ISSN 0006-2979, Biochemistry (Moscow), 2010, Vol. 75, No. 2, pp. 257-263. © Pleiades Publishing, Ltd., 2010.
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Programmed Cell Death in Plants: Protective Effect of Phenolic Compounds against Chitosan and H_2O_2

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Received April 20, 2009
Revision received June 8, 2009

Abstract—Addition of chitosan or H_2O_2 caused destruction of nuclei of epidermal cells (EC) in the epidermis isolated from pea leaves. Phenol, a substrate of the apoplastic peroxidase-oxidase, in concentrations of 10^{-10}-10^{-6} M prevented the destructive effect of chitosan. Phenolic compounds 2,4-dichlorophenol, catechol, and salicylic acid, phenolic uncouplers of oxidative phosphorylation pentachlorophenol and 2,4-dinitrophenol, and a non-phenolic uncoupler carbonyl cyanide *m*-chlorophenylhydrazone, but not tyrosine or guaiacol, displayed similar protective effects. A further increase in concentrations of the phenolic compounds abolished their protective effects against chitosan. Malate, a substrate of the apoplastic malate dehydrogenase, replenished the pool of apoplastic NADH that is a substrate of peroxidase-oxidase, prevented the chitosan-induced destruction of the EC nuclei, and removed the deleterious effect of the increased concentration of phenol (0.1 mM). Methylene Blue, benzoquinone, and N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD) capable of supporting the optimal catalytic action of peroxidase-oxidase cancelled the destructive effect of chitosan on the EC nuclei. The NADH-oxidizing combination of TMPD with ferricyanide promoted the chitosan-induced destruction of the nuclei. The data suggest that the apoplastic peroxidase-oxidase is involved in the antioxidant protection of EC against chitosan and H_2O_2.

DOI: 10.1134/S0006297910020173

Key words: apoplastic peroxidase, programmed cell death, chitosan, hydrogen peroxide, phenolic compounds, protective action, plants, epidermal cells

After plant growth by extension is finished, the primary cell wall is strengthened to oppose the turgor pressure. Between the primary cell wall and plasma membrane the secondary cell wall is built up from cellulose microfibrils consisting of hemicellulose, pectin, and structural glycoprotein cross-links tightly packed in lignin, an insoluble hydroxyphenylpropane polymer. These phenolic compounds are polymerized with involvement of an apoplastic peroxidase, which catalyzes the H_2O_2-dependent formation of phenoxyl radicals in different resonance states responsible for the variety of radical condensation products [1].

In the presence of H_2O_2 and electron donors DH_2, such as phenolic compounds PhOH, peroxidase (Per) catalyzes the peroxidase reaction [2, 3]:

$$H_2O_2 + 2DH_2 \rightarrow 2H_2O + 2DH^{\cdot}, \quad (1)$$

which includes the following stages:

$$Per^{3+} + H_2O_2 + 2H^+ \rightarrow Per^{5+}\ (\text{compound I}) + 2H_2O, \quad (2)$$

$$Per^{5+} + PhOH \rightarrow Per^{4+}\ (\text{compound II}) + PhO^{\cdot} + H^+, \quad (3)$$

$$Per^{4+} + PhOH \rightarrow Per^{3+} + PhO^{\cdot} + H^+. \quad (4)$$

Digitals at Per (here and further) mean the number of oxidative equivalent absent in the iron and heme of the enzyme (more in detail see in [2]).

Peroxidases also catalyze reactions where O_2 is an acceptor of electrons:

$$O_2 + 2DH_2 \rightarrow 2H_2O + 2D. \quad (5)$$

Abbreviations: BQ, *p*-benzoquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCP, 2,4-dichlorophenol; DNP, 2,4-dinitrophenol; EC, epidermal cells; FeCy, potassium ferricyanide; MB, Methylene Blue; PCP, pentachlorophenol; ROS, reactive oxygen species; SA, salicylic acid; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine.

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In these peroxidase-oxidase reactions only some electron donors can be involved, such as NADH, dihydroxyfumarate, indol-3-acetate, and naphthohydroquinones. In addition to reactions 2-4, peroxidase-oxidase also catalyzes the following reactions [2, 3]:

$$Per^{3+} + DH^{\cdot} \rightarrow Per^{2+} + D + H^{+}, \quad (6)$$

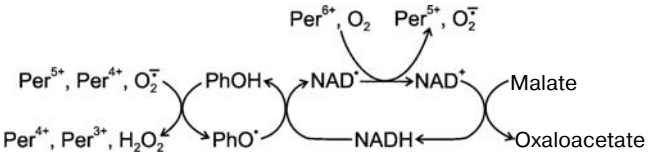
$$Per^{2+} + O_2 + 4H^{+} \rightarrow Per^{6+}\ (\text{compound III}) + 2H_2O, \quad (7)$$

