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Programmed Cell Death in Plants: Protective Effect of Tetraphenylphosphonium and Tetramethylrhodamine Cations Used as Transmembrane Quinone Carriers

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Abstract—Tetraphenylphosphonium (TPP^+) and tetramethylrhodamine ethyl ester ($TMRE^+$) cations used as transmembrane carriers of ubiquinone (MitoQ) and plastoquinone (SkQ, SkQR) in mitochondria prevented at nanomolar concentrations the chitosan- or H_2O_2-induced destruction of the nucleus in epidermal cells of epidermis isolated from pea leaves. The protective effect of the cations was potentiated by palmitate. Penetrating anions of tetraphenylboron (TB^-) and phenyl dicarbaundecaborane also displayed protective effects at micromolar concentrations; the effect of TB^- was potentiated by NH_4Cl. It is proposed that the protective effect of the penetrating cations and anions against chitosan is due to suppression of the generation of reactive oxygen species in mitochondria as a result of the protonophoric effect of the cations plus fatty acids and the anions plus NH_4^+. Phenol was suitable as the electron donor for H_2O_2 reduction catalyzed by horseradish peroxidase, preventing the destruction of cell nuclei. The penetrating cations and anions, SkQ1, and SkQR1 did not maintain the peroxidase or peroxidase/oxidase reactions measured by their suitability as electron donors for H_2O_2 reduction or by the oxidation of exogenous NADH.

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Key words: penetrating ions, tetraphenylphosphonium, tetramethylrhodamine ethyl ester, tetraphenylboron anion, phenyl dicarbaundecaborane anion, cell nucleus destruction, protective effect, plant epidermal cells, chitosan, CN^-

Reactive oxygen species (ROS) include singlet oxygen and the products of one-, two-, and three-electron reduction of O_2: $O_2^{\cdot-}$, H_2O_2, and $OH^{\cdot}$. They exert both negative and positive effects on cells. Due to their high reactivity, they oxidize various substances, such as proteins, nucleic acids, and lipids, resulting in the loss of functional integrity. On the other hand, ROS are implicated in signal pathways regulating growth and development, cell cycle, and programmed cell death (PCD) in plants [1].

In plants, PCD is induced by CN^- [2]. The process is terminated by the protein kinase inhibitor staurosporine [3]. Reduction of plastoquinone at the *o* site of chloroplast cytochrome b_6f-complex likely activates a protein kinase inducing PCD. Plant protein kinases are activated by microbial elicitors such as flagellin (a protein component of bacterial flagella) and fragments of chitin (component of fungal cell wall) [4]. Chitosan, the product of incomplete deacetylation of chitin, is an efficient elicitor. Chitosan caused destruction of epidermal cell (EC) nuclei in pea leaf epidermis incubated under aerobic conditions [5].

In plant cells ROS are generated in mitochondria, chloroplasts, and nuclei, as well as by apoplastic peroxidase (see [6] and literature cited there) and NADPH-oxidase of the plasma membrane [5]. In animal mitochondria ROS are primarily generated by respiratory chain

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $C_{12}TPP^+$, dodecyltriphenylphosphonium; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCP, 2,4-dichlorophenol; DNP, 2,4-dinitrophenol; DPI^+, diphenyleneiodonium; $DTPP^+$, decyltriphenylphosphonium; ECs, epidermal cells; GCs, guard cells; MB, methylene blue; PCB^-, phenyl dicarbaundecaborane; PCD, programmed cell death; ROS, reactive oxygen species; SkQ1, 10-(6′-plastoquinonyl)decyltriphenylphosphonium; SkQ3, 10-(6′-methylplastoquinonyl)decyltriphenylphosphonium; SkQR1, 10-(6′-plastoquinonyl)decylrhodamine 19; TB^-, tetraphenylboron; $TMRE^+$, tetramethylrhodamine ethyl ester; TPP^+, tetraphenylphosphonium.

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complexes I and III [7]. However, in plants an alternative dehydrogenase catalyzing electron transfer from matrix NADH to ubiquinone competes with complex I. Similarly, an alternative oxidase catalyzing electron transfer from ubiquinol to O_2 with formation of H_2O competes with complex III [8]. About 1% of the O_2 absorbed by plant tissues is channeled into ROS production [9]. The process is inhibited by CN^-, a cytochrome oxidase inhibitor [9, 10], upon transition of the mitochondrial respiratory chain from state 4 to state 3 caused by addition of oxidative phosphorylation uncouplers [11-13], activation of mitochondrial uncoupling proteins with fatty acids [14], or activation of alternative oxidase with pyruvate [12, 13]. The O_2^- generated in mitochondria is converted into H_2O_2 and O_2 by Mn-superoxide dismutase, which is present in mitochondrial matrix of plants and animals and is insensitive to CN^- [8].

