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Effect of Exogenous Phenols on Superoxide Production by Extracellular Peroxidase from Wheat Seedling Roots

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Abstract—Competitive and complimentary relationships of various peroxidase substrates were studied to elucidate the enzymatic mechanisms underlying production of reactive oxygen species in plant cell apoplast. Dianisidine peroxidase released from wheat seedling roots was inhibited by ferulate and coniferol, while ferulic and coniferyl peroxidases were activated by *o*-dianisidine. Both ferulate and coniferol, when added together with hydrogen peroxide, stimulated superoxide production by extracellular peroxidase. We suggest that substrate–substrate activation of extracellular peroxidases is important for stress-induced oxidative burst in plant cells.

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Key words: wheat root, superoxide, peroxidase, phenol, extracellular solution

Peroxidase (POD) is one of the main components of the immune system of plants. It is activated under various impacts in different organs of diverse plant species [1-4]. POD is implicated in wound- or pathogen-induced formation of protective barriers composed of lignin and suberin, which are produced due to the H_2O_2-dependent oxidative polymerization of oxycinnamoyl alcohols (*p*-coumarol, coniferol, and sinapol) and formation of ester bonds with cross-linking agents, namely, phenolcarbonic acids (*p*-coumaric, caffeic, ferulic, etc.) [5-8].

Also, various stress agents can result in fast production of reactive oxygen species (ROS) on the surface of plant cells, so-called oxidative burst [9-12]. Most researchers associate POD activation with activity of an antioxidative system. Moreover, POD is offered as a stress marker [13, 14]. POD is in fact a good stress marker, but its functions do not only come to elimination of hydrogen peroxide. Under certain conditions (in particular, in oxidation of pyridine nucleotides and polyphenols) some apoplastic PODs exhibit their oxidase activity by production of superoxide anion radical ($O_2^{\cdot-}$) and H_2O_2 at physiological pH values [15-18]. Thus, under stress conditions POD can play a role of either anti- or prooxidant. ROS are known to play a role of signaling mediators and can control important biological processes [19-21]. Significant activation of $O_2^{\cdot-}$-producing POD during the initial period after root excision from seedlings is supposed to be necessary for development of nonspecific adaptation syndrome in wheat root cells [22]. It was shown that under biotic stress H_2O_2 production by extracellular POD during oxidative burst requires an elevation of apoplastic pH and presence of reducing agents [17]. Nevertheless, problems concerning possible mechanisms and factors providing stimulation of the ROS-producing activity of POD have not yet been resolved.

We previously reported that excised wheat roots can be effectively used as a test system for study of reactivity and adaptive processes in response to adverse factors [23, 24]. The cell surface POD is one of the main sources of $O_2^{\cdot-}$ in roots excised from wheat seedlings [25, 26]. Moreover, weakly associated POD isoforms localized in the apoplastic space of root cells are released into the extracellular solution [27]. It is known that soluble substances, possibly including electron donors, such as ascorbate and phenolic compounds, are released from cells under stress conditions [5, 28]. Coniferol is supposed to stimulate H_2O_2 production [29, 30], and significant accumulation of phenolcarbonic acids, particularly, ferulate, facilitates the cytotoxic effect observed under biotic stress in wheat and rye [31, 32]. The purified extracellular wheat PODs possess high enzymatic activity in

Abbreviations: Co, peroxidase compound; ECS, extracellular solution; $O_2^{\cdot-}$, superoxide anion radical; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutases.

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oxidation of ferulate acid and coniferol [33]. The question arises as to whether phenolic compounds released into the apoplast under stress conditions can "switch" the functioning of peroxidases from peroxidative to oxidative activity and serve as POD substrates in ROS production? The goal of the present work was to study effects of exogenous POD substrates, ferulate and coniferol, on activity and $O_2^{\cdot-}$-producing ability of the extracellular POD from wheat root seedlings.

MATERIALS AND METHODS

Seedlings of spring wheat (*Triticum aestivum* L.), variety Lyuba, grown on 0.25-mM $CaCl_2$ solution for five days were used as the object for study. When the effect of ascorbate on activity of guaiacol POD was examined, the seedlings were grown on distilled water. Immediately after excision from the seedlings, the roots were incubated in a tested solution for 1 h. The solution after root incubation and removal served as the extracellular solution (ECS). The roots were incubated in 0.25 mM $CaCl_2$ for a control or in distilled water in experiments with ascorbate.

Activity of POD (EC 1.11.1.7) in ECS was determined by spectrophotometry with the following substrates: guaiacol (436 nm; $\varepsilon = 25.5\ mM^{-1}\cdot cm^{-1}$), *o*-dianisidine (460 nm; $\varepsilon = 30.0\ mM^{-1}\cdot cm^{-1}$), ferulate (310 nm; $\varepsilon = 16.6\ mM^{-1}\cdot cm^{-1}$), and coniferol (265 nm; $\varepsilon = 7.5\ mM^{-1}\cdot cm^{-1}$). Guaiacol POD was determined as described earlier [22]. In the cases of dianisidine, ferulic, and coniferyl PODs, the reaction mixture contained 0.05-mM sodium citrate buffer, pH 5.4, 1 mM of the corresponding main substrate (*o*-dianisidine, ferulate, or coniferol), and 0.5 ml of ESC in a total volume of 3 ml. When the effect of a second substrate on POD activity was examined, NADH, ascorbate, *o*-dianisidine, ferulate, coniferol, epinephrine, or salicylate was also added. When the combined effect of two additional substrates on dianisidine POD activity was evaluated, the reaction mixture contained 1 mM epinephrine and either 1 or 5 mM ferulate beside the main substrate. Activity of ascorbate peroxidase (EC 1.11.1.11) was determined from decrease in the level of ascorbate determined by spectrophotometry (265 nm; $\varepsilon = 8.24\ mM^{-1}\cdot cm^{-1}$). The reaction mixture (total volume of 3 ml) contained 0.05 mM sodium-phosphate buffer, pH 7.0, 1 mM ascorbate, and 0.5 ml ECS. In all experiments, the peroxidase reaction was initiated by addition of 1 mM H_2O_2.

