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Cyanide-Induced Death of Cells in Plant Leaves

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Abstract—Destruction of guard cell nuclei in epidermis isolated from leaves of pea, maize, sunflower, and haricot bean, as well as destruction of cell nuclei in leaves of the aquatic plants waterweed and eelgrass were induced by cyanide. Destruction of nuclei was strengthened by illumination, prevented by the antioxidant α-tocopherol and an electron acceptor N,N,N′,N′-tetramethyl-*p*-phenylenediamine, and removed by quinacrine. Photosynthetic O_2 evolution by the leaf slices of a C_3 plant (pea), or a C_4 plant (maize) was inhibited by CN^- inactivating ribulose-1,5-bisphosphate carboxylase, and was renewed by subsequent addition of the electron acceptor *p*-benzoquinone.

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Programmed cell death (PCD) is a physiological response to internal or external signals associated with maintenance of tissue homeostasis, development of immunological reactions to pathogens, realization of development program and cell differentiation, response to abiogenic stress factors, and aging [1]. Chemical and physical factors are easier to study than natural biological signals because they cause synchronous and mass death of cells, thus facilitating subsequent analysis of results. We [2] have used cyanide, a PCD inducer in plants, which causes internucleosomal decomposition of nuclear DNA [3, 4]. Cyanide has multiple effects: it inhibits cytochrome *c* oxidase of mitochondria, catalase, peroxidases including ascorbate peroxidase of chloroplasts, and Cu,Zn-superoxide dismutase [5] and inactivates ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) [6].

On evidence of light microscopy, CN^- causes fragmentation and decomposition of nuclei in cells of epidermis isolated from pea leaves [2]. Epidermis is a monolayer composed of structurally and functionally different basal epidermal cells (EC) and stoma guard cells (GC): EC contain mitochondria only, whereas GC, like mesophyll cells, contain both chloroplasts and mitochondria. Light significantly accelerated CN^--induced decomposition of GC nuclei, but had no effect on decomposition of EC nuclei [2]. The pattern of ultrastructural changes indicates that CN^--induced death of GS occurs via apoptosis [7].

Electron acceptors maintaining the Hill reaction in chloroplasts (*p*-benzoquinone, menadione, N,N,N′,N′-tetramethyl-*p*-phenylenediamine –TMPD, etc.) switching on noncyclic electron transfer inhibited by CN^- due to inactivation of Rubisco and subsequent exhaustion of $NADP^+$ prevented CN^--induced apoptosis of GC. Light-activation of GC apoptosis was inhibited by diuron (DCMU), an inhibitor of electron transfer in Photosystem II, stigmatellin, or iodonitrothymol dinitrophenyl ester (DNP-INT), inhibitors of plastoquinol oxidation by Rieske FeS protein in the center *o* of chloroplast cytochrome b_6f complex. The process was prevented by the protein kinase inhibitor staurosporine. It was concluded that GC apoptosis depends on combined effects of two factors: reactive oxygen species (ROS) and plastoquinone acting in center *o* of b_6f complex [8, 9]. Experiments with pea mutants have shown that light stimulation of CN^--induced GC apoptosis and its abolition by DCMU (3-(3′,4′-dichlorophenyl)-1,1-dimethylurea) are associated with activity of Photosystem II [10]. The data are considered as evidence for involvement of chloroplasts in apoptosis.

Abbreviations: BQ) *p*-benzoquinone; DCF) 2′,7′-dichlorofluorescein; DCFH) 2′,7′-dichlorofluorescin; DCMU) 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; EC) basal epidermal cells; FeCy) potassium ferricyanide; GC) stoma guard cells; PCD) programmed cell death; ROS) reactive oxygen species; Rubisco) ribulose-1,5-bisphosphate carboxylase/oxygenase; TMPD) N,N,N′,N′- tetramethyl-*p*-phenylenediamine.