Anaerobic conditions, which prevent ROS production, suppress PCD in plants similarly to antioxidants [3, 5]. There are both enzymatic and nonenzymatic mechanisms of ROS detoxification in the cell [15]. Nonenzymatic antioxidants include redox-buffers ascorbate and glutathione, as well as α-tocopherol, flavonoids, alkaloids, and carotenoids. Enzymes detoxifying ROS include superoxide dismutase, catalase, ascorbate peroxidase, and glutathione peroxidase. Among the members of the large peroxidase family in plants [16], the apoplastic peroxidase implicated in cell wall lignin formation is of particular importance. Lignin is an insoluble polymer composed of hydroxyphenylpropane monomers. Polymerization of this phenolic substance occurs with the action of apoplastic peroxidase catalyzing the H_2O_2-dependent formation of phenoxyl radicals in different resonance states determining a variety of radical condensation products [17]. The apoplastic peroxidase is also implicated in antioxidant defense of cells [18].

Phenol, a substrate of apoplastic peroxidase/oxidase, prevented the chitosan- and H_2O_2-induced destruction of EC nuclei in pea leaf epidermis [18]. The phenolic substances 2,4-dichlorophenol (DCP), catechol, and salicylic acid, phenolic uncouplers of oxidative phosphorylation pentachlorophenol and 2,4-dinitrophenol (DNP), and non-phenolic uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) protected cells from the destructive effect of chitosan [18].

Both chitosan- and CN^--induced PCD in pea leaf epidermis was prevented by ubiquinone or plastoquinone covalently modified with membrane-penetrating cation of decyltriphenylphosphonium ($DTPP^+$) or rhodamine [19], namely, 10-(6′-ubiquinonyl)-$DTPP^+$ (MitoQ), 10-(6′-plastoquinonyl)-$DTPP^+$ (SkQ1), and 10-(6′-plastoquinonyl)decylrhodamine 19 (SkQR1). Half-maximal concentrations for the protective effect of cationic quinone derivatives were in the pico- and nanomolar range. Besides the quinone moiety, these substances contain a cationic component (either $DTPP^+$ or decylrhodamine 19) that plays the role of an electric locomotive [20] transporting uncharged quinones into negatively charged inner surfaces of plasmatic membrane and the inner mitochondrial membrane, in other words, into the cytoplasm and the mitochondrial matrix.

In this work we tested the effect of penetrating cations on programmed cell death in plants and compared it with the effect of penetrating anions and phenolic compounds. The tested substances protected EC nuclei from destruction induced by chitosan or H_2O_2.

MATERIALS AND METHODS

Structures of membrane-penetrating cations and anions used in the experiments are shown on the Fig. 1. This figure also shows structures of plastoquinone cationic derivatives SkQ1, SkQ3, and SkQR1. Low-viscosity chitosan (Fluka, Germany), the product of partial deacetylation of crab shell chitin, is a polydisperse in molecular mass heteropolymer of *N*-acetylglucosamine and glucosamine.

Chitosan (100 μg/ml) and H_2O_2 (100 μM) were used as inducers of EC nucleus destruction in epidermis. Experiments were carried out on epidermis isolated from the bottom side of leaves of 7-15-day-old pea (*Pisum sativum* L., cv. Alpha) plantlets grown under periodic illumination with luminescent lamps (~100 $\mu E \cdot m^{-2} \cdot sec^{-1}$; 16 h light, 8 h dark) at 20-24°C. Epidermis was detached with forceps and placed into distilled water. Vacuum infiltration of epidermis at 1-2 mm Hg for 1-2 min was used for quick delivery of the used reagents into the cells. This treatment did not affect further development of the plantlets. Specimens were placed into polystyrene plates and incubated in distilled water with reagents (composition is given in figure legends) at room temperature in the dark.

Following incubation, specimens were treated with Battaglia's fixative (chloroform–96% ethanol–glacial acetic acid–40% formaldehyde, 5 : 5 : 1 : 1 v/v) for 5 min, washed with ethanol for 10 min for fixative removal, incubated in water for 5 min, and stained with Carracci hematoxylin for 20 min; the stained specimens were then washed with tap water. The cells were examined under a Carl Zeiss Laboval 4 microscope (Germany). The number of cells with destroyed nuclei and cells lacking nuclei was determined from 300-500 cells for each epidermal film.

RESULTS AND DISCUSSION

Figure 2a shows that, like $DTPP^+$-derivatives of quinones [19], TPP^+ prevents chitosan-induced destruction of EC nuclei. Its protective effect was maximal at 10^{-12}-10^{-11} M, decreased with further increase in concen-