$$Per^{3+} + O_2^{\cdot -} + 4H^{+} \rightarrow Per^{6+} + 2H_2O, \quad (8)$$

$$Per^{6+} + DH^{\cdot} \rightarrow Per^{5+} + D + H^{+}. \quad (9)$$

Per^{6+} (compound III) is a catalytically inactive form of the enzyme. Phenolic compounds combined with NADH convert the peroxidase from the Per^{6+} state into the active form [4]. To record peroxidase-oxidase reactions catalyzed by the isolated horseradish peroxidase, the incubation medium, in addition to phenolic compounds (usually 2,4-dichlorophenol) and NADH, is usually supplemented with Methylene Blue (MB), which is supposed to decrease the peroxidase activity of the enzyme and regulate the contents of Per^{2+} converted into the inactive compound III [5].

A reaction succession is proposed which combines peroxidase and peroxidase-oxidase reactions with involvement of NADH [5] (the scheme is based on earlier concepts [3, 4, 6]):



Reduction of NAD^+ by malate is catalyzed by malate dehydrogenase tightly bound with the cell wall [7-9]. Malate is released into the apoplast by the mesophyll and stoma cells [10, 11] and can be absorbed by the stoma cells from the apoplast [12], thus controlling the CO_2-dependent movement of the stomas. Due to the chain mechanism, the peroxidase-oxidase reaction displays pronounced oscillations with complex dynamics [5]. These oscillations can prevent excessive accumulation of reactive oxygen species (ROS) that are lethal for the enzyme [13].

As a provider of ROS the apoplast peroxidase is involved not only in lignification of cell walls but also in realization of the hypersensitive response that is manifested by the local death of plant cells in response to infectious agents or their signaling compounds (elicitors). In response to various elicitors, the stoma guard cells and basic epidermal cells (EC) in the epidermis from tobacco leaves lose viability producing H_2O_2 under the influence of the apoplastic peroxidase and flavin oxidase [14]. The elicitor chitosan (poly(β-1,4)-N-acetylglucosamine), which is a product of the incomplete deacetylation of a cell wall component of fungi (chitin), induced production of ROS and programmed death of EC in the epidermis from pea leaves. This death could be prevented by inhibitors of flavin oxidases diphenyleneiodonium and quinacrine [15].

In animals including humans, a special role among flavin oxidases in production of ROS belongs to NADPH oxidases of the plasma membrane of phagocytes: neutrophils, eosinophils, monocytes, and macrophages [16-18]. NADPH oxidases (NOXs) are structurally subdivided into three groups: 1) NOX1-4, the transmembrane protein gp91phox (a glycoprotein with molecular mass 91 kDa of phagocytic oxidase), which binds NADPH and FAD on the cytoplasmic surface, contains two hemes of the cytochrome *b*558 in the transmembrane part located in the outer and inner monolayers of the membrane, and generates $O_2^{\cdot -}$ on the outer surface; 2) NOX5, the protein gp91phox bound on the cytoplasmic surface with a calmodulin-like domain possessing four Ca^{2+}-binding sites; 3) dual oxidases (DUOXs) consisting of NOX5 and the peroxidase domain on the membrane outer surface. DUOX-like oxidases are also found in plants [19].

The purpose of the present work was to determine the role of the apoplastic oxidase in the chitosan-induced programmed death of plant cells using phenolic compounds as the enzyme substrates. These compounds were shown to protect EC against the deleterious effect of chitosan and H_2O_2.

MATERIALS AND METHODS

Experiments were performed using the epidermis isolated from the lower surface of leaves of 7-15-day-old seedlings of pea *Pisum sativum* L., cv. Alpha. The plants were grown under conditions of periodic illumination with luminescent lamps at the intensity of ~100 $\mu E \cdot m^{-2} \cdot sec^{-1}$ (light for 16 h, darkness for 8 h) at 23-28°C. The epidermis was separated with pincers, placed into distilled water, and supplemented with reagents (they are listed in figure legends). Epidermis slices were treated with chitosan on mixing them in chitosan suspension on a multiposition magnetic stirrer. The epidermis was incubated in polystyrene plates at 22-25°C with reagents added from aqueous solutions.