Protein was determined by the Bradford method [34]. One unit of POD was defined as the amount of the enzyme that oxidizes 1 μmol of *o*-dianisidine per minute. The ability of ECS to produce $O_2^{\cdot-}$ was judged from oxidation of the $O_2^{\cdot-}$ acceptor epinephrine to adrenochrome, which was determined by spectrophotometry (480 nm; $\varepsilon = 4020\ M^{-1}\cdot cm^{-1}$). Specificity of $O_2^{\cdot-}$ formation was confirmed using superoxide dismutase (SOD). The standard reaction medium, total volume 2.34 ml, contained ECS with POD concentration of 0.8 U/ml in 0.05-mM sodium-phosphate buffer, pH 6.8, 1 mM epinephrine, and effectors (H_2O_2, ferulate, and coniferol) in different combinations. The standard incubation time was 20 min.

Experiments were performed in 3-5 biological replications, each with three analytical replications. Data were processed using Microsoft Excel software. Representative data of each experimental series are shown.

The following chemicals were used: ferulic acid, coniferol, $CaCl_2$ (Fluka, Switzerland); *o*-dianisidine (Acros Organics, Belgium); SOD, NADH (Sigma, USA); epinephrine (ICN, USA). All other chemicals of chemical or analytical purity grade were purchased from Reakhim (Russia).

RESULTS

Reducing agents such as ascorbate and NADH inhibit the activity of guaiacol POD. Incubation of roots with 1 mM ascorbate led to complete inhibition of guaiacol peroxidase in ECS (Fig. 1a). Activity of the extracellular dianisidine POD was also completely inhibited both after incubation of roots with 1 mM ascorbate and after addition of ascorbate to ECS (data not shown). Considerable inhibition of guaiacol POD activity in ECS was observed after incubation of roots with 0.5 mM NADH for 1 h. Direct addition of 0.5 mM NADH to ECS led to virtually complete inhibition of the enzyme activity (Fig. 1b). The ferulic POD was activated by addition of 0.1 mM *o*-dianisidine into the reaction mixture; the effect was enhanced with increase of its concentration to 1 mM (Fig. 1c). Similar results were obtained with coniferyl POD: addition of 0.1 mM of *o*-dianisidine resulted in activation of the enzyme, and the effect was more pronounced when the concentration of the second substrate was increased tenfold (Fig. 1d). No ascorbate peroxidase activity was found in ECS.

Activity of dianisidine POD did not change when salicylate in concentrations ranging from 1 μM to 10 mM as the second substrate was added into the reaction medium (Fig. 2a). Addition of either 0.1 mM ferulate (Fig. 2b, column *2*) or 0.1 mM coniferol (Fig. 2c, column *2*) as the second substrate led to considerable decrease in the enzyme activity. Elevation of ferulate concentration by 10 (Fig. 2b, column *3*) and 50 times (Fig. 2d, columns *4* and *5*) did not result in statistically significant change in the activity. Unlike ferulate, the increase in concentration of coniferol from 0.1 to 1 mM resulted in a small but statistically significant elevation of dianisidine POD activity (Fig. 2b, column *3*). Epinephrine taken at the concentration of 0.1 mM inhibited the dianisidine POD activity. This inhibition was more pronounced when the epinephrine concentration was increased to 1 mM (Fig. 2d, columns *2* and *3*), but less expressed in comparison with