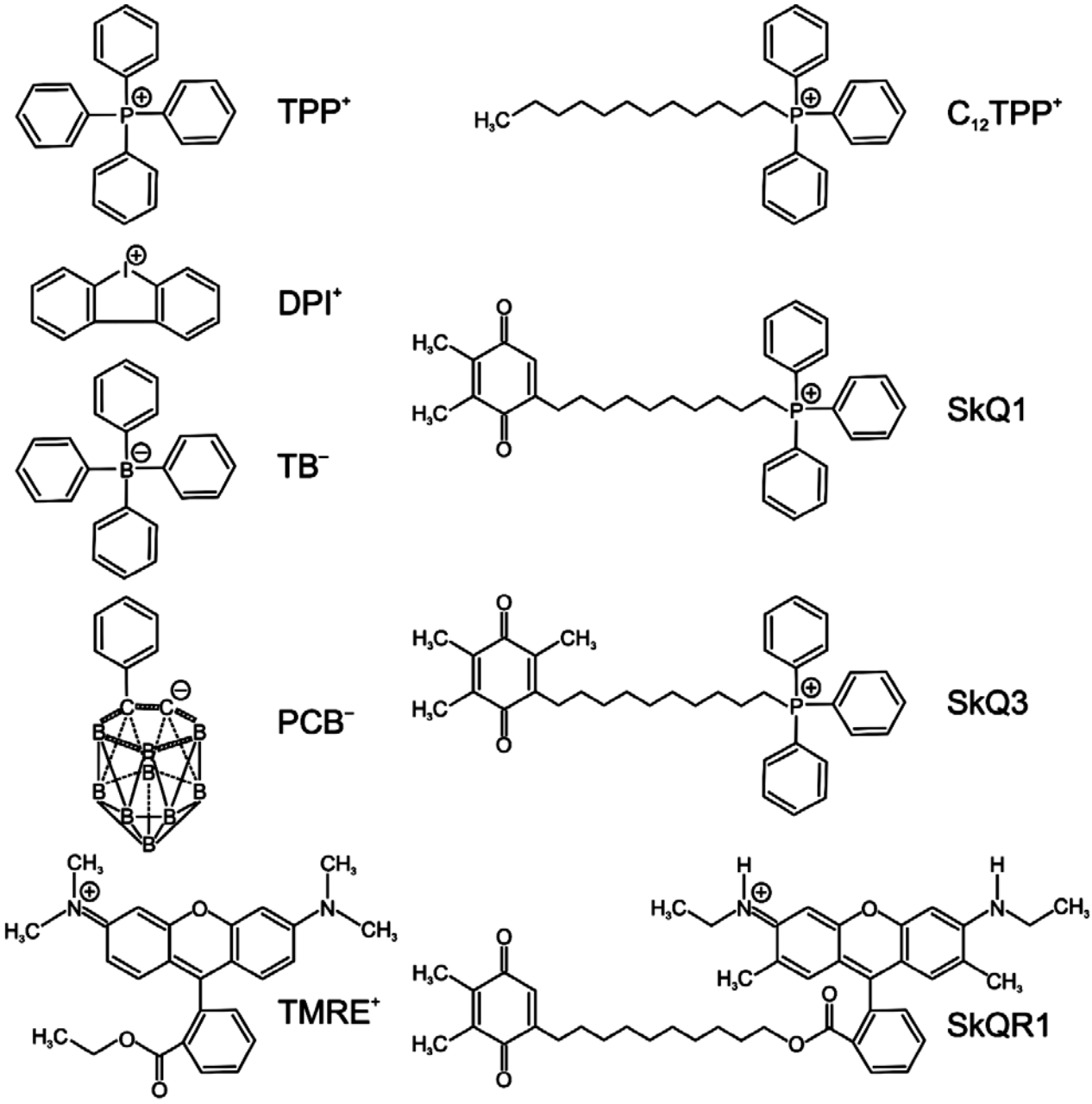

Fig. 1. Structures of tetraphenylphosphonium (TPP^+), dodecyltriphenylphosphonium ($C_{12}TPP^+$), diphenyleneiodonium (DPI^+), tetraphenylboron (TB^-), phenyl dicarbaundecaborane (PCB^-), tetramethylrhodamine ethyl ester ($TMRE^+$), 10-(6′-plastoquinonyl)decyltriphenylphosphonium (SkQ1), 10-(6′-methylplastoquinonyl)decyltriphenylphosphonium (SkQ3), and 10-(6′-plastoquinonyl)decylrhodamine 19 (SkQR1) ions.

tration, and disappeared at 10^{-8} M and higher. Destruction of EC nuclei was also prevented by $C_{12}TPP^+$ and $TMRE^+$ cations with maximum protective effect at 10^{-10}-10^{-9} and 10^{-11}-10^{-8} M, respectively, which disappeared at 10^{-6} M (Fig. 2, b and c). The inhibitor of flavin enzymes diphenyleneiodonium (DPI^+) is also a univalent cation, but it had no effect on the chitosan-induced EC nucleus destruction at concentrations below 10^{-7} M and inhibited it at concentrations above 10^{-7} M (Fig. 2d), likely due to its inhibitory effect on NADPH-oxidase of plasma membrane [5]. We also tested Ca^{2+} cations over a wide range of concentration. They provided no defense from chitosan, but inhibited EC nucleus destruction at 0.1-1 mM [21].