Upon the incubation, the epidermis was treated for 5 min with Battaglia's fixative (a mixture of chloroform, 96%-ethanol, glacial acetic acid, and 40%-formalin at the ratio of 5 : 5 : 1 : 1), washed with ethanol for 10 min to remove the fixative, incubated in water for 5 min, and stained with Carazzi's hematoxylin for 20 min. Stained cells of the epidermis were washed with tap water and studied by light microscopy. Cell death was recorded by destruction of the cell nuclei. The fraction of the cells

with destroyed nuclei and nucleus-free cells was calculated per 250-500 cells studied (in 3-4 fields of the epidermis). Experiments were performed in 2-3 repetitions. Typical data of a repetition are presented.

RESULTS

Chitosan caused death of EC in the epidermis from pea leaves that was detected by destroying the cell nuclei but had no effect on the stoma guard cell nuclei [15]. Phenol as a substrate of peroxidase [5] at the concentration of 10^{-10}-10^{-6} M prevented the chitosan effect on the EC (Fig. 1). The protective effect of phenol decreased with increase in its concentration and reduced to zero at the concentration of 1 mM. Other phenolic compounds, such as 2,4-dichlorophenol (DCP), catechol, and salicylic acid (SA) displayed similar protective effects (Fig. 1). Aqueous and ethanolic extracts of a catechol-containing tea also had protective effects (data not presented). Chitosan-induced destruction of the EC nuclei was prevented by pentachlorophenol (PCP), 2,4-dinitrophenol (DNP), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Fig. 2) used as uncouplers of oxidative and photosynthetic phosphorylation [20]. Their protective effects were observed at lower concentrations than the effect of phenol. The phenolic compound tyrosine did not protect against chitosan (Fig. 3), whereas the protective effect of another phenol, guaiacol, occurred at concentration higher than 10^{-7} M and increased up to 1 mM (Fig. 3).

