the presence of DBP are presented in Fig. 1b: the determinations were performed 5, 10, and 15 min after the seedlings had been submerged into the incubation medium. DBP sharply increased the production of H_2O_2. But one has to keep in mind that H_2O_2 was most likely generated as a result of disproportioning $O_2^{\cdot-}$:

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2.$$

Determinations of H_2O_2 production confirmed once more that DBP accelerated ROS generation by the seedlings.

The effect of DBP on the system of electron transfer in mitochondria isolated from coleoptiles of 5-day-old wheat seedlings was studied. DBF at concentrations of 10^{-4}-10^{-3} M inhibited NADH dehydrogenase. It inhibited mitochondrial respiration under conditions of malic acid oxidation (Fig. 2). Addition into the incubation medium of another substrate, succinic acid, partially overcame the inhibitory effect of DBP on respiration (data not presented). According to data of work [9], inhibition of NADH dehydrogenase has to stimulate production of ROS in mitochondria, in particular, the accumulation of H_2O_2 in the cell. This problem will be considered in detail later.

In the second part of this work, we studied the DBP-induced changes in the ultrastructure of cells and mitochondria by electron microscopy. Margination of chromatin is clearly demonstrated in the sections (Fig. 3). Mitochondria inside vesicles, or mitochondrial vesicles,

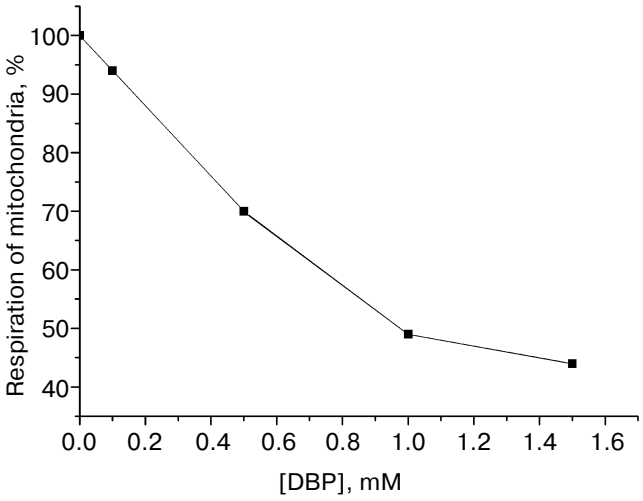


Fig. 2. Inhibition of respiration of mitochondria isolated from coleoptiles in the presence of increasing concentrations of DBP. The points indicate the rate of respiration (in % of the initial) after addition of a certain concentration of the inhibitor.