We also tested penetrating synthetic anions TB^- and PCB^-. These substances, unable of electrophoretically transfer into the cytoplasm or mitochondrial matrix and, contrariwise, rejected by them, also prevented the chi-

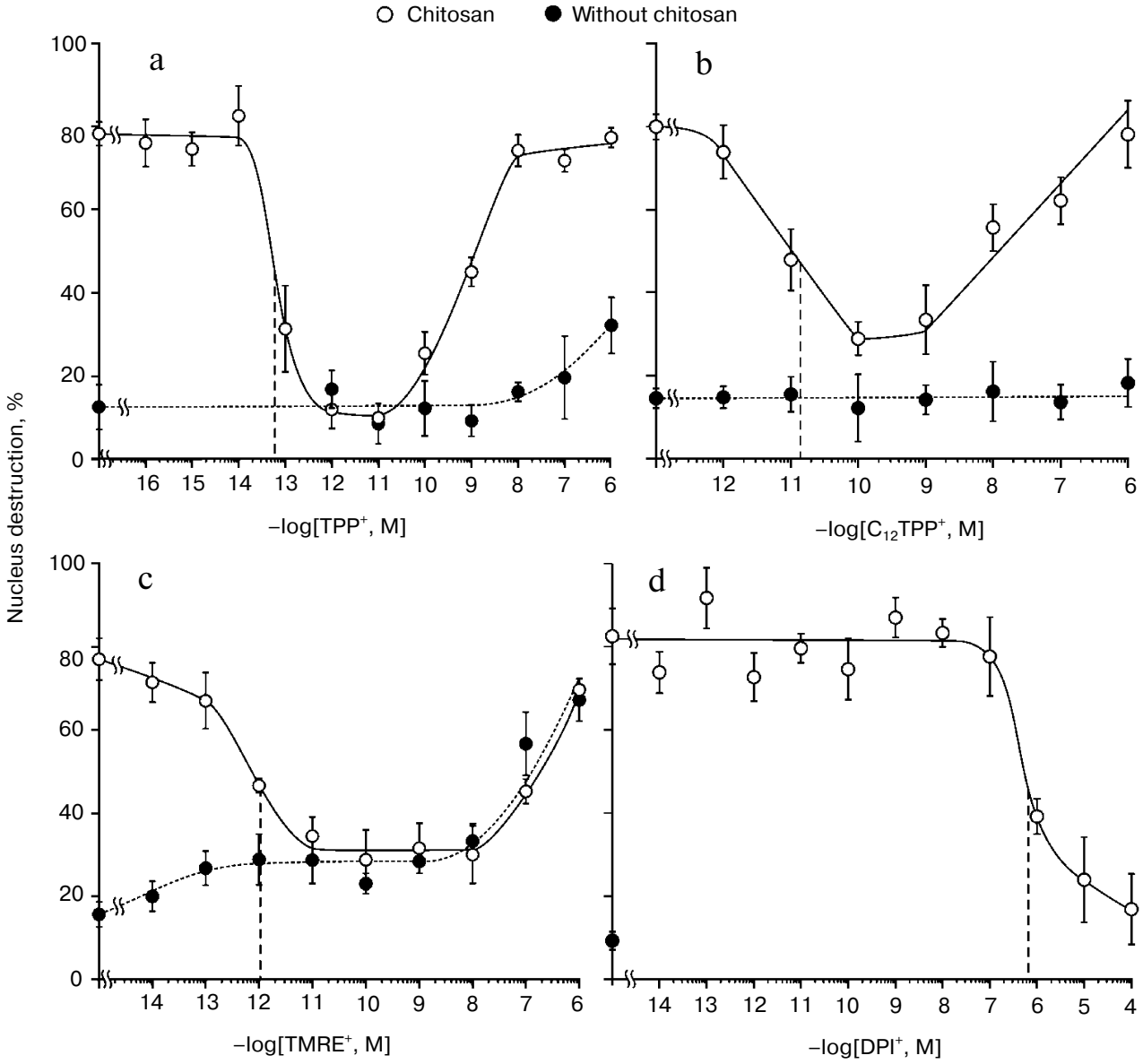

Fig. 2. Effects of TPP^+, $C_{12}TPP^+$, $TMRE^+$, and DPI^+ on the chitosan-induced EC destruction of nuclei in pea leaf epidermis. Epidermal films in 2 ml of distilled water were treated with chitosan (100 μg/ml) on a magnetic stirrer for 30 min, supplied with TPP^+, $C_{12}TPP^+$, $TMRE^+$, or DPI^+, and incubated in the dark for 3 h without stirring. The state of cell nuclei was determined in 300-500 cells in each experiment. Molar concentrations of cations added in the form of bromide salts at which half-maximum protective effect was observed are denoted with dashed perpendiculars to the abscissa.

tosan-induced EC nucleus destruction (Fig. 3). Maximal effect of TB^- and PCB^- was observed at 10^{-7}-10^{-6} M and higher, i.e. at concentrations several orders of magnitude exceeding those of the penetrating cations.

Since penetrating cations are transported across membranes by means of the electric component ($\Delta\psi$) of the driving force for proton transport (Δp), we tested acetate, whose transfer depends on ΔpH. At concentrations 10^{-5}-10^{-4} M Na^+-acetate decreased the chitosan-induced EC nucleus destruction (Fig. 4). The protective effect of acetate disappeared with increase in its concentration to 1-10 mM. Moreover, at these concentrations acetate itself (in the absence of chitosan) caused EC nucleus destruction.

