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RESEARCH PAPERS

Interaction of Apoplastic Peroxidases from Wheat Roots with Nitrite and Nitrate: Intermediates and Products

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Abstract—Peroxidases are widespread in animal and plant tissues, wherein they perform a variety of functions. Peroxidases have a broad specificity for substrates of various chemical structures. Along with hydrogen peroxide, phenolic compounds, and toxic compounds of aromatic nature, nitrogen-containing compounds are substrates for peroxidases. This work is devoted to the study of the role of wheat extracellular peroxidases in the metabolism of nitrogen-containing compounds. It has been shown that partially purified isozymes differing in peroxidase activity are involved in the metabolism of nitrogen-containing compounds. The formation of primary and secondary phenoxyl radicals during the combined oxidation of chlorogenic acid, nitrite, and H_2O_2 was demonstrated. With cooxidation with purified isoenzymes *p*-coumaric acid and nitrite, the formation of 4-hydroxy-3-nitrocinnamic acid was revealed. It is assumed that the same isoforms can participate both in the oxidation of nitrite with the formation of nitrophenol and in the reduction of nitrate. The participation of plant peroxidases in nitrogen metabolism can be represented as a set of reactions for the reduction and/or oxidation of nitrogen of different oxidation states with the formation of active intermediates.

Keywords: *Triticum aestivum*, apoplastic peroxidase, nitrite, nitrate, phenoxyl radicals
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INTRODUCTION

Peroxidase (POX) is a key enzyme of redox metabolism and is involved in both the generation and utilization of reactive oxygen species (ROS) [1, 2]. It is known that POX has a wide substrate specificity for substrates of various chemical structures. Along with hydrogen peroxide, phenolic compounds, and toxic compounds of an aromatic nature, the substrates of POX are nitrogen compounds of various oxidation states: nitrates and nitrites [3–5]. In plants, one of the key processes of nitrogen metabolism is the reduction of nitrates, which occurs both in leaves and roots with the participation of nitrate reductase and nitrite reductase [6]. Along with this, oxidoreductases, in particular, peroxidases, may be involved [7]. The interaction of POX with nitrogen compounds can be accompanied by the formation of charged by-products with an unpaired electron and, therefore, they are reactive. Potentially, this interaction can be accompanied by the formation of reactive nitrogen species (RNS), such as the nitrogen dioxide radical NO_2, dinitrogen tri- and tetroxides N_2O_3, N_2O_4, nitrogen monoxide NO, peroxynitrite $ONOO^-$, S-nitrosoglutathione GSNO, S-nitrosothiol SNO [7, 8]. It is known that RNS play an important role in signal transduction in plants and regulate a wide range of physiological and biochemical reactions [9, 10]. In particular, NO takes part in the regulation of the cell cycle of a plant cell, the processes of differentiation and morphogenesis of plants. It is involved in the transduction of signals that stimulate the synthesis of phytohormones [11]. At present, data have been accumulated on the participation of NO in plant responses to stressors [11, 12]. In addition, RNS oxidize and nitrate nucleic acids, proteins, and lipids [9].

Until now, the role of POX in the metabolism of nitrogen compounds has been poorly studied. In animal cells, it has been shown that some POX are involved in the formation of RNS [8]; in addition, some POX of animal and plant origin can use NO as a substrate [13, 14]. The involvement of individual POX isoforms isolated from agricultural plants, including cereals, in the metabolism of nitrogen compounds has

Abbreviations: ROS—reactive oxygen species; RNS—reactive nitrogen species; POX—peroxidase; ECS—extracellular solution.