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Quinacrine and diphenyliodonium, which are known as inhibitors of NADPH oxidase of cell plasma membrane, in tested concentrations did not influence either respiration or photosynthetic O_2 release by pea leaf slices, but prevented CN^--induced death of GC [11]. It is possible that CN^--resistant NADPH oxidase of GC plasma membrane is a source of ROS causing CN^--induced PCD. When ROS are insufficiently produced, added H_2O_2 stimulated PCD [11].

In the light of these data, a question emerges on the effect of CN^- on cells of other plants. A peculiar interest was to compare plants with C_3- and C_4-photosynthesis, in which fixation of atmospheric CO_2 is maintained by Rubisco and phosphoenolpyruvate carboxylase, respectively. Aquatic plants (hydrophytes) were also examined.

MATERIALS AND METHODS

The objects of the study were stoma guard cells from leaves of pea (*Pisum sativum* L.) cultivar Alpha, sunflower (*Helianthus annuus* L.), haricot bean (*Phaseolus vulgaris* L.), and maize (*Zea mays* L.), as well as cells of aquatic plants waterweed (*Elodea canadensis* Michx.) and eelgrass (*Vallisneria spiralis* L.). For germination, the seeds of pea, sunflower, haricot bean, and maize were maintained in water for 1 day followed by incubation on wet filter paper for 1-2 days [2]. Germinated seeds were rooted in jars filled with tap water and grown hydroponically under continuous illumination with luminescence lamps (light intensity ~1000 lx, temperature 20-24°C). Plantlets at the age of 8-17 days were used in experiments. Experiments with waterweed and eelgrass were performed using fragments of leaves growing on young branches at the distance of 1-2 cm from the top. Their leaves are a cell bilayer and along the edges even a monolayer, allowing comfortable observation of cell nuclei under a light microscope.

Epidermal films from the bottom surface of leaves were separated with forceps and placed into distilled water [2]. Vacuum infiltration of epidermal films for 1-2 min was used for rapid influx of added reagents into the cells [2]. The samples were placed into polystyrene plates and incubated for 24 h in distilled water or in 0.1 M Na^+,K^+-phosphate buffer, pH 7.4, with additives (the composition is given in the figure legends) at room temperature either in dark or under illumination with luminescent lamps at the light intensity of ~1000 lx.

After the incubation, the samples were washed with distilled water for 5 min followed by treatment for 5 min with Battaglia's fixative (mixture of chloroform, 96% ethanol, glacial acetic acid, and 40% formaldehyde, 5 : 5 : 1 : 1). [2]. Then the samples were washed with ethanol for 10 min for removal of the fixative, incubated for 5 min in water and stained with Carazzi's hematoxylin for 20 min. The stained epidermal peels were washed with tap water used for the microscopy. All experiments were replicated twice and in most cases thrice. The number of cells with destroyed nuclei and lack of nuclei was determined from 300-500 inspected cells.

To determine the effect of CN^- on maize mesophyll cells, leaf slices were placed in 2.5 mM KCN, incubated in the light for 1 day, fixed with 3% glutaraldehyde solution in 0.1 M Na^+,K^+-phosphate buffer, pH 7.4, for 2 h at 4°C followed by post-fixation with 1% osmium tetroxide in the same buffer for 1.5 h and dehydrated in ethanol solutions of increased concentration; 70% ethanol solution was saturated with uranyl acetate (1.5%). The prepared material was embedded in Epon-812 resin (Fluka, Switzerland). Semithin slices (~3 μm) were prepared using an LKB III ultramicrotome (LKB, Sweden).

2′,7′-Dichlorofluorescein (DCF) fluorescence was measured on a VersaFluor fluorimeter (Bio-Rad, USA) as described previously [11]. Maize leaf with removed lower epidermis was fixed by its intact surface on a polystyrene plate, submerged in 50-μM DCFH (2′,7′-dichlorofluorescin) diacetate solution, and incubated in the dark for 10 min followed by washing with distilled water; then the leaf was placed into a photometric cell filled with 25 mM Hepes-NaOH, pH 7.2. Fluorescence of DCF was excited with light with λ = 485-495 nm and registered at 515-525 nm.

