ISSN 0006-2979, Biochemistry (Moscow), 2010, Vol. 75, No. 1, pp. 95-100. © Pleiades Publishing, Ltd., 2010.
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Production of Hydrogen Peroxide and Nitric Oxide Following Introduction of Nitrate and Nitrite into Wheat Leaf Apoplast

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Received February 4, 2009
Revision received March 27, 2009

Abstract—Infiltration of wheat (*Triticum aestivum* L.) seedling leaves with excess of nitrate, nitrite, or the NO donor sodium nitroprusside leads to increase both in content of hydroperoxide and activity of peroxidase and decrease in superoxide dismutase (SOD) activity in the leaf apoplast. Polymorphism of extracellular peroxidases and the presence of Cu/Zn-SOD have been shown in apoplast. Using an ESR assay, a considerable increase in the level of NO following infiltration of leaf tissues with nitrite has been demonstrated. These data suggest development of both oxidative and nitrosative stresses in leaves exposed to high levels of nitrate or nitrite. A possible interplay of NO and reactive oxygen species in plant cells is discussed.

DOI: 10.1134/S0006297910010128

Key words: wheat, *Triticum aestivum* L., apoplast, nitrate, nitrite, nitric oxide, hydrogen peroxide, antioxidant enzymes

Nitrogen is one of the main elements required for plant growth and development. It is a component of compounds of various oxidation levels whose interconversions determines its biochemical cycle. Nitrogen ions are also signaling molecules influencing expression of some genes and biosynthesis of enzymes [1]. One of the products of nitrate reduction and nitrite oxidation is nitric oxide (NO), a potent effector of physiological processes [2] that is implicated in protective reactions of plants in response to environmental changes [3, 4].

Biological activity of NO is to be considered in association with that of other substances, such as reactive oxygen species (ROS) [5]. NO interacts with superoxide anion and other free radicals to form highly reactive intermediates. NO and ROS can reciprocally enhance or abate effects of each other on cells depending on the ratio of their intracellular concentrations [6]. The ROS content in plant cells is under multilevel control of antioxidant enzymes. Different isoforms of the antioxidant system enzymes act in chloroplasts, mitochondria, cytosol, and peroxisomes, in which ROS can be produced in the course of redox reactions. Enzymes catalyzing these reactions are also found in the multicomponent compartment apoplast [7]. Unlike closely studied intracellular system of antioxidant enzymes, those protecting apoplast against ROS are far less understood despite the important role of apoplast in both metabolism and signal transduction [8]. A direct injection of putative NO precursors into the intercellular space might lead to overproduction of nitric oxide with development of nitrosative stress and alteration in the redox state of apoplast.

Our goal in this work was to explore formation of NO and H_2O_2, as well as alterations in activity of extracellular superoxide dismutase (SOD) and peroxidases, in response to introduction of high concentrations of nitrate and nitrite into wheat leaf apoplast.

MATERIALS AND METHODS

Winter wheat (*Triticum aestivum* L.) variety Dar Zernograda seedlings were grown for eight days in culture boxes at 23°C under a half-day light regime (12 h light/12 h dark) with energy flow of 100 W/m^2.

Leaves were cut off under water and infiltrated either with 50-mM potassium nitrate, 25-mM calcium nitrate, 50-mM potassium nitrite, or 5-mM sodium nitroprusside for 15 min under a pressure of 80 kPa. Leaves infiltrated with distilled water were used as control. All solutions were

Abbreviations: DETC, sodium diethyldithiocarbamate; NR, nitrate reductase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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adjusted to pH 6.0. The contents of apoplast were extracted by centrifugation for 10 min at 4°C and 600*g* (r_{av} = 6 cm).