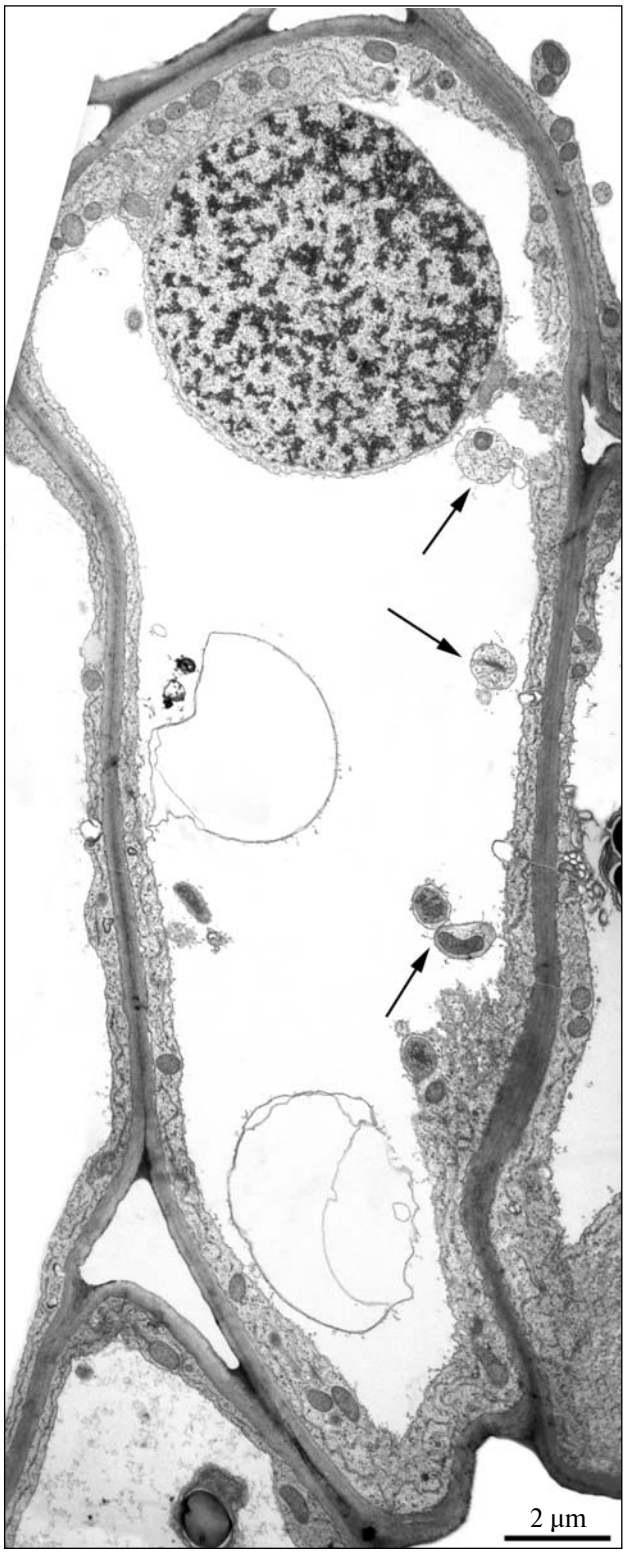


Fig. 3. Electron microphotograph of a parenchyma cell of a 4-day-old seedling coleoptile. Chromatin margination is clearly seen; the arrows indicate cytoplasmic vesicles containing mitochondria.

were earlier detected by us in vacuoles of parenchyma cells of apical zones of 4-day-old wheat seedling coleoptiles [4]. The formation of these structures was followed using ultrathin serial sections: the tonoplast produced the cytoplasm evagination into the vacuole, then some mitochondria displaced there, and these evaginations "laced off". As a result, suspended mitochondria-containing cytoplasmic bodies appeared in the vacuoles. These "vesicular" mitochondria were intact and actively utilized oxygen. They had the same set of cytochromes as normal mitochondria, but were specified by the intense synthesis of heavy ($\rho = 1.718$ g/cm^3) mitochondrial DNA [10, 11].

The cultivation of wheat seedlings in the presence of different concentrations of the gametocide DBP caused structural changes in the parenchyma cells of etiolated seedling coleoptiles similar to changes described earlier. As in the case of $O_2^{\cdot-}$ generation, the ultrastructure of the coleoptile parenchyma cells was most affected in the presence of 10^{-4} M DBP, i.e., the formation of mitochondria-containing vesicles was sharply intensified. The parenchyma cells of these seedlings had a large central vacuole and a small volume of cytoplasm as a narrow parietal layer (Fig. 4, a and b). On the vacuole surface, the cytoplasm formed numerous evaginations of different size with mitochondria inside (1-10 and more, depending on the size of the evaginated cytoplasmic volume). All mitochondria of the cell, except perinuclear ones, were located directly close to the tonoplast (vacuolar membrane) in the cytoplasmic evaginations. In some cells, this cytoplasmic layer boundary to the vacuolar membrane and containing mitochondria was separated by a membrane from the bulk of the cell cytoplasm (Fig. 4a). In vacuoles of these cells there were isolated mitochondria-containing cytoplasmic fragments encircled with a closed membrane. These structures were sometimes completely filled with tightly packed mitochondria (Fig. 4a).

On comparing the cells of an intact aging coleoptile (control, Fig. 5a) and the cells from coleoptile of the seedling grown in the presence of 10^{-4} M DBP (Fig. 4, a and b) one can see that DBP significantly activated the formation of the membrane-delimited isolated mitochondria-containing cytoplasmic fragments and also sharply increased the number of mitochondria and changes their ultrastructure. The mitochondria larger than the control ones had an electron-dense condensed matrix, and sometimes elongated mitochondria with constrictions were found (Fig. 5b).

In the parenchyma cells from coleoptile of the seedlings grown at the higher concentration of DBP (10^{-3} M) the formation of isolated cytoplasmic fragments ejecting mitochondria from the cytoplasm was less pronounced (Fig. 6a) than at 10^{-4} M DBP; thus, by this characteristic and also by the number of mitochondria in the cell they corresponded to cells of the intact coleoptile. However, they were somewhat unlike the control; in the cytoplasm of the coleoptile cells of these seedlings unusual membrane formations appeared consisting of accumulations of closed membrane vesicles with different configuration (Fig. 7, a and b). In this case, large myelin-like structures formed by plasmalemma were also observed Fig. 6b).

In the cells from coleoptile of the wheat seedlings grown in the presence of 10^{-6} M there were no differences in the ultrastructure of control and intact coleoptiles.