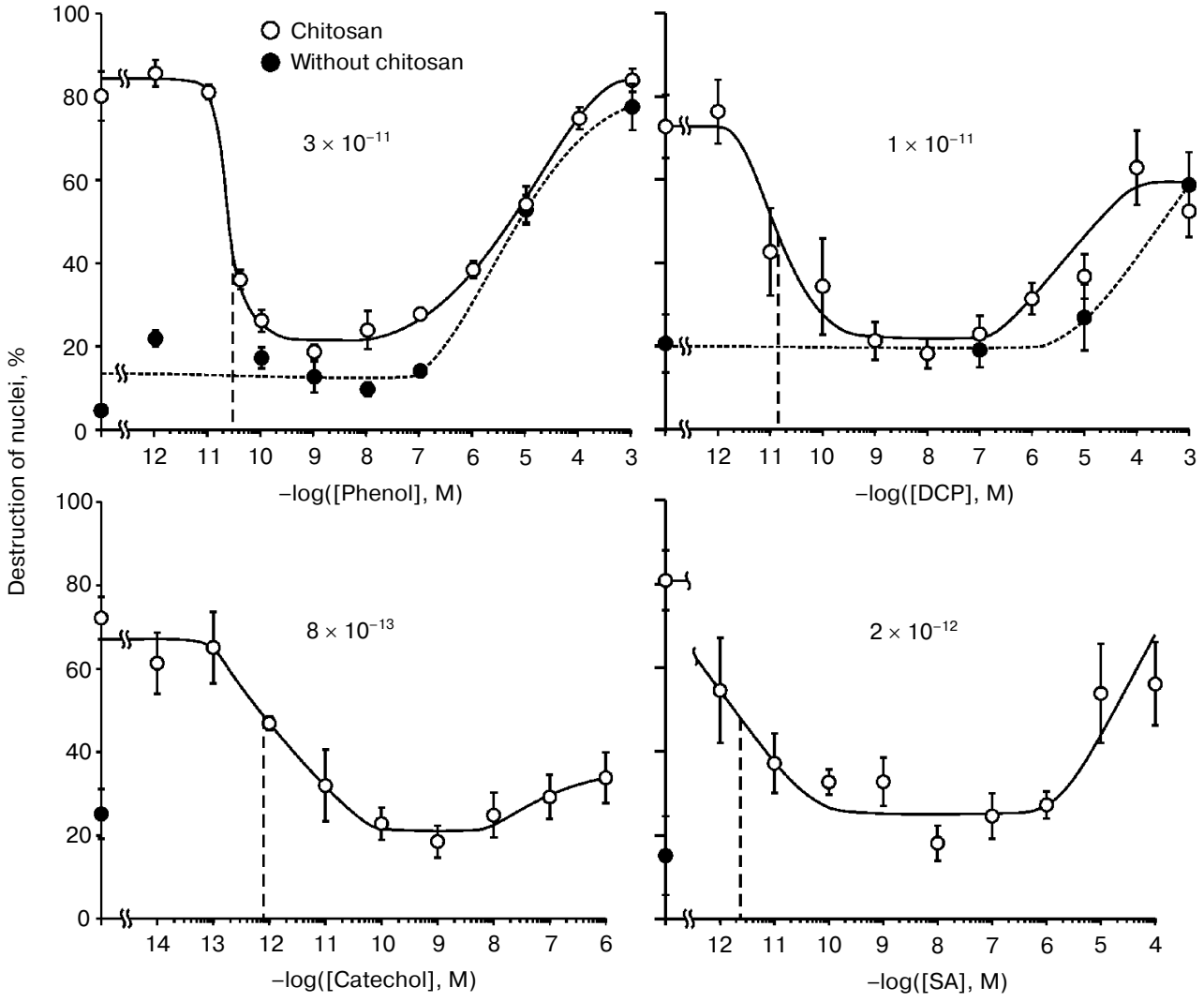
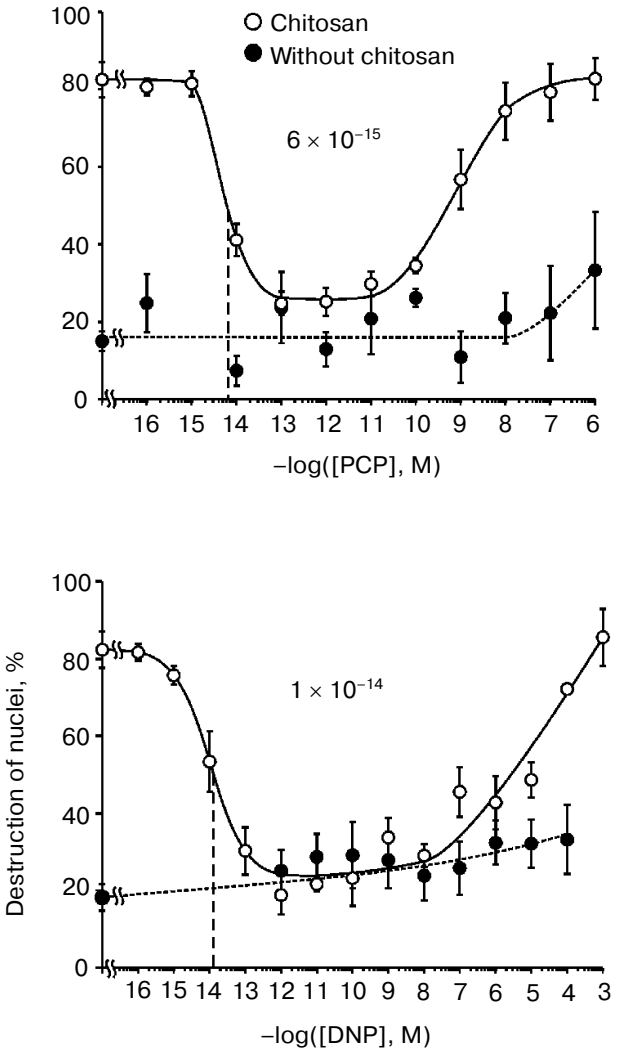


Fig. 1. Effects of phenolic compounds on the chitosan-induced destruction of EC nuclei in the epidermis from pea leaves. The epidermis slices were treated with chitosan (100 μg/ml) on a magnetic stirrer for 30 min, then supplemented with phenol, DCP, catechol, or SA and incubated for 3 h in the dark without mixing. The state of the cell nuclei was recorded in 250-500 cells in each variant of the experiment. Values of C_{50} (concentrations of the phenolic compounds corresponding to the half-maximal protective effect) are shown by vertical dashed lines to the abscissa axis and expressed in M.



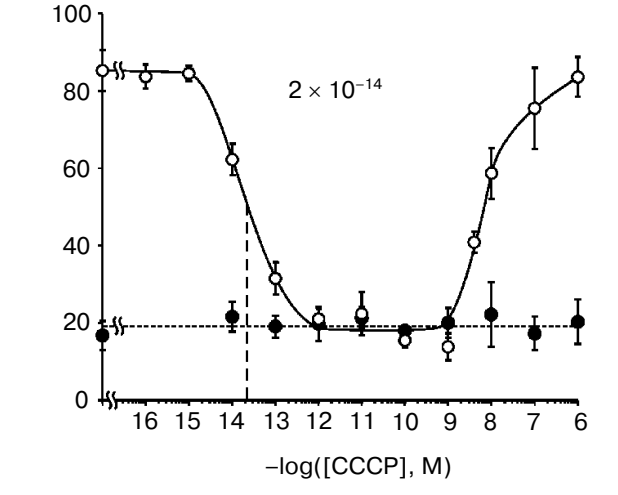


Fig. 2. Effects of protonophore uncouplers PCP, DNP, and CCCP on the chitosan-induced destruction of EC nuclei in the epidermis from pea leaves. The experimental conditions are described in Fig. 1 legend.