Nitric oxide was determined by electron spin resonance (ESR) spectrometry using the mixture of sodium diethyldithiocarbamate (DETC) (0.5 mg/g), ferrous sulfate (0.0375 mg/g), and sodium citrate (0.1875 mg/g) as a spin probe [9]. Interaction of DETC with iron (II) cation results in formation of water-insoluble complex that, when interacting with NO, forms the stable $(DETC)_2$–Fe^{2+}–NO complex detectable by ESR. Following infiltration with nitrate or nitrite solution, the leaves (100 mg) were homogenized in the presence of the spin probe and immediately frozen in liquid nitrogen. The ESR spectra of thus prepared samples were recorded on a Bruker ER-200E-SRS X-range spectrometer (Germany) at 77°K in a double resonator together with the reference sample. Relative amount of $(DETC)_2$–Fe^{2+}–NO was estimated from integral intensity of the ESR signal [10].

Both H_2O_2 contents and antioxidant enzymes were determined in the apoplastic fluid. Hydrogen peroxide was determined using xylenol orange by optical densitometry at 560 nm [11]. Total SOD (EC 1.15.1.1) activity was determined from inhibition of photochemical reduction of nitroblue tetrazolium in the presence of riboflavin and methionine. Optical density at 560 nm was recorded. One unit of SOD activity was defined as the amount of the enzyme causing a 50% decrease in the rate of nitroblue tetrazolium reduction [12]. SOD activity was expressed in arbitrary units/min per mg protein. Activity of extracellular peroxidases (EC 1.11.1.7) was determined from increase in optical density at 590 nm in reaction mixture composed of equal volumes of 0.2 M acetate buffer (pH 5.2), 5 mM benzidine, 90 mM H_2O_2, and the examined apoplastic fluid. Reaction was initiated by addition of H_2O_2. The reaction mixture in which H_2O_2 was replaced by water served as control. Peroxidase activity was calculated according to the method of Boyarkin [13]. The apoplastic fluid samples were evaluated for contamination by cytosolic proteins by measuring the enzymatic activity of an intracellular marker (glucose-6-phosphate dehydrogenase) [14]. Protein was determined by Bradford's method [15].

The apoplastic fluid proteins were separated by nondenaturing PAGE according to the modified (without SDS and 2-mercaptoethanol) Laemmli's protocol [16] in a vertical 6-16% polyacrylamide gel followed by staining with silver nitrate [17]. Isoperoxidases were detected by staining of the gel with 0.05 M benzidine and 30 mM hydrogen peroxide in 0.2 M acetate buffer, pH 5.2 [18]. SOD was visualized by incubation of the gels in 50-mM Tris-HCl buffer, pH 8.0, containing 0.2 mM nitroblue tetrazolium, 5 mM EDTA, and 15 μM riboflavin. Subsequently, the gels were irradiated using daylight fluorescent lamps (total power 40 W) until bands appeared. Protein load was 0.45 μg of peroxidases or 2 μg of SOD per lane.

Each sample was replicated thrice, and each experiment was replicated 4-5 times. The data were statistically processed using Student's *t*-test (with the check for normal distribution of variance). Data are presented as the means and standard errors of the mean. The difference was taken as significant when $P < 0.01$.

The following chemicals were used: $FeSO_4 \cdot 7H_2O$, xylenol orange, nitroblue tetrazolium, and N,N,N′,N′-tetramethylethylenediamine from Sigma (USA); glycine and acrylamide from Panreac (Spain); EDTA from Fluka (Germany); Coomassie blue G-250, glycylglycine, NADP, and riboflavin from AppliChem (Germany). Other chemicals of chemical or analytical purity grade were produced in Russia.