ROS production in mitochondria depends on transmembrane potential. Decrease in $\Delta\psi$ by 10-15% leads to tenfold decrease in the rate of ROS production [22]. Uncouplers of oxidative phosphorylation, being H^+ carriers across membranes, inhibit $\Delta\psi$ and ROS formation in mitochondria [22, 23]. Penetrating cations combined with fatty acid anions have high protonophoric activity [24]. Fatty acid (palmitate, 2 μM) combined with

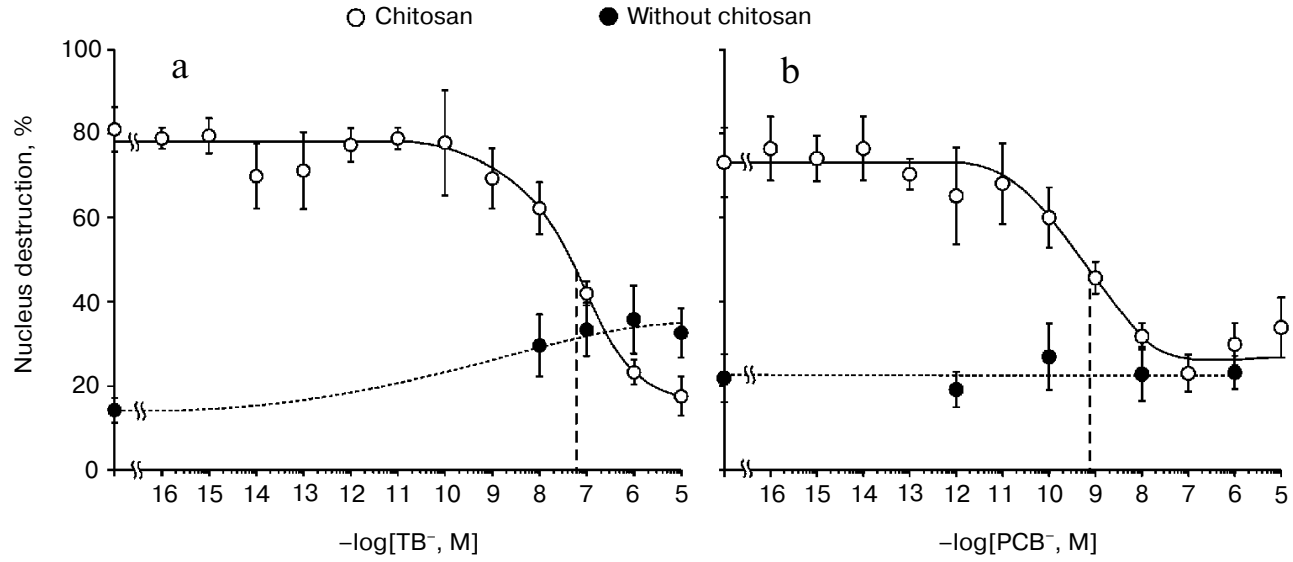

Fig. 3. Effect of TB^- and PCB^- anions on the chitosan-induced EC nucleus destruction in pea leaf epidermis. TB^- and PCB^- were used in the form of Na^+ salts. Experimental conditions are the same as in Fig. 2.

$C_{12}TPP^+$ significantly inhibited the chitosan-induced EC nucleus destruction (Fig. 5a). As for penetrating anions, they can exert uncoupling effect due to their transport across membranes together with NH_4^+. In fact, the protective effect of TB^- against the chitosan-induced EC nucleus destruction is potentiated by addition of NH_4Cl (Fig. 5b). Both endogenous fatty acids and NH_4^+ are undoubtedly present in cells of epidermis isolated from leaves, so the protective effect of penetrating cations and anions is exerted without their addition (Figs. 2 and 3).

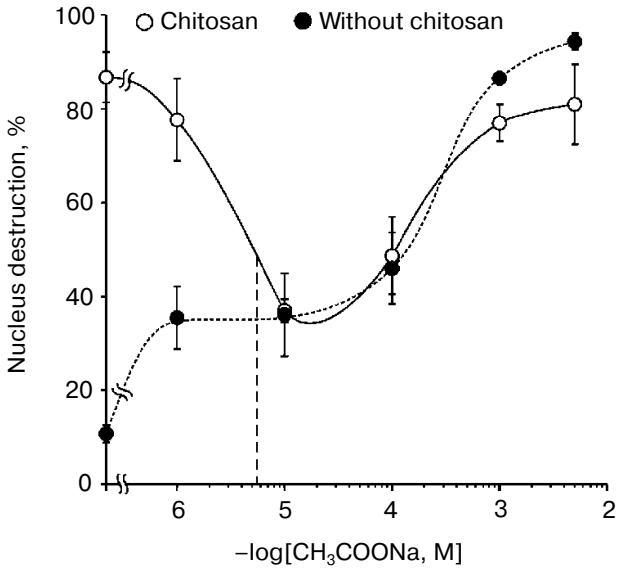

Fig. 4. Effect of Na^+-acetate on the chitosan-induced EC nucleus destruction in pea leaf epidermis. Experimental conditions are the same as in Fig. 2.