Oximetry (uptake and evolution of O_2 by leaf slices) was carried out as previously described [9, 11].

RESULTS

According to the previously reported data [2, 8, 9], CN^- caused destruction of GC nuclei in pea epidermis (Fig. 1a). The effect of CN^- as an apoptosis inducer was enhanced by illumination and inhibited by the electron acceptor TMPD and antioxidant α-tocopherol. Similar data were obtained with GC of maize, sunflower, and haricot bean (Fig. 1, b-d). Quinacrine prevented the CN^--induced decomposition of GC nuclei (Fig. 1, b and d). Earlier, the same effect of quinacrine was demonstrated on GC of pea [11].

Cyanide caused destruction of cell nuclei in waterweed and eelgrass leaves (Fig. 2). Destruction of nuclei increased with increasing CN^- concentration. The maximal effect of CN^- on pea leaf GC was observed at concentration of 2.5 mM [2]. As in the case of the terrestrial plants, CN^--induced destruction of cell nuclei of hydrophytes was higher in the light than in the dark; TMPD and α-tocopherol removed the effect of CN^- (Fig. 2).

Figure 3 illustrates O_2 uptake by maize leaf slices caused by oxidation of intracellular substrates (endogenous respiration) in mitochondria. Illumination causes O_2 evolution associated with electron transfer from H_2O to $NADP^+$ in chloroplasts. As in pea [11], the photosynthetic O_2 evolution by maize leaf slices is inhibited by