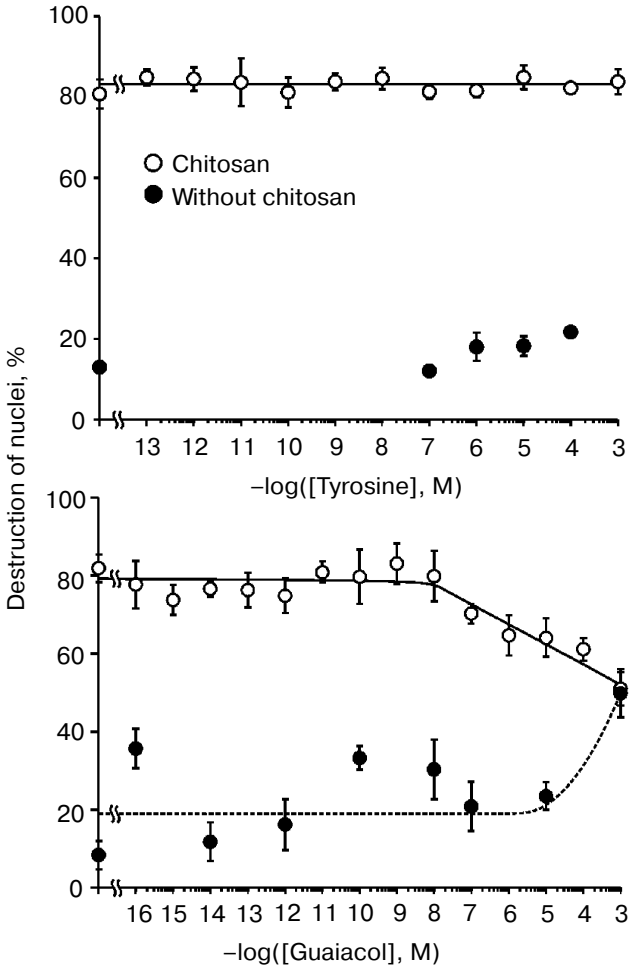


Fig. 3. Effects of tyrosine and guaiacol on the chitosan-induced destruction of EC nuclei in the epidermis from pea leaves. The experimental conditions are described in Fig. 1 legend.

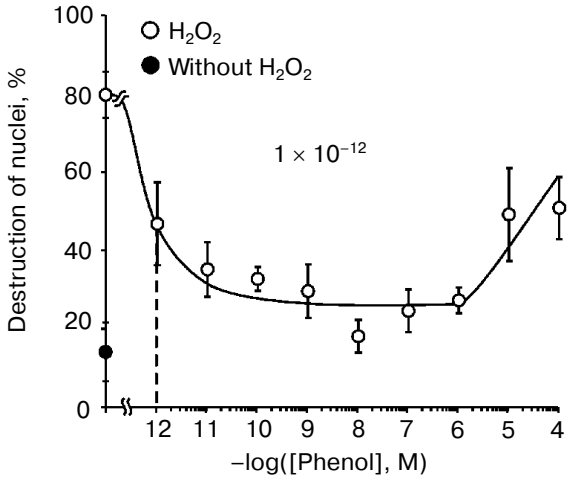


Fig. 4. Effect of phenol on the H_2O_2-induced destruction of EC nuclei in the epidermis from pea leaves. The epidermis slices were supplemented with 100 μM H_2O_2 and phenol and incubated in the dark for 3 h. Other conditions of the experiment are described in Fig. 1 legend.