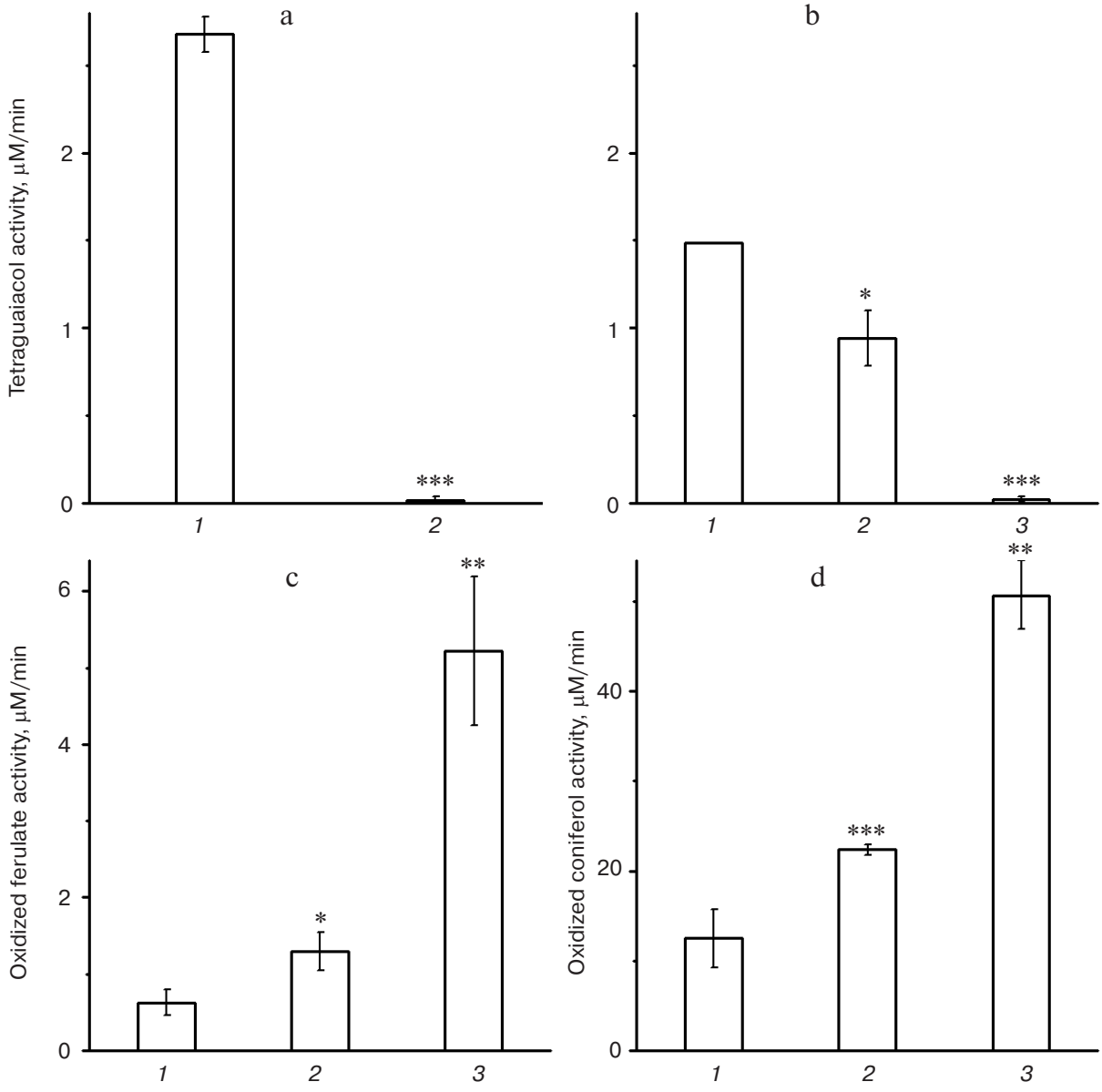

Fig. 1. Activity of tetraguaiacol (a, b), ferulate (c), and coniferol (d) PODs in ECS in the presence of various substances. *1*) Control (distilled water) (a), 0.25 mM $CaCl_2$ (b-d); *2*) after incubation of roots for 1 h with either 1 mM ascorbate (a) or 0.5 mM NADH (b); *3*) with 0.5 mM NADH (b); *2*, *3*) with 0.1 and 1 mM *o*-dianisidine, respectively (c, d). Here and in Figs. 2 and 3, the difference is significant at $P < 0.05$ (*), < 0.01 (**), and < 0.001 (***).

effect of 1 mM ferulate (Fig. 2d, columns *3* and *4*). Considerable inhibition of the dianisidine POD activity was also observed when 1 mM epinephrine and 1 mM ferulate were added simultaneously (Fig. 2d, column *6*). This inhibition was more pronounced than that caused by 1 mM epinephrine only and less pronounced than that caused by 1 mM ferulate only (Fig. 2d, columns *3*, *4*, and *6*). However, the joint inhibitory effect of 1 mM epinephrine and 5 mM ferulate on the dianisidine POD activity was exactly the same as the inhibitory effect of 5 mM ferulate only (Fig. 2d, columns *5* and *7*).

Both H_2O_2 and phenolic compounds serving as both peroxidase substrates and possible factors enhancing free radical production were used in experiments on the $O_2^{\cdot-}$-producing capability of POD determined from oxidation of epinephrine. Addition of 1 mM H_2O_2 to ECS had no effect on epinephrine oxidation (Fig. 3a, column *2*). Addition of either 1 or 10 mM ferulate to ECS containing 1.6 U/ml of POD led to stimulation of $O_2^{\cdot-}$ production (Fig. 3a, columns *3* and *4*). Note that neither 5 nor 10 mM ferulate had an effect on the $O_2^{\cdot-}$-producing capability of ECS containing 0.8 U/ml POD (Fig. 3b,