H_2O_2 alone was not effective as an inducer of guard cell (GC) apoptosis, but it considerably stimulated the process in combination with CN^- [25]. H_2O_2 caused destruction of EC nuclei that was prevented by TPP^+ and $C_{12}TPP^+$ (Fig. 6). Their protective effect was maximal at 0.1-1 and 1-100 nM, respectively, and decreased with further increase in concentration.

The penetrating cation concentrations C_{50} at which the chitosan- and H_2O_2-induced EC nucleus destruction twofold decreased were in the femto- and picomolar ranges (Table 1). The C_{50} values for penetrating anions TB^- and PCB^- were higher. Protective effect from chitosan was also demonstrated for phenol and a structural analog of rhodamines, fluorescein, containing like phenols and unlike rhodamines a free hydroxyl group. The C_{50} value for acetate was 5.5 μM. We also tested methionine, whose residues in mitochondrial proteins possibly provide defense against ROS [26, 27]. One can see in Table 1 that protective effect of acetate and methionine are many orders lower than that of other tested substances.

We reported earlier that apoplastic peroxidase is implicated in antioxidant defense of ECs from chitosan and H_2O_2 [18]. In connection with this, we tested the above substances as electron donors (DH_2) in the reaction of H_2O_2 reduction catalyzed by horseradish peroxidase:

$$H_2O_2 + 2\ DH_2 \rightarrow 2\ H_2O + 2\ DH^{\bullet}. \qquad (1)$$

Unsuitability of DH_2 was detected from O_2 release in response to following addition of catalase:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2. \qquad (2)$$

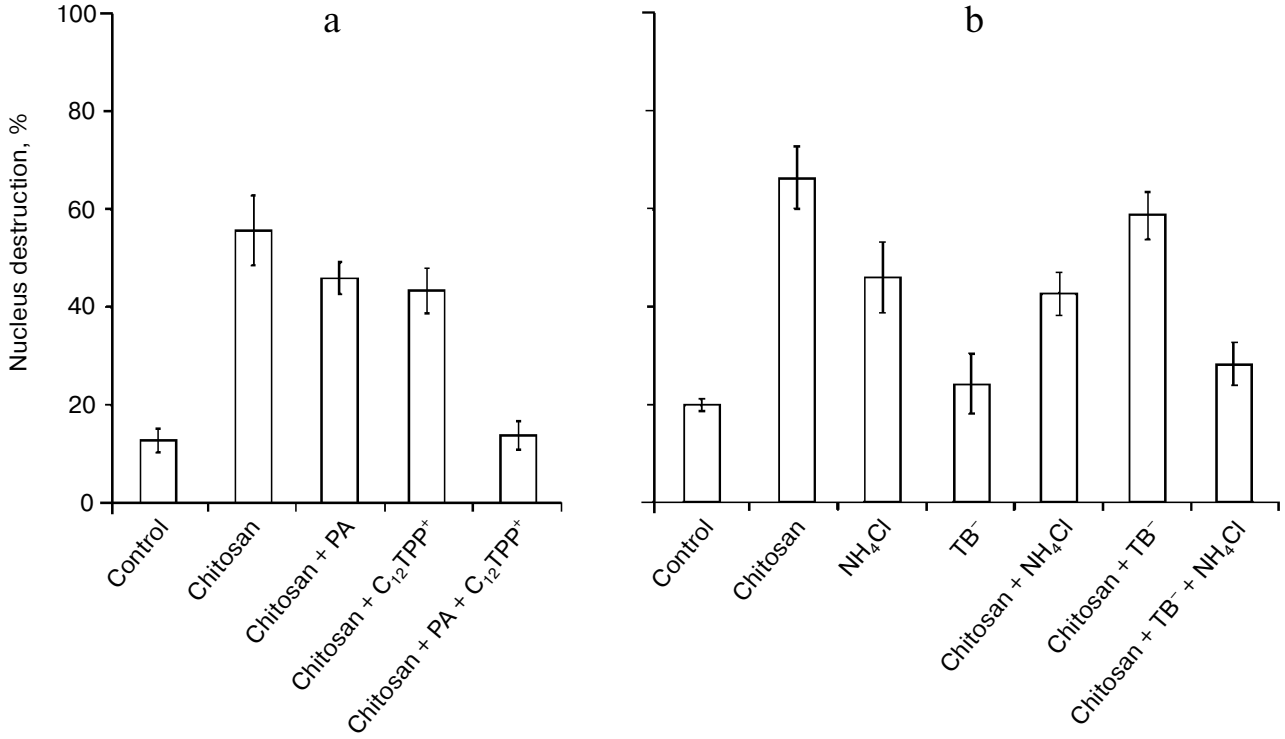

Fig. 5. Combined effects of palmitic acid (PA) with $C_{12}TPP^+$ (a) and NH_4Cl with TB^- (b) on the chitosan-induced EC nucleus destruction in pea leaf epidermis. Experimental conditions are the same as in Fig. 2. Additives: 2 μM PA, 10^{-10} M $C_{12}TPP^+$, 10^{-5} M NH_4Cl, and 10^{-6} M TB^-.