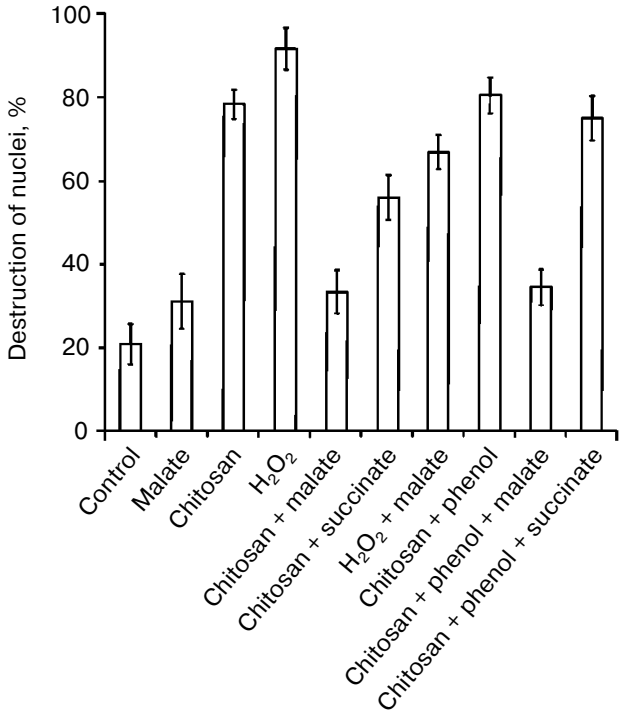


Fig. 5. Effect of malate as a substrate of malate dehydrogenase on the chitosan- and H_2O_2-induced destruction of EC nuclei in the epidermis from pea leaves. Epidermis slices were treated with chitosan (100 μg/ml) on a magnetic stirrer for 30 min or with 100 μM H_2O_2 for 3 min, then were supplemented with 5 mM succinate, 5 mM malate, and 0.1 mM phenol and incubated for 3 h in the dark without mixing. Other conditions of the experiment are described in Fig. 1 legend.

Along with chitosan, hydrogen peroxide also effectively induced the programmed death of EC [15]. Phenol at the concentration of 10^{-11}-10^{-6} M prevented the H_2O_2-induced destruction of the EC nuclei (Fig. 4). A further increase in the phenol concentration was associated with disappearance of its protective effect.

Malate added as a substrate of the apoplastic malate dehydrogenase prevented the chitosan-induced destruction of EC nuclei and abolished the damaging effect of the increased concentration of phenol (0.1 mM) (Fig. 5, compare with Fig. 1). The protective effect of succinate in the presence of chitosan was lower than the effect of malate. Succinate did not abolish the damaging effect of 0.1 mM phenol (Fig. 5). Malate decreased the apoptosis-inducing effect of H_2O_2 on the EC.

Methylene Blue (MB), a cofactor of peroxidase-oxidase reactions [5], decreased the chitosan-induced destruction of EC nuclei (Fig. 6). The E'_0 value of MB is 11 mV [21]. Other redox agents, such as *p*-benzoquinone (BQ) and N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD) (values of their E'_0 are, respectively, 239 and 270 mV), were also tested. The two compounds similarly to MB abolished the destructive effect of chitosan (Fig. 6). The protective effects of these three agents