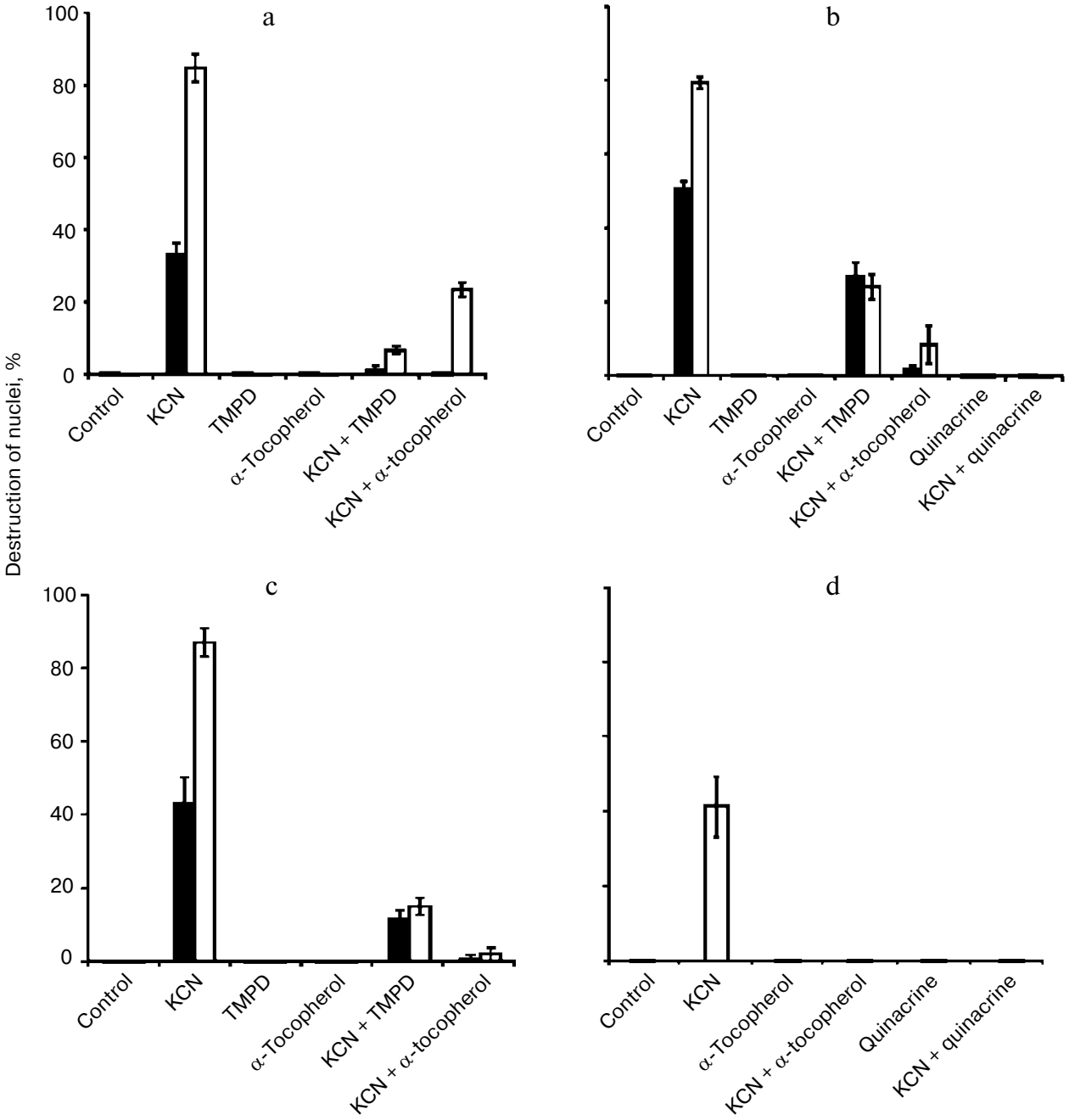

Fig. 1. Effect of different agents on the CN^--induced destruction of GC nuclei in leaf epidermis of pea (a), maize (b), sunflower (c), and haricot bean (d) in the dark (black columns) and in the light (light columns). Additives: 2.5 mM KCN, 100 μM TMPD or α-tocopherol, 50 μM quinacrine.

CN^-, and, with following addition of ferricyanide and *p*-benzoquinone, a membrane-penetrating electron acceptor in the Hill reaction, is replaced with light-dependent DCMU-sensitive O_2 evolution. In their oxymetric parameters, leaf slices of maize, a plant with C_4-photosynthesis (Fig. 3), are similar to that of pea, a plant with C_3-photosynthesis [11].

Oxidation of non-fluorescent 2′,7′-dichlorofluorescin (DCFH) to fluorescent DCF is an indicator of ROS production in cells [12, 13]. Addition of menadione to slices of maize leaves with removed lower epidermis led to formation of DCF in cells pre-loaded with non-fluorescent DCFH diacetate (Fig. 4). Being reduced by components of the photosynthetic [14, 15] and respiratory [16, 17] electron transport chains, menadione semiquinone undergoes spontaneous oxidation by O_2 with formation of $O_2^{\cdot-}$, which in turn forms H_2O_2 with participation of superoxide dismutase. Menadiol is also oxidized

by O_2 with formation of H_2O_2 [16, 18]. Increase in DCF fluorescence caused by menadione was enhanced by a following addition of H_2O_2 (Fig. 4a) and inhibited by nitroblue tetrazolium (NBT) (Fig. 4b) oxidizing $O_2^{\cdot-}$ [19] and thereby preventing H_2O_2 generation. Quinacrine inhibited DCF response caused by both menadione and H_2O_2 (Fig. 4, c and d). Similar results were previously obtained from experiments with epidermal peels from pea leaves [11].

Figure 5a shows a longitudinal section of maize lamina: one can see that the vascular bundle sheath cells are more than two times longer than the mesophyll cells. So, the probability of appearance of nuclei on the lateral section of the leaf is lower for sheath cells than for mesophyll cells. Figure 5 (b-d) shows a characteristic anatomy of a C_4 plant leaf: vascular bundles are surrounded by dense layer of big sheath cells, around which a spongy layer of mesophyll cells is localized. Cyanide caused decomposition of nuclei both in mesophyll cells (Fig. 5, c and d) and in GC (Fig. 1b).