RESULTS AND DISCUSSION

The ESR spectra of the paramagnetic $(DETC)_2$–Fe^{2+}–NO complex in leaf specimens demonstrated a characteristic hyperfine triplet pattern with g-factor 2.038 that is determined by an unpaired electron (with spin S = 1/2) on the nitric oxide molecule. Analysis of these spectra revealed a substantial increase in NO production following the introduction of potassium nitrite into the apoplast: the intensity of signal from the $(DETC)_2$–Fe^{2+}–NO complex increased by 183% in comparison with control (Fig. 1). In contrast, nitrate caused only slight (by 18%) elevation of the integral ESR signal. One of the enzymes implicated in NO production is NAD(P)H: nitrate reductase (NR) (EC 1.7.1.2), which can also act as nitrite reductase [19-21]. The nitrite/nitrate ratio and

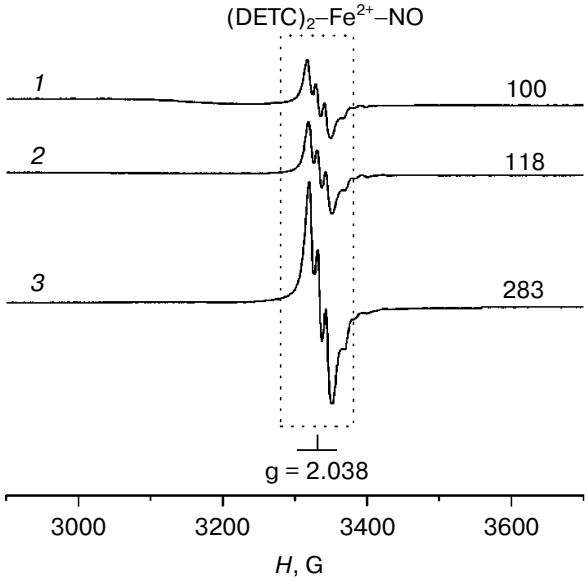

Fig. 1. ESR signal of mononitrosyl complexes of iron with diethyldithiocarbamate in wheat seedling leaf tissues infiltrated with distilled water (*1*), nitrate (50 mM) (*2*), and nitrite (50 mM) (*3*). Integral intensities of signals (in percent) are presented on the right.

overall (nitrite + nitrate) level are key determinants of NR-induced NO production in plant tissue [22]. In our experiments, 15-min action of nitrate might be insufficient for generation of significant amounts of NO because a considerable NO production delay was observed when nitrate instead of nitrite was used as the substrate [23].

Other NO sources in the plant cell are proteins similar to animal NO synthases involved in conversion of L-arginine to L-citrulline and nitric oxide [20, 24]. A plasmalemma-bound root-specific nitrite:NO oxidoreductase is another protein implicated in NO production. Unlike nitrate reductase, it uses cytochrome *c* as electron donor *in vitro*, and its pH optimum is more acidic [25]. Recent data suggest involvement of organelles in NO production. Planchet and coworkers [26] demonstrated that tobacco mitochondria form NO from nitrite, whereas soybean chloroplasts were able to use both arginine and nitrite for NO formation [24].

Under the conditions of our experiment, nitric oxide produced within the cell can go out to the intercellular space due to diffusion across the outer lipid membrane (plasmalemma). Finally, NO_2^- in the apoplast can undergo nonenzymatic reduction to NO. This reaction occurs only at low pH. In this aspect, apoplast is a preferential compartment for NO formation [27]. In thale cress (*Arabidopsis thaliana*) plants infected by *Pseudomonas syringae*, NO was initially accumulated in the extracellular space and only then it appeared in the cytoplasm of surrounding cells [28].

Signaling functions of NO depend on a set of conditions such as the rates of NO production and diffusion and redox status of the cell. NO production can be accompanied by accumulation of superoxide anion or hydrogen peroxide [29]. Exogenous NO modulates the level of H_2O_2 in leguminous guard cells [30, 31] and corn mesophyll cells [32]. Nitric oxide can influence activities of SOD, catalase, ascorbate peroxidase, and glutathione peroxidase in leaves of various plants [32-34]. In turn, ROS can elevate NO level via its influence on activity of NO-producing enzymes [5, 33]. For instance, the H_2O_2-induced NO production was found in mung bean (*Phaseolus aureus*) leaf cells [35]. H_2O_2 causes accumulation of nitric oxide in guard cells of *Vicia faba* and *Arabidopsis* [21, 30]. Removal of H_2O_2 using antioxidants or NADPH-oxidase inhibitors prevented NO formation. ROS and NO can react with each other. Particularly, a reaction between NO and superoxide forms peroxynitrite ($ONOO^-$) [36].