not been shown. It is agricultural plants that undergo significant stress when applying high doses of nitrogen fertilizers, which leads to the accumulation of nitrogenous compounds and products of their metabolism in plants and, ultimately, in the body of animals and humans that consume these plants with food. Huang et al. [15] showed that a commercial preparation of horseradish POX catalyzes the formation of NO from hydroxyurea in the presence of H_2O_2. In addition, transgenic tobacco plants overexpressing the peroxidase gene *swpa4* had an increased level of NO [16]. Sakihama et al. [5] demonstrated the nitration of plant phenols using a commercial preparation of horseradish POX. The nitration reaction of phenols by horseradish POX was studied in detail by Kong et al. [17], the kinetic characteristics were analyzed, and the ping-pong mechanism of two-substrate enzymatic reaction (double substitution mechanism) was proposed. These data indicate the complex nature of the interaction of plant POX, nitrogen-containing compounds, and phenolic POX substrates with the formation of biologically active intermediates. In this regard, the aim of this study was to identify the products of the interaction of extracellular POX of wheat roots with phenols in the presence of nitrogen compounds. To achieve this goal, the research objectives included (1) determining an intermediate product of a radical nature formed as a result of joint POX-mediated oxidation of phenols and nitrites and (2) identifying the final product of this reaction.

MATERIALS AND METHODS

Object. Our research object was seedlings of spring wheat (*Triticum aestivum* L.) of the Kazanskaya Jubileynaya variety grown in a solution of 0.25 mM $CaCl_2$ at 23°C, 12-h-light period and energy flux density of 100 W/m^2. Roots of 5-day-old seedlings immediately after cutting off from seedlings were infiltrated with Tris-HCl buffer (50 mM pH 7.4) at a pressure of 80 kPa for 30 s. Postinfiltration buffer (extracellular solution, ECS) was used to isolate proteins. Cytoplasmic contamination with ECS was determined by the activity of the marker cytoplasmic protein glucose-6-phosphate dehydrogenase (EC 1.1.1.49) [18]. The protein content was measured by the Bradford method [19].

Purification of proteins. Extracellular proteins were precipitated $(NH_4)_2SO_4$ (30–80%) and centrifuged at 23500 *g* for 20 min. The resulting precipitate was resuspended in Tris-HCl buffer (50 mM pH 7.4) and dialyzed against Tris-HCl buffer (25 mM pH 9.0) overnight at 4°C.

Ion-exchange chromatography was carried out on a column (1.5 × 13 cm) with DEAE-Sephadex A-50 equilibrated with Tris-HCl buffer (25 mM pH 9.0). The bound proteins were eluted in a linear NaCl concentration gradient (0–0.6 M) in the same buffer at a rate of 0.4 mL/min. The volume of the fractions was 1 mL. The analysis of the homogeneity of peroxidase isoforms in each chromatographic fraction was carried out by electrophoresis under nondenaturing conditions, without the addition of Na-DDS and mercaptoethanol, in 12% PAGE followed by staining with benzidine to visualize peroxidase activity (see below). The molecular weight of proteins was determined using a wide range of molecular weight markers (Bio-Rad, United States) and gel staining Coomassie G250. For further analysis, the samples were concentrated by ultrafiltration on an Ultracel-10K membrane (Merck Millipore, United States).

Isoelectric focusing of proteins was performed in PAAG using ampholines pH 3.5–10 (LKB, Sweden). To determine the isoelectric point of proteins, a set of standards IEF-M1A (3.6–9.3) (Sigma, United States) was used.

The activity of peroxidase isoenzymes in the gel was revealed by staining with 0.05 M benzidine solution with 30 mM H_2O_2 in acetate buffer (0.2 mM, pH 5.2).

Determination of enzyme activity. POX activity (EC 1.11.1.7.) was measured spectrophotometrically (PerkinElmer, United States) using benzidine as a substrate (ε_{590} = 34 mM^{-1} cm^{-1}). The reaction mixture with a total volume of 3 mL consisted of acetate buffer (100 mM, pH 5.3), 1 mM H_2O_2, 1 mM benzidine, and 0.5 mL of the sample. Determination of the activity of peroxidases for oxidation of *p*-coumaric acid (ε_{285} = 17.787 mM^{-1} cm^{-1}) was carried out under the following conditions: K-phosphate buffer (35 mM, pH 7.4), 0.1 mM *p*-coumaric acid, 1 mM H_2O_2, and 0.05 mL of the sample; the total volume of the reaction mixture was 0.5 mL. The nitrate reductase activity of POX was measured by the rate of accumulation of the product of the enzymatic reaction, nitrite [20]. The reaction mixture with a total volume of 0.5 mL consisted of 30 mM Na-phosphate buffer pH 8.0, 100 mM KNO_3, 0.64 mM $K_2S_2O_5$, 0.64 mM sodium diethyldithiocarbamate, and 0.05 mL of the sample. The mixture was incubated for 20 min at room temperature, the reaction was stopped by adding 0.05 mL of glacial acetic acid, then 0.55 mL of Griss reagent was added and kept for 20 min. The amount of the resulting product was measured spectrophotometrically at 527 nm (PerkinElmer, United States).