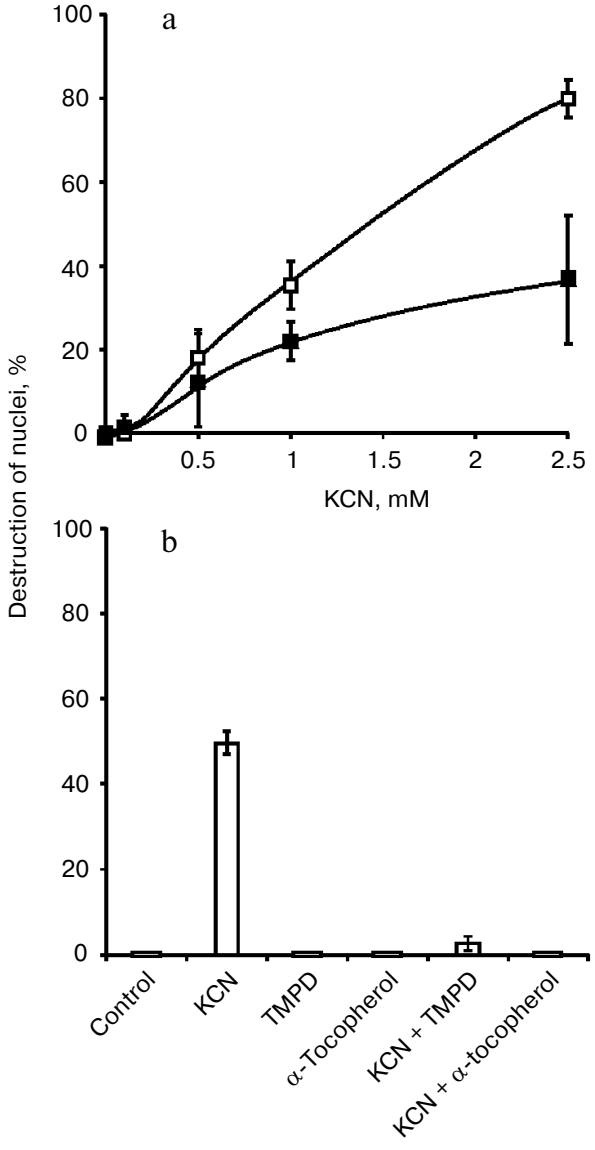

Fig. 2. Cyanide-induced destruction of nuclei in leaf cells of waterweed in the dark (closed squares) and in the light (open squares) (a) and eelgrass in the light (b). Additives: 2.5 mM of KCN, 100 μM of TMPD or α-tocopherol.

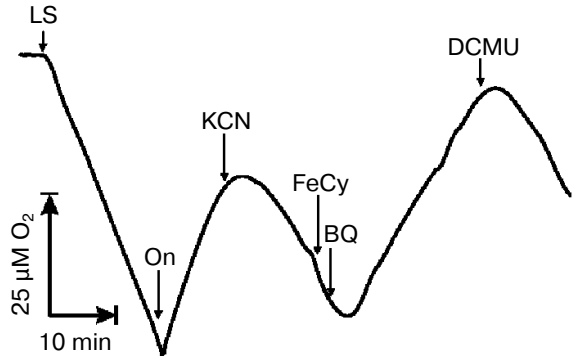

Fig. 3. Oxygen uptake and evolution by maize leaf slices (LS). Incubation medium: 10 mM Hepes-NaOH, pH 7.0, LS with chlorophyll content of 30 μg/ml. Additives: 2.5 mM KCN, 3 mM ferricyanide (FeCy), 100 μM *p*-benzoquinone (BQ), 20 μM DCMU. On, turning on the light. Initial rates of respiration and photosynthetic O_2 evolution (without additives) were 7-8 μmol O_2/mg chlorophyll per hour.