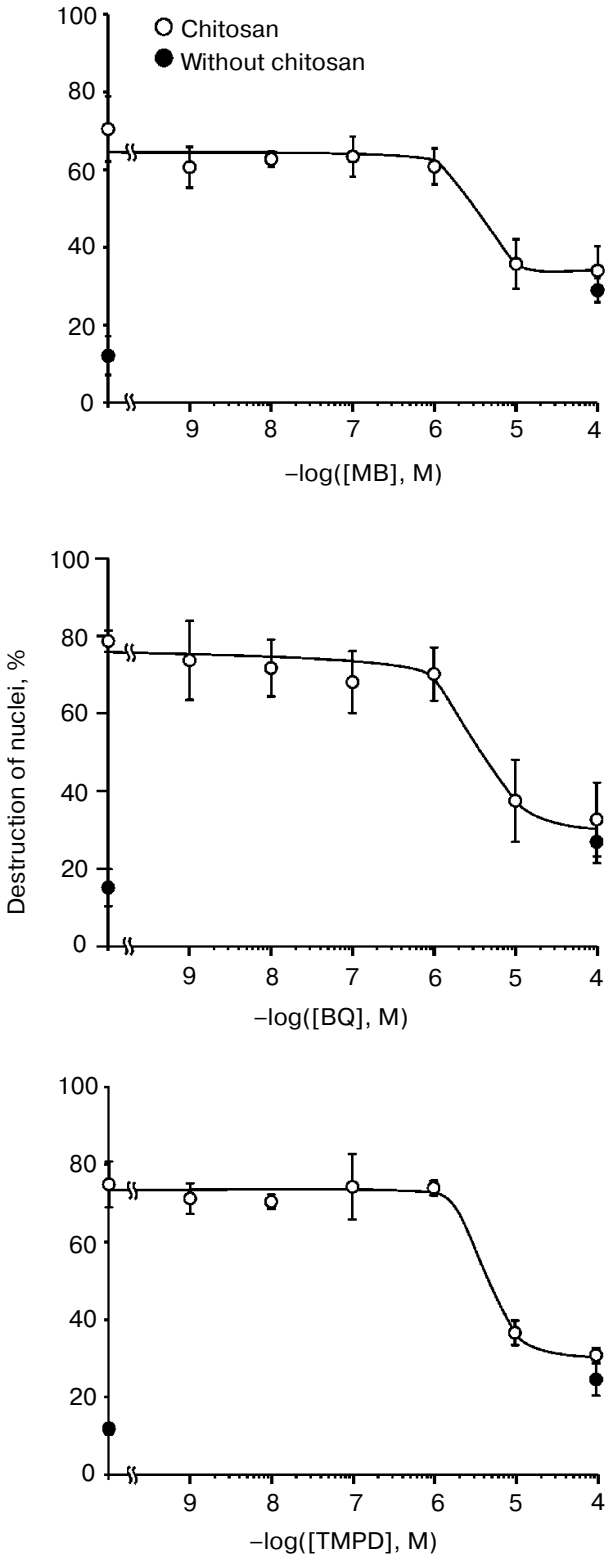


Fig. 6. Effects of MB, BQ, and TMPD on the chitosan-induced destruction of EC nuclei in the epidermis from pea leaves. Epidermis slices were treated with chitosan (100 μg/ml) on a magnetic stirrer for 30 min, supplemented with MB, BQ, or TMPD, and incubated for 5 h in the dark without mixing. Other conditions of the experiment are described in Fig. 1 legend.

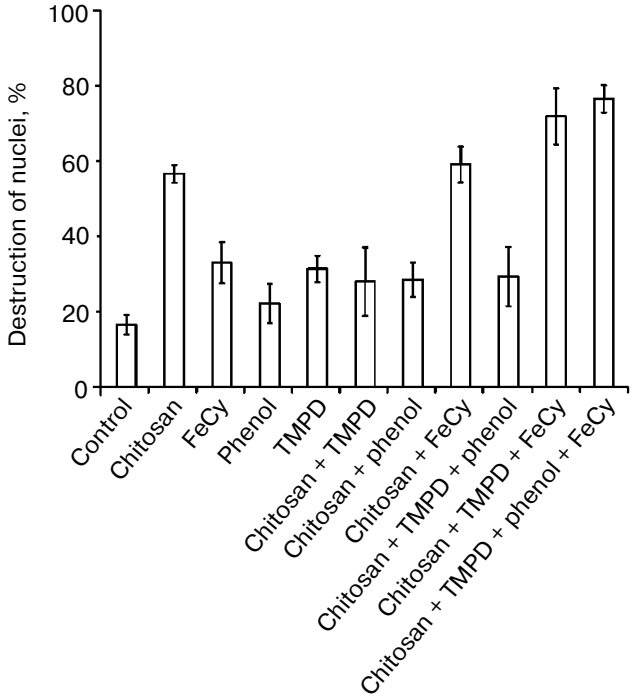


Fig. 7. Effects of potassium ferricyanide (FeCy), phenol, and TMPD on the chitosan-induced destruction of EC nuclei in epidermis from pea leaves. Epidermis slices were treated with chitosan (100 μg/ml) on a magnetic stirrer for 30 min, then supplemented with 3 mM FeCy, $1.06 \cdot 10^{-8}$ M phenol, and 0.1 mM TMPD and incubated for 5 h in the dark without mixing. Other conditions of the experiment are described in Fig. 1 legend.

achieved saturation at the concentrations of 10^{-5}-10^{-4} M.

Because the apoplastic malate dehydrogenase is a supplier of NADH, influence of the apoplastic NADH transition into the oxidized form on the chitosan-induced death of EC was studied in the final experiments. Spectrophotometry at 340 nm revealed that TMPD combined with ferricyanide oxidized NADH. Ferricyanide itself had no influence, but combined with TMPD or with phenol it increased the chitosan-induced destruction of EC nuclei (Fig. 7).

DISCUSSION

Phenolic compounds used as substrates of peroxidase-oxidase reactions [5] prevented the chitosan-induced destruction of EC nuclei with C_{50} values in the range of 10^{-11}-$8 \cdot 10^{-13}$ M (Fig. 1). Phenol protected against H_2O_2 with the C_{50} value of 10^{-12} M (Fig. 4). Phenolic uncouplers of oxidative phosphorylation PCP and DNP and a non-phenolic uncoupler CCCP displayed higher protection, with C_{50} values of ~10^{-14} M (Fig. 2). These compounds act not only as peroxidase-oxidase substrates but also as uncouplers inhibiting the ROS production in the respiratory chain in plant mitochondria [15, 22, 23]. The isolated lignin peroxidase catalyzes oxidation of PCP to 2,3,5,6-tetrachloro-*p*-benzoquinone with the equimolar production of Cl^- [24]. The involvement of peroxidase-oxidase in the EC protection against chitosan and H_2O_2 is evidenced by data on the programmed cell death prevention by malate (which is a substrate of malate dehydrogenase) (Fig. 5), by MB (which seems to support the optimal regimen for the catalytic action of peroxidase-oxidase) [5], and also by BQ and TMPD (Fig. 6). The shortage of NADH upon its oxidation under the influence of TMPD and ferricyanide increased the chitosan-induced destruction of EC nuclei (Fig. 7).