DISCUSSION

Electron microscopy data have shown that the gametocide DBP activates in cells a specific process—a native (without destruction of the cell) ejection of mitochondria from the cytoplasm into the vacuole. These mitochondria are enclosed within vesicles that contain a small amount of cytoplasm. This process, which may be called "mitochondrial exocytosis", was detected by us earlier [4] during spontaneous apoptosis in wheat coleoptiles in the early stages of the seedling development. In our experiments, DBP did not change the character of spontaneous apoptosis in the wheat coleoptiles but significantly activated this process. It should be emphasized that formation of mitochondrial vesicles (enhancement of apoptosis) and generation of ROS were stimulated by the same concentration of DBP. To reveal such a correlation was the main purpose of the present work.

It was tempting, based on the findings and literature data, to outline causal-consequence relationships between the effect of DBP on the first complex of the mitochondrial respiratory chain, generation of the "external" superoxide anion, and apoptosis.

The suggested scheme is based on the biphasic mechanism of induction of ROS (and streams of calcium ions), which was considered in works [3, 12]. Inhibition of activity of the mitochondrial respiratory chain (complex I) is known to be accompanied by production of small amounts of H_2O_2 [9]. Therefore, it was suggested that suppression by DBP of the complex I activity should trigger the process resulting in accumulation of H_2O_2 in the cytoplasm and activation (according to [13]) of Ca^{2+} ejection from the reticulum. The increase in the Ca^{2+} content in the cytoplasm activates the second cascade of ROS induction, i.e., activation of Ca^{2+}-dependent NADPH oxidase [14]. This is associated with switching on synthesis of the "external" $O_2^{\cdot-}$, and its accumulation has been directly recorded in our experiments. As shown in [3], external ROS (H_2O_2) open in the plasma membrane lanthanum-sensitive calcium channels. This has to be associated with entrance into the cell of many calcium ions from the environment.

The induction of apoptosis observed by us seems to be associated with opening in mitochondria of a nonspe-