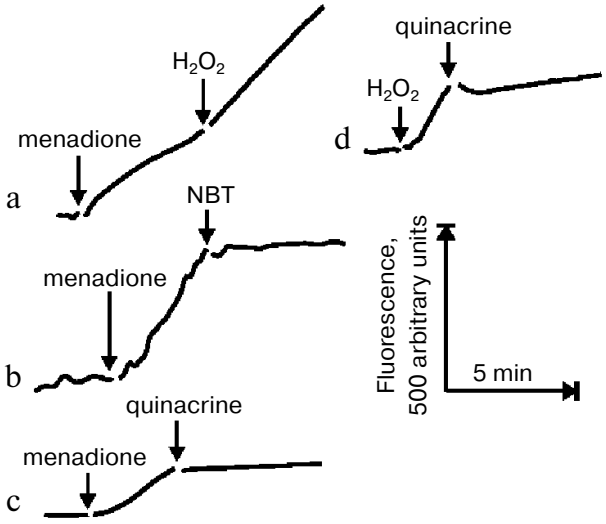

Fig. 4. Generation of DCF from DCFH diacetate added to maize leaves with removed lower epidermis. Additives: 100 μM menadione, 100 μM H_2O_2, 200 μM nitroblue tetrazolium (NBT), 50 μM quinacrine.

DISCUSSION

We have reported previously that CN^- causes decomposition of both GC and EC nuclei in pea leaf epidermis