The oxidation of phenol to phenoxyl radicals ($PhOH \rightarrow PhO^{\bullet} + e^- + H^+$) is characterized by high values of E'_0. The E'_0 values for phenol and DCP are 875 and 902 mV, respectively [5], and for catechol E'_0 is 530 mV [25]. According to the literature data, one-electron reduction reactions $Per^{5+} \rightarrow Per^{4+}$ and $Per^{4+} \rightarrow Per^{3+}$ are characterized by values of E'_0 in the range of 879-965 and 869-992 mV [5]. The E'_0 values for the reaction $O_2^{\bullet-} + e^- + 2H^+ \rightarrow H_2O_2$ are similar: 890-940 mV. For the reaction $Per^{6+} + e^- \rightarrow Per^{5+}$ the presence of $NAD^{\bullet}$ is necessary, which is oxidized to NAD^+ with $E'_0 = -922$ mV [5]. The data under consideration meet interactions of the apoplastic peroxidase-oxidase with phenols, NADH, and malate dehydrogenase (Scheme).

Peroxidase-oxidase reactions of oxidation of phenolic compounds are essentially similar to reactions of the water-oxidizing complex of photosystem II of chloroplasts and cyanobacteria. The uncouplers PCP, CCCP, and 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole are oxidized by the oxygen-evolving complex of photosystem II [26]. They are so-called ADRY (acceleration of the deactivation reactions of the water-splitting enzyme system Y) reagents, which compete with H_2O as an electron donor for the water-oxidizing complex and inhibit the photosynthetic evolution of O_2. ADRY reagents include compounds with different structure: anilinothiophenes, phenylhydrazone derivatives (including CCCP), nitrophenols (including DNP), indophenols, and diphenylamines [26]. All these compounds contain OH- or NH-groups and are protonophore uncouplers of oxidative and photosynthetic phosphorylation. On oxidation by the oxygen-evolving complex (the E'_0 value of the reaction site of P680, which is a primary donor of electrons in the photosystem II of chloroplasts and cyanobacteria, is 1.1-1.3 V [27-30]), ADRY reagents are reduced by the membrane pool of plastoquinone and thus support the cyclic transfer of electrons in photosystem II [26, 31]. The role of ADRY reagents in the electron transport with involvement of the water-oxidizing complex and photosystem II reminds in many aspects the role of phenols in the peroxidase-oxidase reactions where phenols are cyclically oxidized by Per^{5+}, Per^{4+}, and $O_2^{\bullet-}$ and are reduced concomitantly with NADH oxidation to $NAD^{\bullet}$.

Among the phenolic compounds studied, tyrosine did not protect against chitosan. The protective effect of guaiacol was weak (Fig. 3). As an amino acid, tyrosine can be used in biosynthesis. Similarly to coniferyl alcohol, guaiacol (2-methoxyphenol) is a substrate in the synthesis of lignin. A hypersensitive response accompanied by thickening of the cell wall, or lignification, can result in deficiency of phenolic substrates for peroxidase-oxidase and thus convert the enzyme into the inactive state, Per^{6+} (compound III).

Thus, with involvement of the apoplastic peroxidase and malate dehydrogenase, phenolic compounds protected EC against the deleterious effect of chitosan and H_2O_2 inducing the hypersensitive response of the plant cells. A phenolic substrate has only to enter into contact with the apoplastic peroxidase-oxidase. And on contact, the oxidation of phenols induced by chitosan or by addition of H_2O_2 is replaced by their cyclic reduction in the peroxidase-oxidase reactions (Scheme), which support the protective antioxidant effect of the added phenols. No prior concentrating of the substrate-antioxidant is needed. Moreover, to realize the protective effect, the substrate does not need to cross barriers. ADRY reagents PCP, DNP, or CCCP [26] have to cross at least one membrane barrier to inhibit the photosynthetic oxidation of H_2O because the oxygen-evolving complex is located on the inner surface of thylakoids in chloroplasts or cyanobacteria.

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