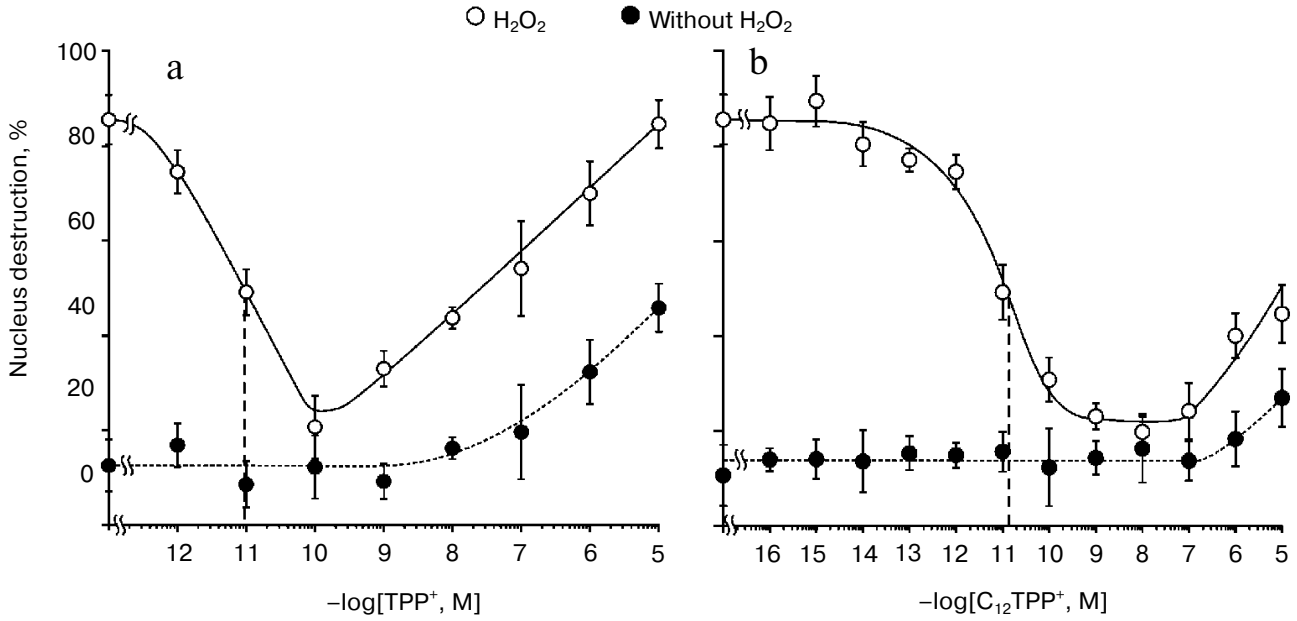

Fig. 6. Effect of TPP^+ and $C_{12}TPP^+$ on the H_2O_2-induced EC nucleus destruction in pea leaf epidermis. Epidermal films were incubated with TPP^+, $C_{12}TPP^+$, and 100 μM H_2O_2 for 3 h in the dark.

One can see in Table 2 that peroxidase substrates include phenol, 2,4-dichlorophenol (DCP), and fluorescein. Other substances, including 10-(6′-methylplastoquinonyl)decyltriphenylphosphonium (SkQ3) and 2′,7′-dichlorofluorescin diacetate (DCFH-DA), did not support the peroxidase reaction.

Besides reaction (1), peroxidases (Per) also catalyze reactions in which O_2 acts as an electron acceptor [28,

Table 1. Concentrations (C_{50}) of various agents providing 50% inhibition of chitosan- or H_2O_2-induced EC nucleus destruction in pea leaf epidermis

PCD inducer	Protective agent	C_{50}, M
Chitosan	TPP^+	6.0×10^{-14}
	$C_{12}TPP^+$	1.4×10^{-11}
	$TMRE^+$	1.1×10^{-12}
	fluorescein	8.1×10^{-10}
	TB^-	6.0×10^{-8}
	PCB^-	5.1×10^{-10}
	phenol	3.1×10^{-11}
	acetate	5.5×10^{-6}
	methionine	1.0×10^{-4}
H_2O_2	TPP^+	9.2×10^{-12}
	$C_{12}TPP^+$	1.4×10^{-11}
	phenol	1.0×10^{-12}

Table 2. Electron donors for H_2O_2 reduction catalyzed by horseradish peroxidase. The substance given in the table (0.15 mM), 10 U/ml horseradish peroxidase, and 0.15 mM H_2O_2 were added into a polarographic cell containing 50 mM Na^+-phosphate buffer, pH 7.0, and incubated for 3-5 min. Release of O_2 suggesting the presence of H_2O_2 in the solution was detected after addition of 20 U/ml catalase

Tested substance	Suitability of tested substance as donor in peroxidase reaction
Phenol	+
DCP	+
Fluorescein	+
DNP	–
SkQ3	–
$TMRE^+$	–
PCB^-	–
DCFH-DA	–
MB	–