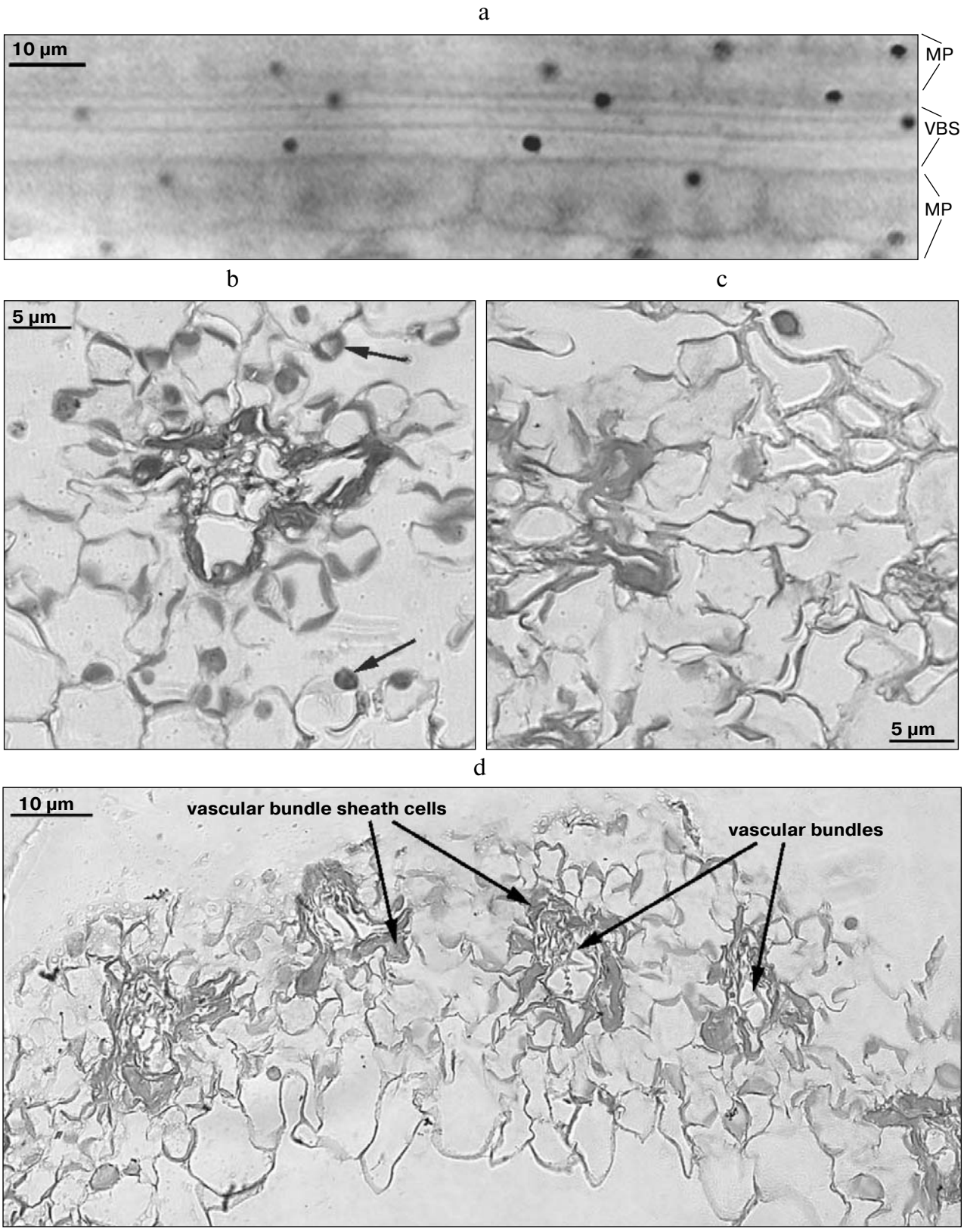

Fig. 5. Light microscopy of maize leaf sections upon the influence of CN^-: a) longitudinal, b, c, d) lateral sections of the leaf. Maize leaf slices were treated with 2.5 mM KCN and incubated in the light for 24 h: a, b) control; c, d) KCN. MP, mesophyll; VBS, vascular bundle sheath cells.

[2, 8, 9]. The data on ultrastructural changes [7] and internucleosomal DNA fragmentation [4] showed an apoptotic mechanism of CN^--induced death of GC. Resemblance of the action of the antioxidant α-tocopherol and TMPD, which interacts with both photosynthetic electron transport chain of chloroplasts and respiratory chain of mitochondria (Figs. 1 and 2), suggests that CN^--induced decomposition of nuclei in cells of other plants also occurs via apoptosis dependent on ROS and regulated by redox state of plastoquinone in the cytochrome b_6f complex of chloroplasts [8, 9]. The data on prevention of CN^--induced destruction of nuclei by quinacrine (Fig. 1) and lack of its effect on both mitochondrial respiration and photosynthetic O_2 evolution by chloroplasts in pea leaf slices [11] demonstrate that ROS for realization of apoptosis seem to be generated by NADPH oxidase of cell plasma membrane. The same result was obtained with maize leaves (Fig. 4) and pea leaf epidermis [11]: quinacrine inhibited the H_2O_2-dependent fluorescent response of DCF. These data confirm a suggestion that H_2O_2, either added or generated in the presence of menadione, activates NADPH oxidase of plasma membrane. ROS-induced ROS release is known in animals [20].

The data of particular interest are that, like in pea [11], photosynthetic O_2 evolution by maize leaf slices (Fig. 3) is inhibited by CN^- inactivating Rubisco. Fixation of CO_2 in C_4 plants (maize) occurs via carboxylation of phosphoenolpyruvate in mesophyll cells to produce oxaloacetate, which is reduced by NADPH to malate. Malate is transported to neighboring vascular bundle sheath cells, where it undergoes $NADP^+$-dependent decarboxylation with participation of malic enzyme to form pyruvate and CO_2, which is fixed again by Rubisco. Cyanide inactivates Rubisco [6] and thereby stops photosynthetic O_2 evolution (Fig. 3) not only by vascular bundle sheath cells, but also by mesophyll, which, being functionally associated with sheath, suffers a deficit of pyruvate (due to the inhibition of malic enzyme) and deficit of $NADP^+$ for non-cyclic electron transfer from H_2O (due to the inhibition of reaction oxaloacetate → malate). The electron-acceptor pair ferricyanide + BQ remedies the situation (Fig. 3) switching on the electron transport from H_2O to BQ both in mesophyll and vascular bundle sheath.