In experiments to assay inhibition of enzymatic reactions, inhibitors were added to the incubation mixture prior to the addition of the enzyme.

Determination of phenoxyl radical. The phenoxyl radical was detected by the method of electron paramagnetic resonance (EPR) [21]. The reaction mixture consisted of Tris-HCl buffer (20 mM, pH 7.4), 1 mM chlorogenic acid, 5 mM H_2O_2, and 0.05 mL of purified enzyme. Experiments were performed in the presence or absence of 5 mM KNO_2. To trap short-lived primary radicals, 0.1 mL of 1 M $ZnSO_4$ was added [22]. The EPR spectra of the prepared samples were

recorded on an EMX X-band spectrometer (Bruker, Germany) at room temperature.

Identification of nitrophenol. Analysis of the nitration reaction *p*-coumaric acid peroxidases were carried out according to the modified protocol of Sakihama et al. [5]. The reaction medium consisted of K-phosphate buffer (25 mM, pH 7.4), 10 mM KNO_2, 0.2 mM *p*-coumaric acid, 1 mM H_2O_2, and 0.05 mL of purified enzyme. The formation of 4-hydroxy-3-nitrocinnamic acid was monitored spectrophotometrically (PerkinElmer, United States) by increasing the optical density at 440 nm.

Identification of the nitration product of *p*-coumaric acid peroxidases was carried out using high performance liquid chromatography (HPLC, Gilson, United States). Reaction mixture (K-phosphate buffer (20 mM, pH 7.4), 100 mM KNO_2, 2 mM *p*-coumaric acid, 10 mM H_2O_2) incubated with 0.1 mL purified enzyme for 60 min and centrifuged at 5000 *g* for 5 min. The resulting supernatant was subjected to chromatographic separation using a C-18 reverse phase column (Ultrasphere ODS, 4.6 × 250 mm, Beckman, United States). The bound substance was eluted with K-phosphate buffer (50 mM, pH 7.0). The elution rate was 0.5 mL/min, and the volume of the injected sample was 100 μL. The yield of substances was monitored by absorption at 314 and 440 nm using a diode array detector (Shimadzu SPD-M20A, Japan). The chromatographic profile of the product was compared to that of the standard. The standard 4-hydroxy-3-nitrocinnamic acid was synthesized as liquid yellow needle crystals from 4-hydroxy-3-nitrobenzaldehyde and malonic (propanedioic) acid according to the Freund protocol [23]. Confirmation of structure standard was carried out by the method of ^{1}H-NMR (CD_3OD, 600 MHz, Bruker, Germany).

The experiments were carried out in three to five biological replicates, and each variant had three analytical replicates. The data were statistically processed using the Microsoft Office Excel 2010 program. The tables show mean values and their standard errors.

RESULTS

In the ECS of wheat roots, proteins were found with peroxidase (16.9 mmol/min per mg of protein) and nitrate-reducing (3.3 mmol/min per mg of protein) activities. The presence of only trace amounts of cytoplasmic contamination (<0.99%) indicates the presence of only apoplastic proteins in the solution. The isozyme spectrum of apoplastic wheat peroxidases was represented by five anionic and cationic isoforms with molecular weights from 31 to 72 kDa (Fig. 1) and isoelectric points from 3.6 to 7.9 (data not shown). Partial cleaning by sedimentation $(NH_4)_2SO_4$ (30–80%) and ion-exchange chromatography revealed the presence of two main POX isoforms: POX1 with a molecular weight of 31 kDa and pI 3.6 and POX2 with a molecular weight of 65 kDa and pI 6.9 (Fig. 1). It was found that the neutral POX2 isoform exhibited a higher peroxidase activity in the oxidation of benzidine (19 times) and the natural substrate, *p*-coumaric acid (24 times), as well as nitrate-reducing activity (7 times) in comparison with POX1 (Table 1). Both the peroxidase and nitrate-reducing activities of POX1 and POX2 were inhibited by NaN_3 and KCN. Azide suppressed the nitrate-reducing activity of POX1 and POX2 more effectively than peroxidase activity (Table 2). Cyanide effectively inhibited both enzymatic reactions, and to a greater extent in POX2 than in POX1.