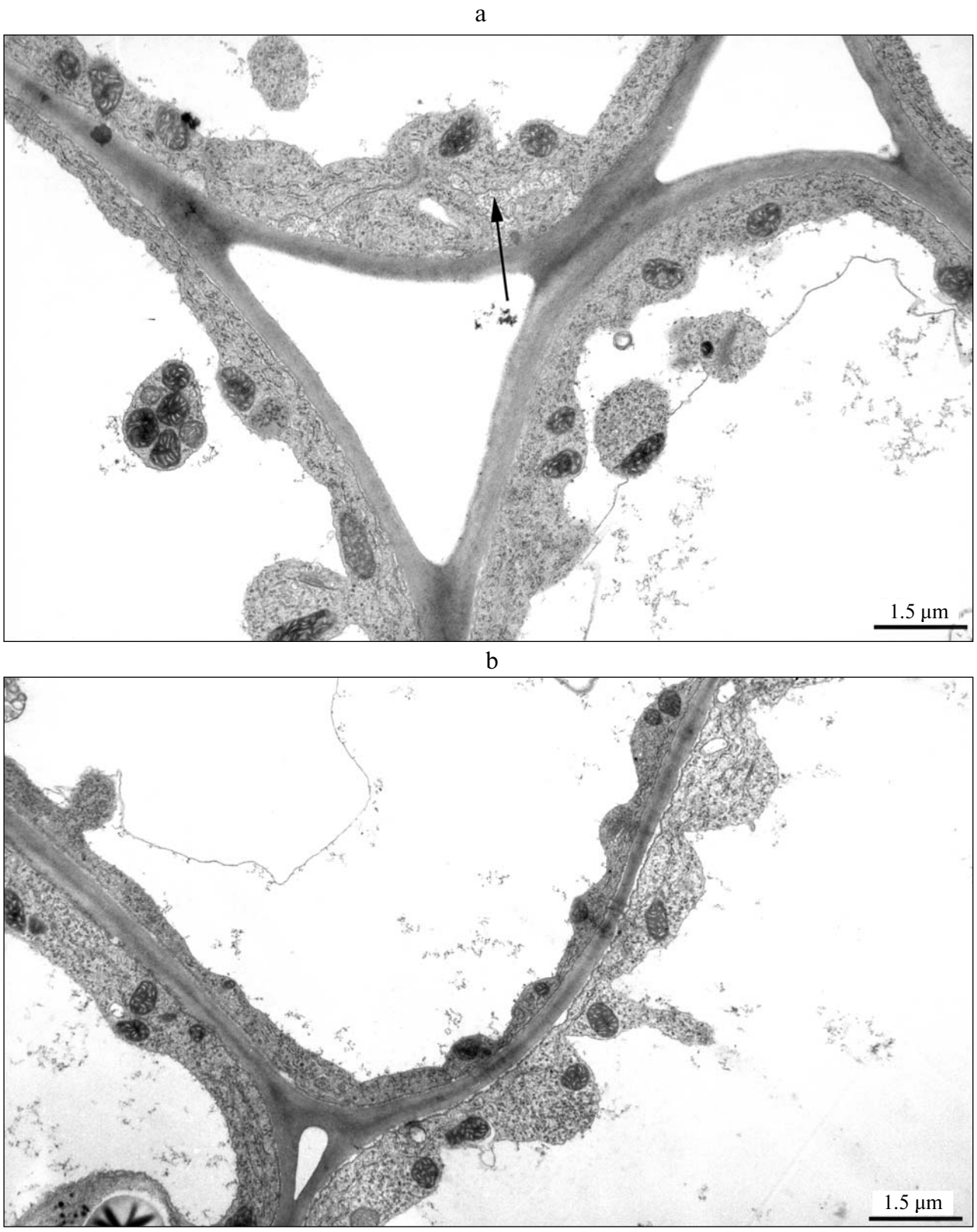
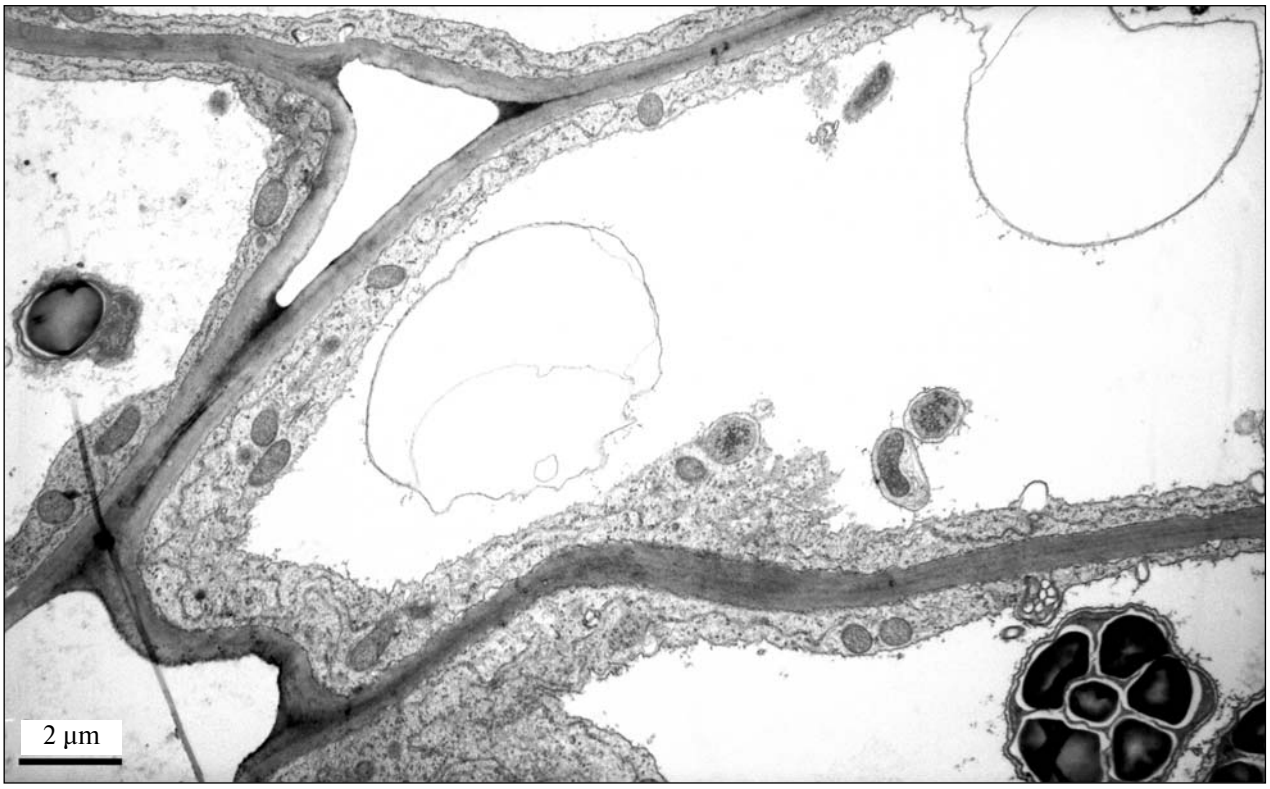