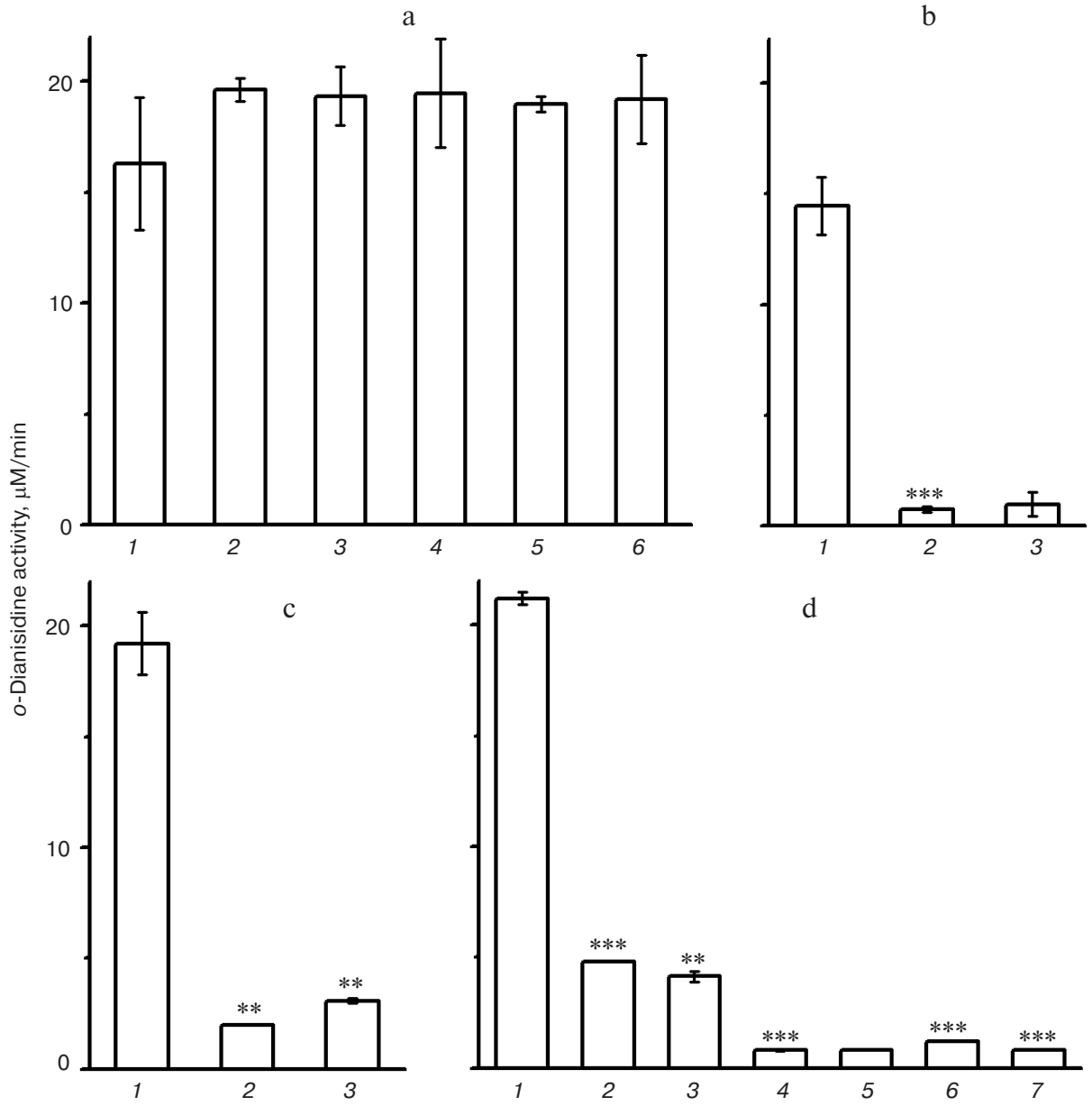

Fig. 2. Activity of *o*-dianisidine POD in ECS in the presence of 0.25 mM $CaCl_2$ (control) (*1*) (a-d), 1, 10, 100, 1000, and 10,000 μM salicylate (*2-6*, respectively) (a), 0.1 and 1 mM ferulate (*2* and *3*, respectively) (b), 0.1 and 1 mM coniferol (*2* and *3*, respectively) (c), 0.1 and 1 mM epinephrine (*2* and *3*, respectively) (d), 1 and 5 mM ferulate (*4* and *5*, respectively) (d), and 1 and 5 mM ferulate together with 1 mM epinephrine (*6* and *7*, respectively) (c).

columns *2* and *3*). The linear stimulating effect on the $O_2^{\cdot-}$-production by ECS was observed under combined action of H_2O_2 and ferulate taken at varied concentrations (Figs. 3a (columns *5* and *6*), 3b (columns *4* and *5*), and 3c (columns *2-4*)). Considerable stimulation of $O_2^{\cdot-}$-production was also observed under the combined action of 1 mM coniferol and 1 mM H_2O_2 (Fig. 3c, columns *5* and *6*).

The $O_2^{\cdot-}$-producing activity of ECS in control was inhibited by 74 ± 7% by addition of 250 U/ml SOD. Unfortunately, we could not confirm $O_2^{\cdot-}$ production in the reaction medium containing ferulate and H_2O_2 by inhibitory analysis because of the interaction between SOD and ferulate in the presence of H_2O_2.

The $O_2^{\cdot-}$-producing activity of POD in the presence of H_2O_2 insignificantly increased with increase in concentration of $O_2^{\cdot-}$ acceptor (epinephrine) in the incubation medium from 1 to 2 mM (Fig. 3d, columns *1* and *3*). Similar effect was observed under the combined action of 10 mM ferulate and 1 mM H_2O_2 (Fig. 3d, columns *2* and *4*). The difference became significant with elevation of epinephrine concentration to 4 mM (Fig. 3d, columns *1*,