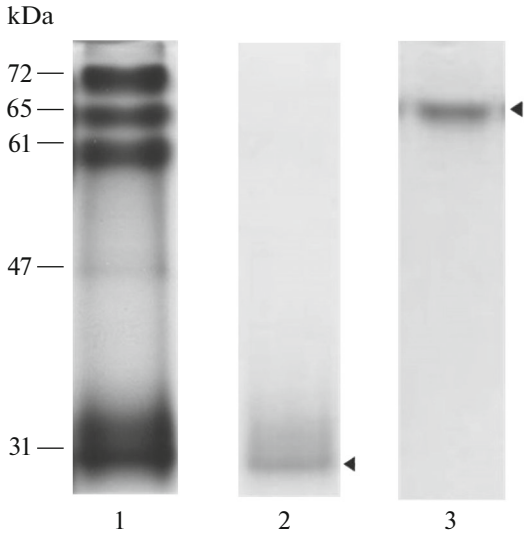

Fig. 1. Isozyme spectrum of wheat extracellular POX: (1) crude extract; (2) partially purified POX1; (3) partially purified POX2.

Oxidation of phenolic substrates by POX is accompanied by the formation of a number of intermediate

Table 1. Peroxidase and nitrate-reducing activities of POX1 and POX2

Isoforms	Molecular weight, kDa	Peroxidase activity		Nitrate-reducing activity, mmol/(mg protein min)
		oxidation of benzidine mmol/(mg protein min)	oxidation *p*-coumaric acid, mmol/(mg protein min)	
POX1	31	2.3 ± 0.1	0.2 ± 0.0	0.8 ± 0.1
POX2	65	43.5 ± 1.7	3.6 ± 0.1	5.5 ± 0.3

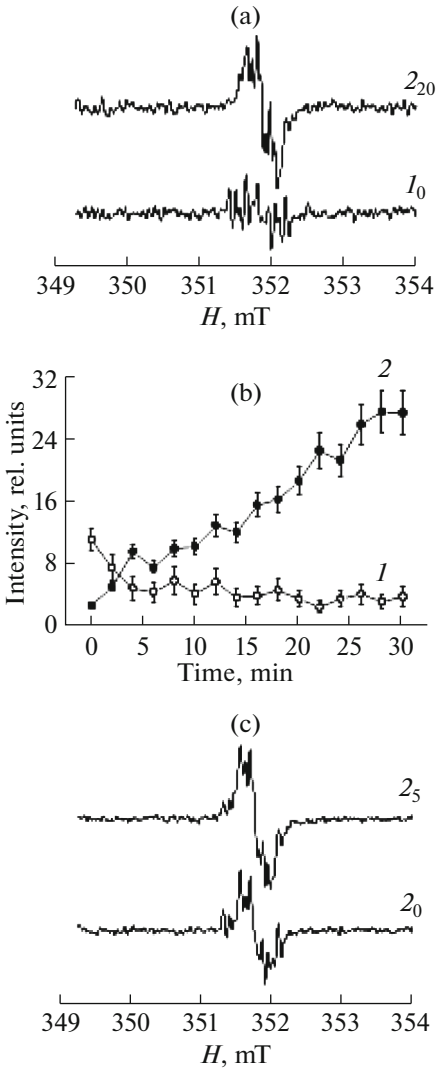

Fig. 2. Formation of phenoxyl radicals during the oxidation of chlorogenic acid by POX2. (a) In the oxidation of chlorogenic acid by POX2 without KNO_2, (b) dynamics of accumulation of secondary radicals without KNO_2, (c) during the oxidation of chlorogenic acid by POX2 with the addition of 5 mM KNO_2. (*1*) Primary radical (g = 2.0044); 1_0—0 min; (*2*) secondary radical (g = 2.00415), 2_0, 2_5, 2_{20}—0, 5, 20 min, respectively.