Fig. 4. Electron microphotographs of parenchyma cells from coleoptile of the 4-day-old seedlings grown in the presence of 10^{-4} M DBP. a) The arrow indicates separation with a membrane of the cytoplasmic layer boundary to the tonoplast. b) Numerous evaginations of the cytoplasm on the surface of the cellular vacuole, with mitochondria located inside are shown, i.e., potential "mitochondrial vesicles".

a



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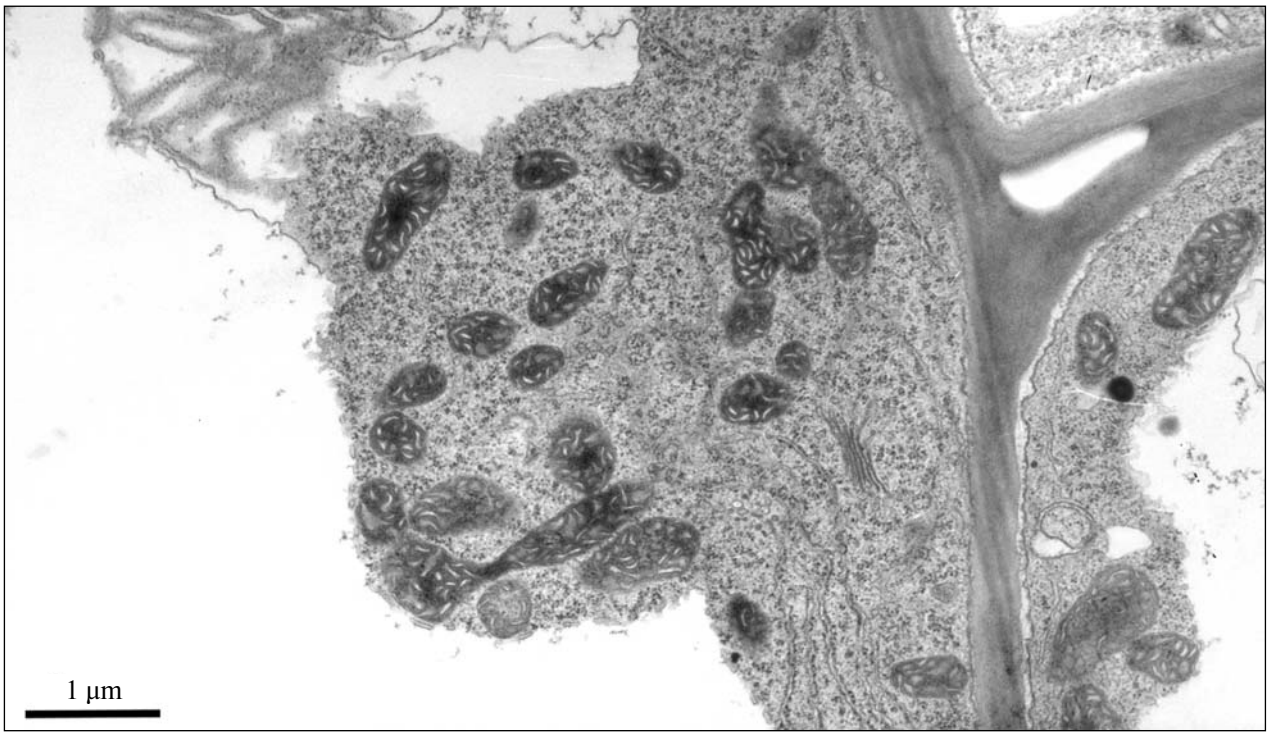
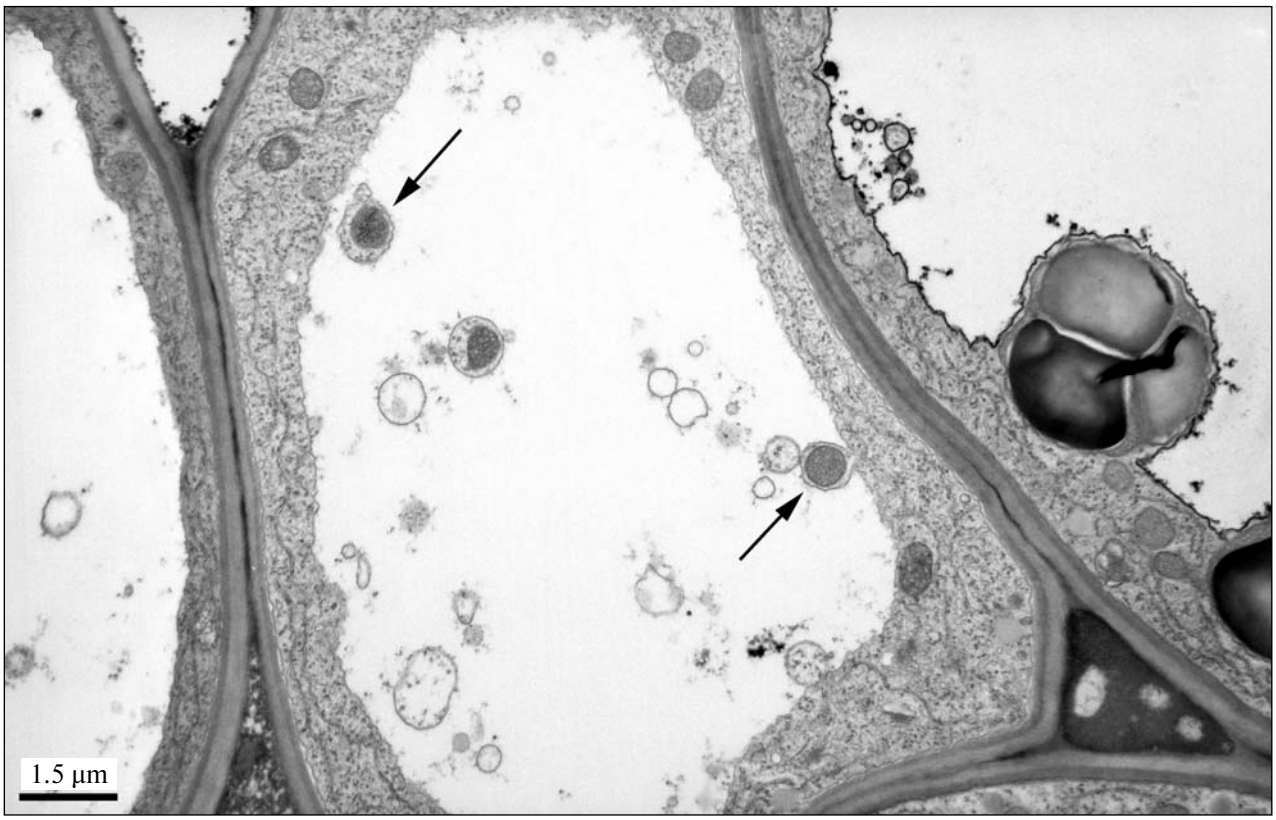