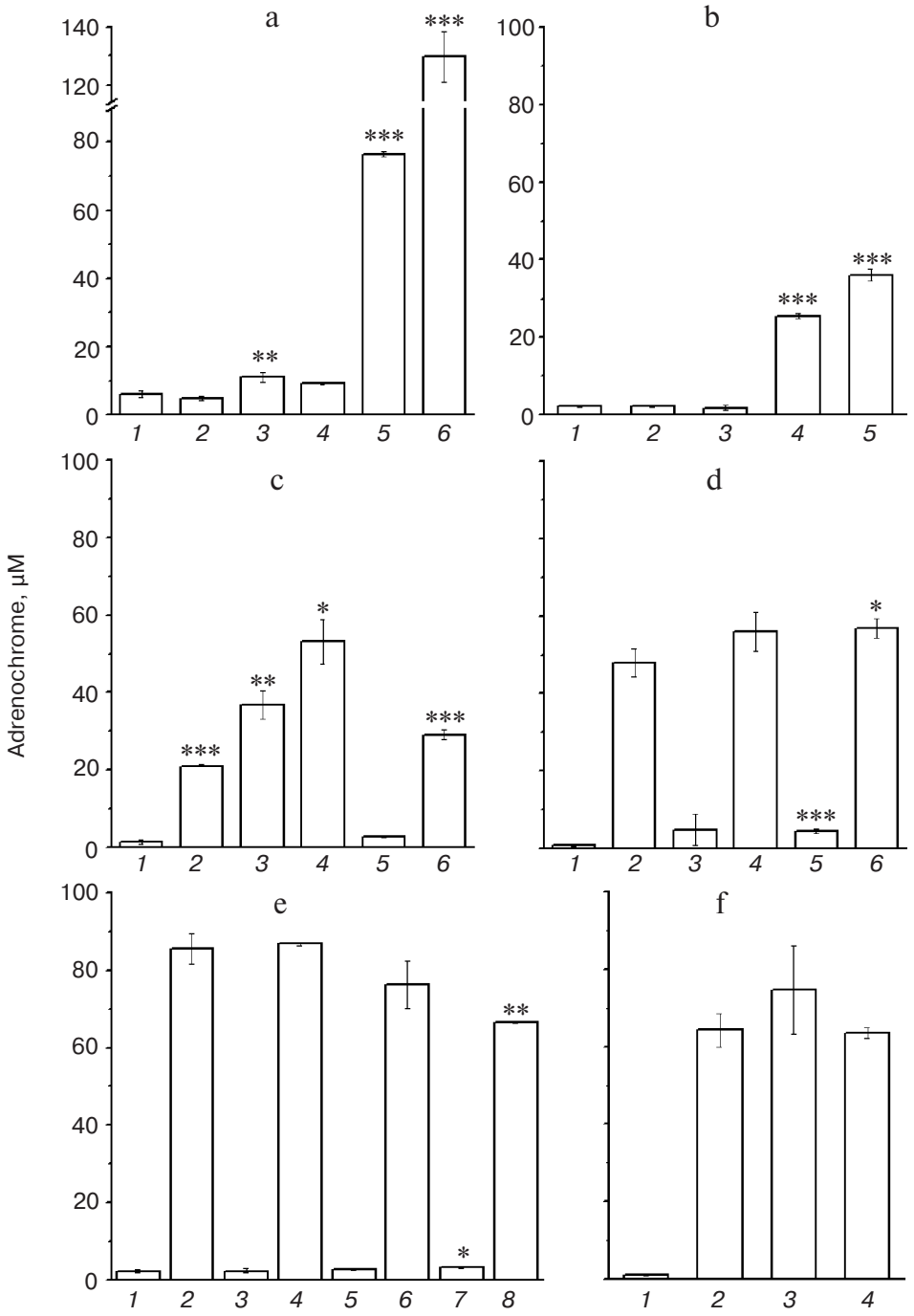

Fig. 3. Superoxide production by ECS estimated from adrenochrome accumulation under the action of various effectors. The reaction medium (total volume of 2 ml) containing 0.05-mM sodium-phosphate buffer, pH 6.8, 1.6 U/ml (a) or 0.8 U/ml (b-f) POD, and effectors was supplemented with 335 μl epinephrine solution (final concentration in the medium 1 mM). The final concentration of epinephrine was increased to 2 and 4 mM in variants *3*, *4* and *5*, *6* (d), respectively. Oxidation of $O_2^{\cdot-}$ acceptor was detected after 20 min of incubation. The incubation time was extended to 40, 60, and 80 min for variants *3*, *4* (e), *5*, *6* (e), and *7*, *8* (e), respectively, and to 45 min for variant *f*. The following effectors were added to the reaction medium: a-c, f) 0.25 mM $CaCl_2$ (control) (*1*); a) 1 mM H_2O_2 (*2*), 1 and 10 mM ferulate (*3* and *4*, respectively); *5*, *6*) the same as *3*, *4* but with addition of 1 mM H_2O_2; b) 5 and 10 mM ferulate (*2* and *3*, respectively); *5*, *6*) the same as *3*, *4* but with addition of 5 mM H_2O_2; c) 0.78, 5, and 10 mM ferulate together with 1 mM H_2O_2 (*2-4*, respectively), 1 mM coniferol (*5*); *6*) the same as *5* but with addition of 1 mM H_2O_2; d) 1 mM H_2O_2 (*1*, *3*, *5*), 10 mM ferulate together with 1 mM H_2O_2 (*2*, *4*, *6*); e) 1 mM H_2O_2 (*1*, *3*, *5*, *7*), 10 mM ferulate together with 1 mM H_2O_2 (*2*, *4*, *6*, *8*); f) 5 mM ferulate together with 1 mM H_2O_2 (*2*); *3*) the same as *2* but with addition of 5 mM ferulate together with 1 mM H_2O_2 after 15 min of incubation; *4*) the same as *2* but with two additions of 5 mM ferulate together with 1 mM H_2O_2 after 15 and 30 min of incubation, respectively.

2, *5*, and *6*). Moreover, when the incubation time was increased from 20 to 40-80 min, the $O_2^{\cdot-}$ production in the presence of H_2O_2 significantly increased only after 80 min of incubation (Fig. 3e, columns *1*, *3*, *5*, and *7*). Alternatively, the combined effect of 10 mM ferulate and 1 mM H_2O_2 resulted in gradual decrease in the $O_2^{\cdot-}$ production, which only became significant after 80 min of incubation (Fig. 3e, columns *2*, *4*, *6*, and *8*). Repeated addition of the same substrates to the reaction medium containing 5 mM ferulate and 1 mM H_2O_2 every 15 min did not result in increase of the $O_2^{\cdot-}$ production (Fig. 3f).

DISCUSSION

Earlier, we found that the incubation of roots with 1 mM ascorbate and 0.5 mM NADH led to increase in formation of extracellular $O_2^{\cdot-}$ [25, 35]. Since the cell surface POD is one of the main sources of $O_2^{\cdot-}$ in roots, there is reason to suppose that these reductants influence the peroxidase activity. The absence of ascorbate peroxidase activity is indirect evidence for the absence of release of intracellular enzymes into ECS during the root incubation. Besides, we have shown that cytosolic contamination of ECS of excised roots comprised only 1.4% [33]. The ECS POD belongs to the class III of secreted plant PODs weakly oxidizing ascorbate but possessing broad substrate specificity [36-38]. NADH and ascorbate are known to be "slow" substrates for class III PODs, whereas phenols, including guaiacol and *o*-dianisidine, are the "rapid" ones. When substrates with drastically different reactivity underwent simultaneous oxidation with POD, the effects of their mutual activation or inhibition are observed [39]. The activation of oxidation of a slowly oxidized substrate and either partial or complete inhibition of a rapidly oxidized substrate (activator) occur [38, 39]. POD is known to be capable of $O_2^{\cdot-}$ production in the presence of reductants and small amount of H_2O_2, according to the scheme of Chance (cited by [40]):