products, including phenoxyl radicals. The formation of primary and secondary phenoxyl radicals with characteristic EPR spectra was revealed during the oxidation of partially purified POX2 of wheat, a natural phenolic substrate, chlorogenic acid (Fig. 2a). Similar results were shown with POX1 (data not shown). A few minutes after the start of the reaction, the EPR spectrum changed, the primary radicals were transformed into secondary ones (Fig. 2b). The dynamics of the accumulation of secondary radicals in the presence of POX indicates their gradual accumulation, while the addition of nitrite led to the formation of secondary radicals instantaneously (Fig. 2c). In the absence of H_2O_2, no radical formation occurred (data not shown).

Spectrophotometric analysis of the products of the KNO_2 and *p*-coumaric acid POX2 cooxidation of wheat roots revealed the formation of a nitro-derivative compound. The increase in optical density at 440 nm, according to Sakihama et al. [5] indicates the formation of 4-hydroxy-3-nitrocinnamic acid (Fig. 3). Similar results were obtained for POX1 (data not shown).

Chromatographic analysis of the products of the KNO_2 and *p*-coumaric acid POX2 cooxidation reaction of wheat roots revealed the formation of a nitro-derivative compound (Fig. 4). Without adding KNO_2 (Fig. 4) or in the absence of POX (data not shown), the formation of nitrophenol did not occur. Comparison of the optical absorption spectra of the synthesized standard 4-hydroxy-3-nitrocinnamic acid and the nitro derivative of the oxidation product of POX revealed the identity of the shape of the spectra with characteristic absorption maxima in the region of 314 and 440 nm at neutral pH (Figs. 4a, 4b).

DISCUSSION

The accumulation of nitrogen-containing compounds and products of their metabolism is very toxic to plants and animals. Along with nitrate and nitrite reductases that metabolize these compounds, POX oxidoreductases can also utilize nitrogen-containing compounds, which leads to the formation of nitro derivatives of phenolic compounds. In this article, we have demonstrated that apoplastic POXs of wheat roots can partici-

Table 2. Inhibition of peroxidase and nitrate-reducing activities of POX1 and POX2 in wheat

Inhibitor	Concentration, mM	Peroxidase activity, % of control		Nitrate-reducing activity, % of control	
		POX1	POX2	POX1	POX2
NaN_3	0.1	85 ± 3	98 ± 2	3 ± 1	6 ± 1
NaN_3	1	52 ± 4	48 ± 2	0	0
KCN	0.1	16 ± 4	1 ± 0	15 ± 2	2 ± 0
KCN	1	13 ± 3	0	3 ± 1	1 ± 0

Peroxidase and nitrate-reducing activities in the control (100%) are shown in Table 1.

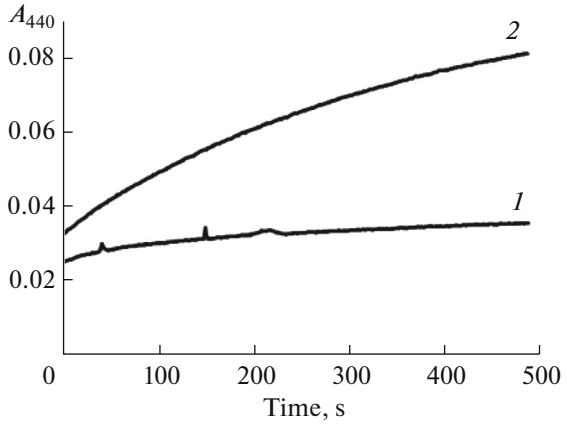

Fig. 3. Formation of a nitro derivative *p*-coumaric acid ($\lambda = 440$ nm) with the addition of KNO_2. (*1*) Without KNO_2; (*2*) with the addition of KNO_2.

pate in the metabolism of nitrogen-containing compounds, jointly oxidizing nitrites and phenols, in particular, with the formation of 4-hydroxy-3-nitrocinnamic acid, and also probably by reducing nitrates to nitrites.