Fig. 5. Electron microphotographs of the parenchyma cells of 4-day-old seedling coleoptile. a) Region of the parenchyma cell from an intact aging coleoptile. b) Fragment of the cell cytoplasm from coleoptile of a seedling grown in the presence of 10^{-4} M DBP; mitochondria are accumulated in the region of the cytoplasm evagination.

a



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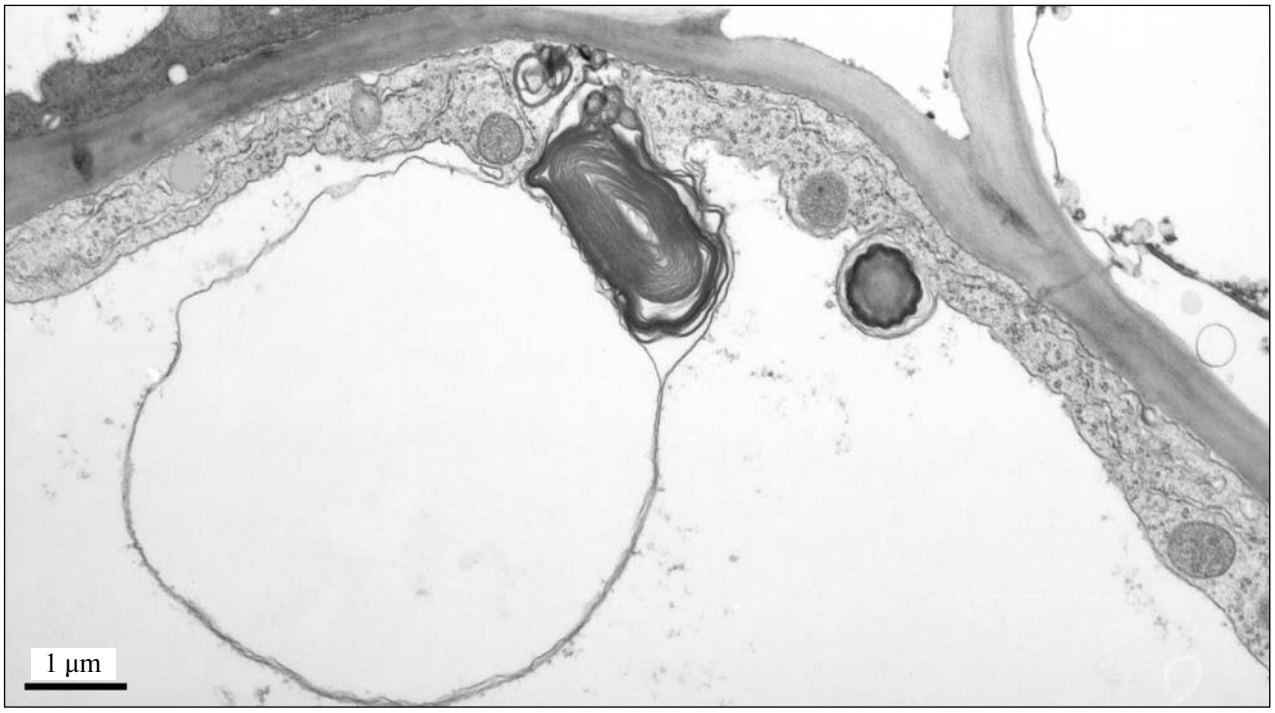