$$\text{Fe(III)} + H_2O_2 \rightarrow \text{CoI} + H_2O, \quad (1)$$

$$\text{CoI} + RH \rightarrow \text{CoII} + R^{\bullet}, \quad (2)$$

$$\text{CoII} + RH \rightarrow \text{Fe(III)} + R^{\bullet}, \quad (3)$$

$$R^{\bullet} + O_2 \rightarrow R^{+} + O_2^{\cdot-}. \quad (4)$$

Thiols, ascorbate, NAD(P)H, and phenols are supposed to act as reductants [41-43]. It remains unknown which substances can serve as substrates for POD when $O_2^{\cdot-}$ is formed *in vivo*. It has been shown in a model system that horseradish POD oxidizes NADH to form $O_2^{\cdot-}$ [41, 44]. Interaction of the substrate with POD leads to formation of reductant radical that can interact with oxygen to produce $O_2^{\cdot-}$ [41, 44], which is in agreement with our previously reported data [35]. Thus, despite the stimulatory effect of NADH and ascorbate on the $O_2^{\cdot-}$ production by roots, these substrates inhibited activity of the extracellular POD when either guaiacol or *o*-dianisidine served as oxidized substrates (Fig. 1). It is likely that enhancement of oxidation when two substrates are simultaneously oxidized by POD primarily implicates reductants rather than guaiacol or *o*-dianisidine. We anticipate that emergence of POD substrates with dramatically different reactivity in apoplast under stress conditions is one of the prerequisites of the oxidative burst. Besides, addition of some phenols is known to result in decrease in concentration of the oxygenated lowly active ferroperoxidase CoII (Eq. (3)), which also can lead to activation of NADH oxidation [39, 41, 45]. Thus, NADH is a good candidate for activated substrate in $O_2^{\cdot-}$ formation by peroxidase on the surface of wheat root cells. Some researchers suppose the presence of NADH in plant cell apoplast [46, 47], but without direct evidence. Ascorbate is another candidate. Unlike NADH, it can be transported from cytoplasm and accumulate in apoplast under stress conditions [48-51]. Ascorbate is known to exhibit either anti- or prooxidant features depending on substrate concentration and oxidative reaction conditions [52]. There are no data in the literature on mechanisms of immediate interaction of ascorbate or its oxidized forms with O_2 followed by $O_2^{\cdot-}$ formation. It is possible that $O_2^{\cdot-}$ formation occurs via a series of intermediates. It was shown, for example, that the oxidation of ascorbate by POD in apoplast is mediated by oxidized intermediates of phenolic acids [49]. The phenolic radicals oxidized in POD-dependent reactions are supposed to undergo autooxidation to form $O_2^{\cdot-}$ and H_2O_2 [38, 53].

Phenolic compounds, being electron donors and reductants for POD by nature, are one-electron aromatic substrates of plant PODs [54]. Moreover, phenolic acids are regarded as more effective electron donors than is ascorbate [49]. There is just a chance that phenols can serve as the main activators in $O_2^{\cdot-}$ formation by peroxidase on the cell surface. The phenolic compounds (ferulic acid and coniferol) found in ECS [55], as well as NADH and ascorbate, are slow POD substrates compared with *o*-dianisidine. Earlier, we have shown that another phenolic compound, salicylate (*o*-hydroxybenzoate), added to the roots causes elevation of both POD activity in ECS and $O_2^{\cdot-}$ production [22, 56]. When salicylate is used as substrate, POD produces $O_2^{\cdot-}$ via formation of free salicylate radical [57]. In our experiments, addition of salicylate to ECS had no significant effect on activity of dianisidine peroxidase (Fig. 2a). Similarly, the addition of salicylate to purified extracellular PODs did not stimulate their guaiacol oxidizing activity [26]. It is likely that in wheat roots salicylate does not compete with *o*-dianisidine and guaiacol and is not an immediate substrate of extracellular POD in $O_2^{\cdot-}$ formation. One can assume that the $O_2^{\cdot-}$-stimulating effect of salicylate added to roots is mediated

by other factors, particularly, by its detergent-like activity and inhibition of catalase [22, 58].

The peroxidase system implicating POD + H_2O_2 + phenolcarbonic acids possesses high radical-producing ability and is known to form ROS [59]. Earlier, it was found that 1 mM H_2O_2 added to either roots or ECS after removal of roots stimulates $O_2^{\cdot-}$ production [22, 56]. In the present work, we found that exogenous H_2O_2 taken at the same concentration (1 mM) had no stimulating effect on $O_2^{\cdot-}$ production if ESC was diluted (the extracellular POD activity was 1.6 U/ml) (Fig. 3a). Ferulate slightly but significantly elevated the level of $O_2^{\cdot-}$ (Fig. 3a), whereas simultaneous addition of H_2O_2 and ferulate resulted in many-fold elevation of $O_2^{\cdot-}$ production by POD (Fig. 3, a-c). Thus, both qualitative and quantitative proportions of interacting components are important for the ROS production in the peroxidase system.

Epinephrine, the acceptor used for $O_2^{\cdot-}$ detection, can also serve as a POD substrate [60]. Here two questions emerge: what substrate of the extracellular wheat root POD is epinephrine in terms of oxidation rate, and whether the stimulating effect of epinephrine on $O_2^{\cdot-}$ production by the system ferulate/coniferol + H_2O_2 + ECS (Fig. 3c) is an artifact of epinephrine oxidation by peroxidase or epinephrine is an actual $O_2^{\cdot-}$ acceptor? Since SOD, the enzyme directly detoxifying superoxide, interacts with ferulate in the presence of H_2O_2, the inhibitory analysis fails to prove $O_2^{\cdot-}$ production in the reaction medium containing ferulate and H_2O_2.

The absorption maxima of the *o*-dianisidine and epinephrine oxidation products are close (460 and 480 nm, respectively). So, detection of any of the products from enhancement of light absorption would be inaccurate if both substrates are present simultaneously. We have shown that dianisidine POD activity is inhibited by so small concentration of epinephrine as 0.1 mM (Fig. 2d). Epinephrine itself appears to remain non-oxidized, because at higher concentration (1 mM) it led to slight but significant, decrease in the level of oxidized products (Fig. 2d). Thus, despite the inhibitory effect of epinephrine on the dianisidine POD activity, *o*-dianisidine does not activate epinephrine oxidation, that is, taken together these substrates do not manifest features characteristic of slowly/rapidly oxidized substrate pairs. It is likely that the mechanism of epinephrine oxidation by peroxidase differs from the mechanism of oxidation of slow substrates such as ferulate and coniferol. Ferulate at concentration of 1 mM inhibits the dianisidine POD activity to a greater degree than does epinephrine at the same concentration (Fig. 2d). Possibly, ferulate is a slower substrate than epinephrine. The combined inhibitory effect of 1 mM ferulate and 1 mM epinephrine on the dianisidine POD activity was greater than that of 1 mM epinephrine only and slightly but significantly lesser than that of 1 mM ferulate only. It is likely that the combined effect of 1 mM ferulate and 1 mM epinephrine is accompanied by slight oxidation of epinephrine, resulting in distortion of the spectrum of oxidized *o*-dianisidine products. We suppose that the increasing the concentration of ferulate to 5 mM prevents this "side" oxidation of epinephrine, because the inhibition level in presence of both 5 mM ferulate and 1 mM epinephrine reached that observed in presence of 5 mM ferulate only (Fig. 2d). It is possible that ferulate does not activate epinephrine oxidation by peroxidase, even in the absence of *o*-dianisidine.