As is known, the oxidation of POX of phenolic substrates is accompanied by the formation of a number of intermediate products, including phenoxyl radicals, which can be detected by EPR [21, 24]. In the present work, the formation of phenoxyl radicals by apoplastic POXs of wheat was detected when chlorogenic acid was used as a phenolic substrate (Fig. 2). During the oxidation of chlorogenic acid by apoplastic POX, the primary phenoxyl radicals were gradually transformed into more stable secondary radicals. Earlier, the formation of primary and secondary radicals of chlorogenic acid was shown using a commercial preparation of horseradish POX [21]. According to Sakihama et al. [24], the half-life of primary phenoxyl radicals is 45 s, and the half-life of secondary radicals is already 20 min. An increase in the lifetime of phenoxyl radicals due to interaction with bivalent metals leads to the manifestation of prooxidant properties of radicals, and, as a consequence, lipid peroxidation and DNA damage [24]. Along with this, it is known that phenoxyl radicals can exhibit antioxidant properties, taking part in the detoxification of H_2O_2, hydroxyl radical, singlet oxygen, since they are intermediate products in the oxidation of phenols by peroxidases [24]. Subsequently, phenoxyl radicals are either reduced to phenol with the help of ascorbate or monodehydroascorbate reductase [21], or, in the presence of nitrites and peroxidase, they are nitrated to form a nitro derivative [5]. In our experiments, the addition of nitrite to the reaction mixture induced the instantaneous formation of secondary phenoxyl radicals even in the absence of POX (Fig. 2). It is possible that the generation of secondary phenoxyl radicals occurs in a nonenzymatic way. Considering that, in the absence of nitrite, the conversion of primary radicals and the accumulation of secondary radicals occurs gradually, it can be assumed that nitrite sharply increases the toxicity of the action of phenoxyl radicals.

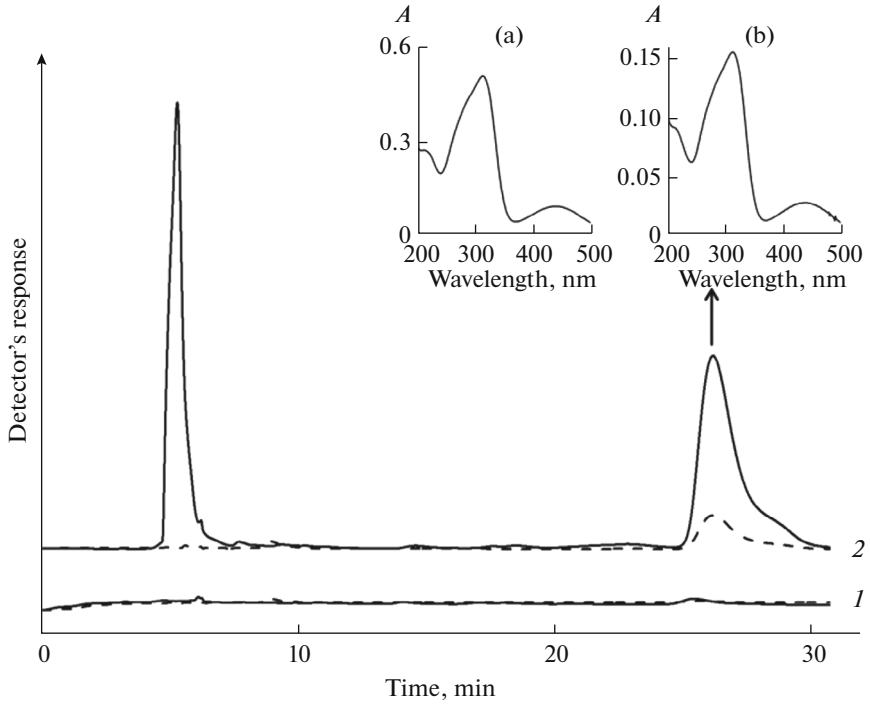

Fig. 4. Chromatographic analysis of the products of the KNO_2 and *p*-coumaric acid oxidation reaction by wheat POX2. The solid line is 314 nm, the dashed line is 440 nm. (*1*) Control; (*2*) KNO_2. The insets show (a) the optical absorption spectra of the 4-hydroxy-3-nitrocinnamic acid standard and (b) the oxidation product of *p*-coumaric acid.