Fig. 6. Electron microphotographs of parenchyma cells from coleoptile of seedlings grown in the presence of 10^{-3} M DBP. There were no evaginations of the cytoplasm. a) The arrows indicate isolated mitochondria-containing cytoplasmic fragments. b) The myelin-like structure formed by the plasmalemma.

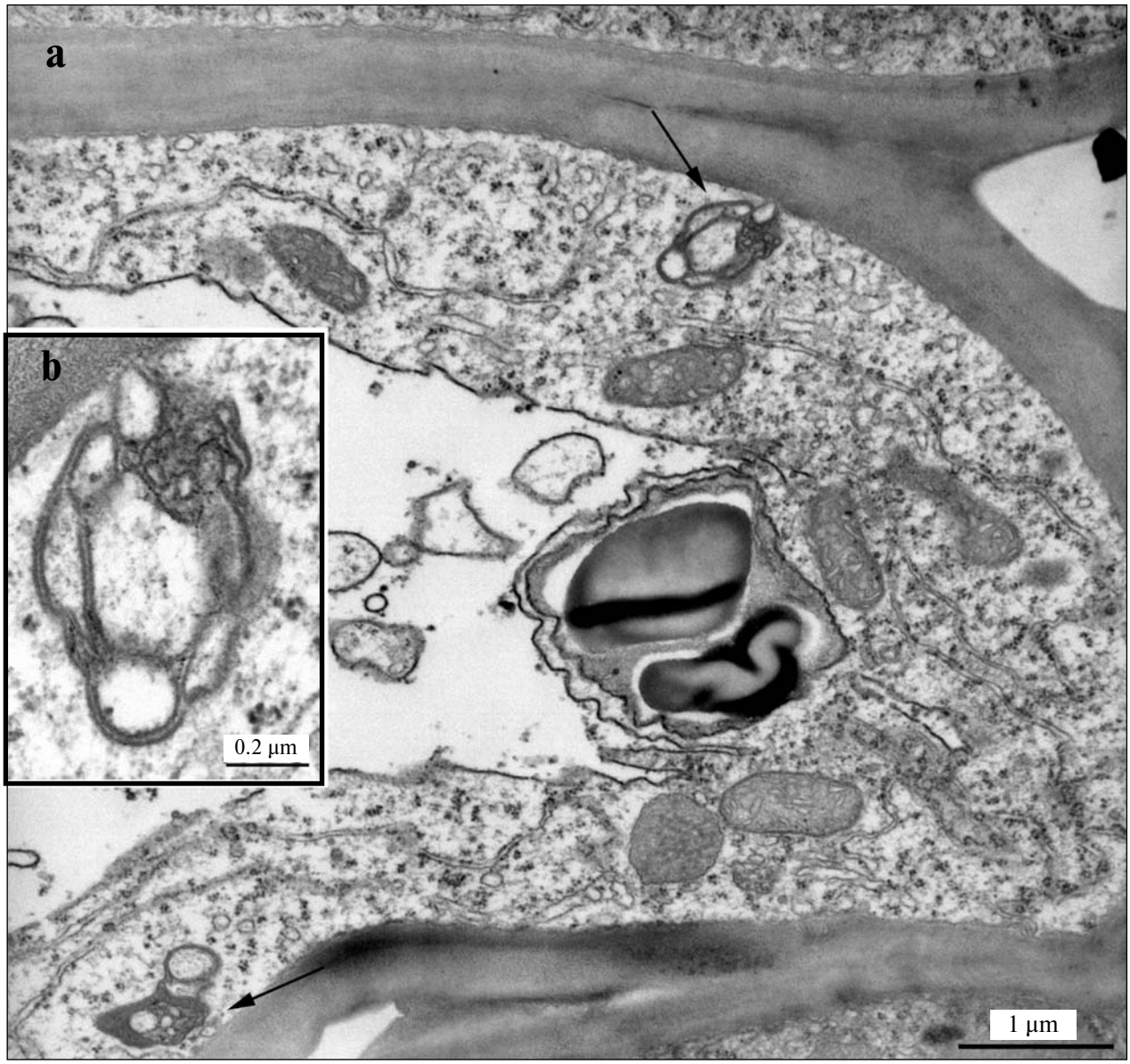

Fig. 7. Electron microphotographs of parenchyma cells from coleoptile of seedlings grown in the presence of 10^{-3} M DBP. a) The arrows indicate unusual membrane formations in the cell cytoplasm. b) Ultrastructure of the membrane formations arising in the cytoplasm under the influence of 10^{-3} M DBP at higher magnification. The ultrastructure of mitochondria was virtually not different from normal.

cific pore under the influence of high Ca^{2+} concentration in the cytoplasm [15, 16] and, respectively, with ejection of cytochrome *c* from the mitochondria [17].

Although this scheme is conventional, it shows that biochemical processes that can provide for the correlation observed between the efficiency of inducing the "external" $O_2^{\cdot-}$ and intensity of apoptosis really exist in the cell.

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REFERENCES

1. Jabs, T. (1999) *Biochem. Pharmacol.*, **57**, 231-245.
2. Frahry, G., and Schopfer, P. (2001) *Planta*, **212**, 175-183.
3. Mahalingam, R., and Fedoroff, N. (2003) *Physiol. Plant.*, **119**, 56-68.

4. Bakeeva, L. E., Kirnos, M. D., Aleksandrushkina, N. I., Kazimirchyuk, S. V., Shorning, B. Yu., Zamyatnina, V. A., Yaguzhinsky, L. S., and Vanyushin, B. F. (1999) *FEBS Lett.*, **457**, 122-125.
5. Shorning, B. Yu., Smirnova, E. G., Yaguzinsky, L. S., and Vanyushin, B. F. (2000) *Biochemistry (Moscow)*, **65**, 1357-1361.