When studying $O_2^{\cdot-}$-producing ability, we also found that neither increase in incubation time nor addition of ferulate and H_2O_2 led to substantial stimulation of epinephrine oxidation (Fig. 3, e and f). The insignificant stimulation of $O_2^{\cdot-}$ production by 1 mM H_2O_2 observed only after 80 min of incubation is possibly due to the autooxidation of epinephrine by atmospheric oxygen, whereas its noticeable decrease under the combined action of 1 mM H_2O_2 and 10 mM ferulate is due to the beginning of reaction product decay for long incubation (Fig. 3d). Thus, when taking that epinephrine oxidation is the consequence of POD enzymatic activity and independent of the incubation time, the substrate concentration would be saturating, and the enzyme inactivated by the reaction product. However, the difference (several times) between the inactivating product concentrations under the action of 1 mM H_2O_2 or combination of 1 mM H_2O_2 and 5 or 10 mM ferulate (Fig. 3, e and f) remains unexplainable. Moreover, when the epinephrine concentration was increased four times, significantly increased accumulation of the reaction product occurred in both cases, with 1 mM H_2O_2 and with 1 mM H_2O_2 added together with 10 mM ferulate, approximately by the same value (Fig. 3d). Partial epinephrine oxidation by peroxidase probably occurred in our experiments (data on incomplete inhibition of the reaction by SOD also supports this supposition), as well as $O_2^{\cdot-}$ quenching by epinephrine. In connection with this, although it is impossible to give an exact quantitative estimation of the formed $O_2^{\cdot-}$, one can judge either increase or decrease in POD capability of $O_2^{\cdot-}$ formation in comparison with control.

We cannot exclude that the system ferulate + H_2O_2 + POD could activate a certain factor facilitating $O_2^{\cdot-}$ production, whose depletion prevented further progress of the reaction. Therefore, following addition of the substrates (H_2O_2 and ferulate) or prolongation of the incubation time had no effect (Fig. 3, e and f). The mechanism of antibiotic effect of phenolcarbonic acids under biotic stress is found to be exclusively due to the toxic effect of ROS formed while the oxidation of these substances occurs [59]. The accumulation of phenols, such as ferulate, was found in resistant wheat and rye varieties under biotic stress [31, 32]. The natural phenols found in ECS under wound stress of wheat roots [55] can probably serve as POD substrates under distinct conditions when $O_2^{\cdot-}$ is formed. It is likely that the primary response reactions of plant cells can be realized via a general unspecific mech-

anism independently on the nature (biotic or abiotic) of a stress factor.

The acceleration of oxidation of a slowly oxidized substrate by a rapidly oxidized one is known to be a general feature of peroxidase systems, which is typical for both peroxidase and oxidase as well as oxygenase functions of this enzyme [39]. We suppose that appearance of some rapidly oxidized POD substrate on the cell surface, which stimulates the oxidation of slowly oxidized substrates, is necessary for activation of oxidation of slowly oxidized substrates (phenols) by peroxidase with subsequent rapid ROS production (oxidative burst) under stress. This supposition requires further studies.