POX of various origins is characterized by the simultaneous oxidation of several substrates [25]. The final product of the joint conversion of phenolic compounds and nitrites, which occurs through the formation of intermediate phenoxyl radicals, is the nitro derivatives of phenols [5]. In our work, during the combined oxidation of coumaric acid and nitrite with purified POX1 and POX2, the formation of 4-hydroxy-3-nitrocinnamic acid was found (Fig. 3). In the absence of POX, the appearance of this nitro derivative is not observed. The content of nitrocinnamic acid among other reaction products is small (Fig. 4), but this acid has an important protective value. For example, it is known that the addition of a nitro group to tyrosine, and to a lesser extent to tryptophan, can lead to a change in the physicochemical properties of proteins and their functions. Thus, the addition of a nitro group causes a noticeable shift in the pKa of the hydroxyl group from 10.07 in tyrosine to 7.50 in 3-nitrotyrosine and increases the hydrophobicity of tyrosine, which affects the activity of the protein. The presence of 3-nitrotyrosine is associated with a wide range of diseases in humans and animals [26]. In plants, tyrosine nitration is involved in various metabolic processes, as well as in responses to the action of abiotic and biotic stressors [27, 28]. Hydroxycinnamic acids are structurally similar to tyrosine, and their nitration prevents the nitration of amino groups of proteins [5]. Thus, the formation of nitrophenols as a result of the combined oxidation of phenols and nitrites by POX can reduce the nitration of proteins.

Earlier, we showed that POX was activated in wheat leaves when plants were placed in the dark. Interestingly, when an excess of nitrate was added, no significant activation of POX was observed [29]. It is possible that the decrease in the POX activity for the oxidation of phenols in the presence of nitrate in the dark is associated with the interaction of the nitrate with the POX active site. It is known that nitrate inhibits the formation of compound E1 (an intermediate semioxidized form of the enzyme) by binding inside the heme "pocket" of POX [30]. Earlier, it was suggested that POX is involved in the reduction of nitrates. Peive et al. [3] found the nitrate-reducing activity of POX in all organs of fodder beans, with a higher level in the roots. In our experiments, the extracellular POX of wheat roots also exhibited nitrate-reducing activity with the formation of nitrite. Interestingly, the nitrate-reducing activity of root POX was more sensitive to the inhibitor of heme-containing enzymes azide than the phenol oxidase activity of POX (Table 2). Similar results were obtained using a commercial preparation of POX horseradish by Ivanova et al. [20]. The sensitivity to cyanide of both activities of wheat POX in our experiments was the same (Table 2). It can be assumed that, despite the fact that the main nitrate-reducing enzyme in plant cells is nitrate reductase, the competitive relationship of H_2O_2 and nitrate lead to the "switching on" of the nitrate-reducing activity of POX under certain conditions, for example, with excessive supply of plants with nitrates or other stresses.

Thus, the participation of POX in nitrogen metabolism can be represented as a set of reactions for the reduction and oxidation of nitrogen with different degrees of oxidation. Under certain conditions, nitrates entering the roots can be metabolized into nitrites with the help of root POX, which can subsequently be rapidly reduced by nitrite reductase to ammonia or, through POX, can be incorporated into nitro derivatives of phenolic compounds. In addition, as shown by EPR data, root POX is involved in the formation of primary and secondary phenoxyl radicals, which are intermediates in the formation of nitrophenols. In this work, we have shown for the first time the participation of the same POX isoforms in wheat roots both in the possible reduction of nitrate and in the oxidation of nitrite with the formation of nitrophenol. Currently available data indicate that POX can regulate the concentration of RNS. In particular, it cannot be ruled out that POX-mediated nitrogen metabolism in plants is accompanied by the formation of RNS, which has a large regulatory effect. Future research will shed light on the involvement and physiological roles of plant POXs in the formation and metabolism of RNS, such as NO_2 and NO, as by-products of cooxidation of nitrogen-containing compounds and phenols.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflicts of interest.

Statement on the welfare of humans or animals. This article does not contain any studies involving humans or animals performed by any of the authors.

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