Thus, non-directly, via vascular bundle sheath, cyanide as inducer of PCD runs down the mesophyll cells and causes destruction of their nuclei (Fig. 5).

REFERENCES

1. Samuilov, V. D., Oleskin, A. V., and Lagunova, E. M. (2000) *Biochemistry (Moscow)*, **65**, 873-887.
2. Samuilov, V. D., Lagunova, E. M., Beshta, O. E., and Kitashov, A. V. (2000) *Biochemistry (Moscow)*, **65**, 696-702.
3. Wang, H. L. J., Bostock, R. M., and Gilchrist, D. G. (1996) *Plant Cell*, **8**, 375-391.
4. Dzyubinskaya, E. V., Kiselevsly, D. B., Lobysheva, N. V., Shestak, A. A., and Samuilov, V. D. (2006) *Biochemistry (Moscow)*, **71**, 1120-1127.
5. Asada, K. (1999) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **50**, 601-639.
6. Ishida, H., Shimizu, S., Makino, A., and Mae, T. (1998) *Planta*, **204**, 305-309.
7. Bakeeva, L. E., Dzyubinskaya, E. V., and Samuilov, V. D. (2005) *Biochemistry (Moscow)*, **70**, 972-979.
8. Samuilov, V. D., Lagunova, E. M., Dzyubinskaya, E. V., Izyumov, D. S., Kiselevsky, D. B., and Makarova, Ya. V. (2002) *Biochemistry (Moscow)*, **67**, 627-634.
9. Samuilov, V. D., Lagunova, E. M., Kiselevsky, D. B., Dzyubinskaya, E. V., Makarova, Ya. V., and Gusev, M. V. (2003) *Biosci. Rep.*, **23**, 103-117.
10. Samuilov, V. D., Lagunova, E. M., Gostimsky, S. A., Timofeev, K. N., and Gusev, M. V. (2003) *Biochemistry (Moscow)*, **68**, 912-917.
11. Samuilov, V. D., Kiselevsky, D. B., Sinitsyn, S. V., Shestak, A. A., Lagunova, E. M., and Nesov, A. V. (2006) *Biochemistry (Moscow)*, **71**, 384-394.
12. LeBel, C. P., Ischiropoulos, H., and Bondy, S. C. (1992) *Chem. Res. Toxicol.*, **5**, 227-231.
13. Wrona, M., Patel, K., and Wardman, P. (2005) *Free Rad. Biol. Med.*, **38**, 262-270.
14. Hauska, G. (1977) in *Encyclopedia of Plant Physiology* (Trebst, A., and Avron, M., eds.) Vol. 5, Springer Verlag, Berlin, pp. 253-265.
15. Samuilov, V. D., Barsky, E. L., and Kitashov, A. V. (1997) *Biochemistry (Moscow)*, **62**, 909-913.
16. Cadenas, E., Boveris, A., Ragan, C. I., and Stoppani, A. O. (1997) *Arch. Biochem. Biophys.*, **180**, 248-257.
17. Kolesova, G. M., Karaukhova, L. S., and Yaguzhinsky, L. S. (1991) *Biokhimiya*, **56**, 1779-1786.
18. Yamashoji, S., Ikeda, T., and Yamashoji, K. (1991) *Biochim. Biophys. Acta*, **1059**, 99-105.
19. Auclair, C., and Voisin, E. (1985) in *CRC Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., ed.) CRC Press, Inc. Boca Raton, Florida, pp. 123-132.
20. Zorov, D. B., Filburn, C. R., Klotz, L.-O., Zweier, J. L., and Sollott, S. J. (2000) *J. Exp. Med.*, **192**, 1001-1014.