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REFERENCES

1. Delalonde, M., and Coumans, M. P. (1998) *Plant Growth Regul.*, **26**, 123-130.
2. Tekchandani, S., and Guruprasad, K. N. (1998) *Plant Sci.*, **136**, 131-137.
3. Velikova, V., Yordanov, I., and Edreva, A. (2000) *Plant Sci.*, **151**, 59-66.
4. Walz, C., Juenger, M., Schad, M., and Kehr, J. (2002) *Plant J.*, **31**, 189-197.
5. Andreeva, V. A. (1988) *Peroxidase Enzyme* [in Russian], Nauka, Moscow.
6. Melon, J. F. (1991) *Plant Physiol.*, **95**, 14-20.
7. Bernards, M. A., and Razem, F. A. (2001) *Phytochemistry*, **57**, 1115-1122.
8. Gorshkova, T. A. (2007) *Plant Cell Wall as a Dynamic System* [in Russian], Nauka, Moscow.
9. Moran, J. F., Becana, M., Iturbeormaetxe, I., Frechilla, S., Klucas, R. V., and Apariciotejo, P. (1994) *Planta*, **194**, 346-352.
10. Prasad, T. K. (1996) *Plant J.*, **10**, 1017-1026.
11. Laloi, C., Apel, K., and Danon, A. (2004) *Curr. Opin. Plant Biol.*, **7**, 323-328.
12. Torres, M. A., and Dangl, J. L. (2005) *Curr. Opin. Plant Biol.*, **8**, 397-403.
13. Roitto, M., Ahonen-Jonnarth, U., Lamppu, J., and Huttunen, S. (1999) *Eur. J. For. Path.*, **29**, 399-410.
14. Burchanova, G. F. (2006) *Anionic Peroxidases as Components of Plant Resistance against Phytopathogenic Fungi*: Candidate's dissertation [in Russian], Institute of Biochemistry and Genetics, Ufa Scientific Center of the Russian Academy of Sciences, Ufa.
15. Merzlyak, M. N. (1989) in *Advances in Science and Technology. Plant Physiology* [in Russian], Vol. 6, VINITI, Moscow, pp. 1-168.
16. Ros Barcelo, A. (2000) *Curr. Top. Phytochem.*, **3**, 197-202.
17. Bolwell, G. P., Bindschedler, L., Blee, K. A., Butt, V. S., Davies, D. R., Gardner, S., Gerrish, C., and Minibayeva, F. (2002) *J. Exp. Bot.*, **53**, 1367-1376.
18. Kawano, T. (2003) *Plant Cell Rep.*, **21**, 829-837.
19. Gamalei, I. A., Klyubin, I. V., Arnautova, I. P., and Kirpichnikova, K. M. (1999) *Tsitologiya*, **41**, 394-399.
20. Shorning, B. Yu., Smirnova, E. G., Yaguzhinsky, L. S., and Vanyushin, B. F. (2000) *Biochemistry (Moscow)*, **65**, 1357-1361.
21. Skulachev, V. P. (2006) *Apoptosis*, **11**, 473-485.
22. Chasov, A. V., Gordon, L. K., Kolesnikov, O. P., and Minibaeva, F. V. (2002) *Tsitologiya*, **44**, 691-696.
23. Gordon, L. K. (1992) *Physiol. Biochem. Cult. Plants (Kiev)*, **24**, 128-133.
24. Alekseeva, V. Ya., Gordon, L. K., Nikolaev, B. A., and Tsentsevitskii, A. N. (1993) *Physiol. Biochem. Cult. Plants (Kiev)*, **25**, 335-340.
25. Chasov, A. V. (2002) *Generation of Superoxide Anion and Peroxidase Activity under Modification of Cell Membrane Conductivity in Wheat Root Cells*: Candidate's dissertation [in Russian], Kazan Institute of Biochemistry and Biophysics, Kazan Scientific Center of the Russian Academy of Sciences, Kazan.
26. Minibayeva, F. V., Mika, A., and Luthje, S. (2003) *Protoplasma*, **221**, 67-72.
27. Chasov, A. V., Kolesnikov, O. P., Minibaeva, F. V., and Gordon, L. K. (2005) *Vestnik Kharkov Nats. Agr. Univ. Ser. Biol.*, **2**, 29-34.
28. Luk'yanova, L. D., Balmukhanov, B. S., and Ugolev, A. T. (1982) *Oxygen-Dependent Processes in the Cell and Its Function* [in Russian], Nauka, Moscow.
29. Rogovin, V. V., Murav'ev, R. A., Akimov, V. S., and Bavykina, I. V. (1987) *Fiziol. Rast.*, **34**, 1181-1185.
30. Pomar, F., Caballero, N., Pedreno, M. A., and Ros Barcelo, A. (2002) *FEBS Lett.*, **529**, 198-202.
31. Chigrin, V. V. (1986) *Zh. Obshch. Biol.*, **47**, 310-326.
32. Komarova, Ye. P., and Davidovich, L. A. (1997) *Fiziol. Rast.*, **44**, 749-755.
33. Minibaeva, F., Kolesnikov, O., Chasov, A., Beckett, R. P., Lüthje, S., Vylegzhanina, N., Buck, F., and Böttger, M. (2009) *Plant, Cell Environ.*, **32**, 497-508.
34. Bradford, M. (1976) *Anal. Biochem.*, **72**, 248-254.
35. Minibayeva, F. V., Kolesnikov, O. P., and Gordon, L. K. (1998) *Protoplasma*, **205**, 101-106.
36. Welinder, K. G., and Gajhede, M. (1993) in *Plant Peroxidases. Biochemistry and Physiology* (Welinder, K. G., Rasmussen, S. K., Penel, C., and Greppin, H., eds.) Rochat-Baumann, Inprimerie Nationale, Geneva, pp. 35-42.
37. Mehlhorn, H., Lelandais, M., Korth, H. G., and Foyer, C. H. (1996) *FEBS Lett.*, **378**, 203-206.
38. Takahama, U. (2004) *Phytochem. Rev.*, **3**, 207-219.
39. Lebedeva, O. V., and Ugarova, N. N. (1996) *Izv. Ros. Akad. Nauk. Ser. Khim.*, **1**, 25-32.
40. Mika, A., Minibayeva, F., Beckett, R. P., and Luthje, S. (2004) *Phytochem. Rev.*, **3**, 173-193.
41. Halliwell, B. A. (1978) *Planta*, **140**, 81-99.
42. Vianello, A., and Macri, F. (1991) *J. Bioenerg. Biomembr.*, **23**, 409-423.
43. Bolwell, G. P., and Wojtaszek, P. (1997) *Physiol. Mol. Plant Pathol.*, **51**, 347-366.
44. Lebedeva, O. V., and Ugarova, N. N. (1997) *Biochemistry* (Moscow), **62**, 212-216.
45. Mader, M., and Fussl, R. (1982) *Plant Physiol.*, **70**, 1132-1134.
46. Elstner, E. F., and Heupel, A. (1976) *Planta*, **130**, 175-180.

47. Gross, G. G., Janse, C., and Elstner, E. F. (1977) *Planta*, **136**, 271-276.
48. Castillo, F. J., and Greppin, H. (1988) *Environ. Exp. Bot.*, **28**, 231-238.
49. Takahama, U., and Oniki, T. (1992) *Plant Cell Physiol.*, **33**, 379-387.
50. Horemans, N., Foyer, C. H., and Asard, H. (2000) *Trends Plant Sci.*, **5**, 263-267.
51. Smirnoff, N. (2000) *Curr. Opin. Plant Biol.*, **3**, 229-235.
52. Zenkov, N. K., Lankin, V. Z., and Menshchikova, E. B. (2001) *Oxidative Stress. Biochemical and Pathophysiological Aspects* [in Russian], MAIK Nauka/Interperiodika, Moscow.
53. Takahama, U., and Oniki, T. (2000) *J. Plant Res.*, **113**, 301-309.
54. Gazaryan, I. G., Khushpul'yan, D. M., and Tishkov, V. I. (2006) *Usp. Biol. Khim.*, **46**, 303-322.
55. Kolesnikov, O. P., Kavieva, A. A., Mityashina, S. Y., Chasov, A. V., Gordon, L. K., and Minibayeva, F. V. (2006) *Proc. 2nd Int. Symp. "Signal Systems of the Plant Cell: the Role in Adaptation and Immunity"*, Kazan, pp. 186-187.
56. Minibayeva, F. V., Gordon, L. K., Kolesnikov, O. P., and Chasov, A. V. (2001) *Protoplasma*, **217**, 125-128.
57. Kawano, T., and Muto, S. (2000) *J. Exp. Bot.*, **51**, 685-693.
58. Tarchevsky, I. A., Maksyutova, N. N., Yakovleva, V. G., and Grechkin, A. N. (1999) *Fiziol. Rast.*, **46**, 23-28.
59. Averianov, A. A. (1991) *Usp. Sovrem. Biol.*, **111**, 722-737.
60. Adak, S., Bandyopadhyay, U., Bandyopadhyay, D., and Banerjee, R. K. (1998) *Biochemistry*, **37**, 16922-16933.