29]. A few electron donors are suitable for these reactions called peroxidase/oxidase reactions. These donors include NADH, dihydroxyfumarate, indole-3-acetate, and naphthohydroquinones. The $NAD^\cdot$ radical produced in reaction (1) is a potent reductant that undergoes spontaneous oxidation with O_2:

$$NAD^\cdot + O_2 \rightarrow NAD^+ + O_2^{\cdot-}. \quad (3)$$

The E_0' value for the $NAD^\cdot/NAD^+$ couple is –922 mV [30]. $NAD^\cdot$ is an electron donor in the reaction [28]:

$$Per^{3+} + NAD^\cdot \rightarrow Per^{2+} + NAD^+, \quad (4)$$

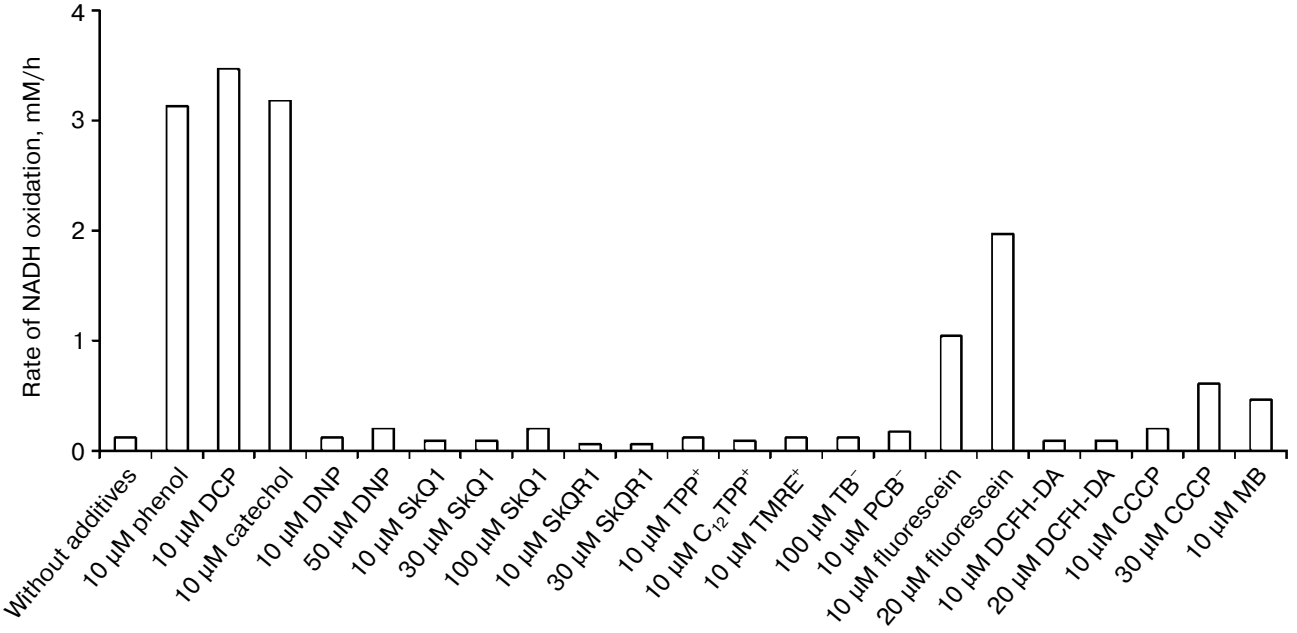

Fig. 7. Effect of various substances on NADH oxidation by peroxidase. Horseradish peroxidase (10 U/ml), 0.5 mM H_2O_2, and the tested substance were added to 0.1 mM NADH solution in 50 mM phosphate buffer, pH 7.0. NADH oxidation was determined from the change in optical density at 340 nm using molar extinction coefficient 6200 $M^{-1} \cdot cm^{-1}$.

where the figures at Per reflect the number of deficient electrons.

Peroxidase/oxidase reactions occur with implication of Per^{2+} and Per^{3+}:

$$Per^{2+} + O_2 + 4\ H^+ \rightarrow Per^{6+} + 2\ H_2O, \quad (5)$$

$$Per^{3+} + O_2^{\cdot-} + 4\ H^+ \rightarrow Per^{6+} + 2\ H_2O. \quad (6)$$

Per^{6+} is a catalytically inactive form of the enzyme. Phenolic substances combined with NADH transform the state Per^{6+} into the active peroxidase form [31]. NADH is oxidized to NAD$^{\cdot}$ by phenyl radical produced in reaction (1), and NAD$^{\cdot}$ reduces Per^{6+} to Per^{5+}, as discussed earlier [18].

Figure 7 illustrates the effect of various substances on NADH oxidation by horseradish peroxidase. Among the tested substances, only phenol, DCP, catechol, fluorescein, and, to less extent, CCCP and MB supported NADH oxidation. Penetrating cations and anions, as well as cationic derivatives of plastoquinone, were ineffective. These data are consistent with the data presented in Table 2, suggesting that the tested penetrating cations and anions and cationic derivatives of quinones do not support the peroxidase reaction.

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