6. Auclair, C., and Voisin, E. (1987) in *CRC Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., ed.) CRC Press, Inc., Boca Ration, Florida, pp. 123-132.
7. Smirnova, E. G., Lyubimov, Yu. I., Malinina, T. G., Lyubimova, E. Yu., Aleksandrushkina, N. I., Vanyushin, B. F., Kolesova, G. M., and Yaguzhinsky, L. S. (2002) *Biochemistry (Moscow)*, **67**, 1271-1275.
8. Reynolds, E. (1963) *J. Cell Biol.*, **17**, 208-212.
9. Turrens, J. F., and Boveris, A. (1980) *Biochem. J.*, **191**, 421-427.
10. Aleksandrushkina, N. I., Zamyatnina, V. A., Bakeeva, L. E., Seredina, A. V., Smirnova, E. G., Yaguzhinsky, L. S., and Vanyushin, B. F. (2004) *Biochemistry (Moscow)*, **69**, 285-294.
11. Bakeeva, L. E., Zamyatnina, V. A., Shorning, B. Yu., Aleksandrushkina, N. I., and Vanyushin, B. F. (2001) *Biochemistry (Moscow)*, **66**, 850-859.
12. Lecourieux, D., Mazars, C., Pauly, N., Ranjeva, R., and Pugin, A. (2002) *Plant Cell*, **14**, 2627-2641.
13. Hara, Y., Wakamori, M., Ishii, M., Maeno, E., Nishida, M., Yoshida, T., Yamada, H., Shimizu, S., Mori, E., Kudoh, J., Shimizu, N., Kurose, H., Okada, Y., Imoto, K., and Mori, Y. (2002) *Mol. Cell.*, **9**, 163-173.
14. Banfi, B., Tirone, F., Durussel, I., Knisz, J., Moskwa, P., Molnar, G. Z., Krause, K.-H., and Cox, J. A. (2004) *J. Biol. Chem.*, **279**, 18583-18591.
15. Tassani, V., Biban, C., Toninello, A., and Siliprandi, D. (1995) *Biochem. Biophys. Res. Commun.*, **207**, 661-667.
16. Castilho, R. F., Kowaltowski, A. J., Meinicke, A. R., Bechara, E. J. H., and Vercesi, A. E. (1995) *Free Rad. Biol. Med.*, **18**, 479-486.
17. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell*, **91**, 479-489.