The introduction of potassium and calcium nitrates or direct NO sources (potassium nitrite and sodium nitroprusside) into the intercellular space resulted in elevation of H_2O_2 level (most prominent with nitrite) in wheat seedling leaf apoplast (Fig. 2a). Elevation of hydrogen peroxide production following infiltration of apoplast with nitrate or nitrite is likely due to development of oxidative stress.

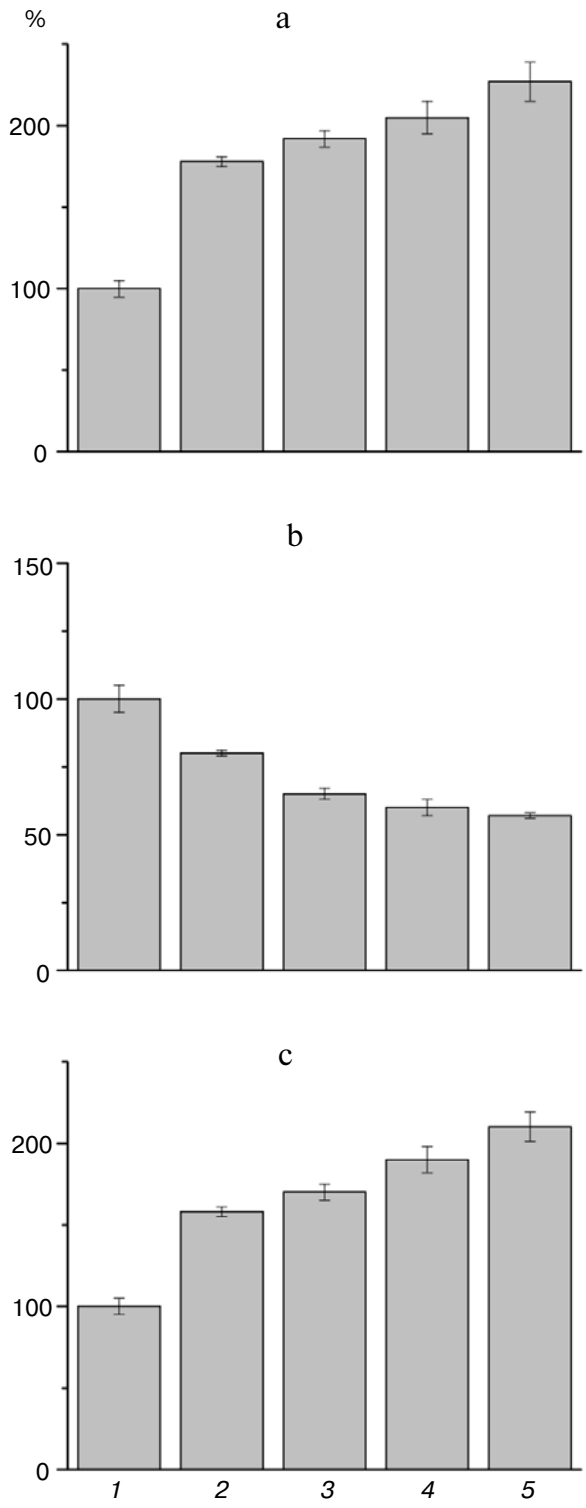

Fig. 2. Extracellular H_2O_2 level (a) and activities of SOD (b) and peroxidases (c) in apoplast of wheat seedling leaves infiltrated with nitrates or sodium nitrite or nitroprusside (% of control): *1*) H_2O; *2*) KNO_3 (50 mM); *3*) $Ca(NO_3)_2$ (25 mM); *4*) sodium nitroprusside (5 mM); *5*) KNO_2 (50 mM). Control samples contained 0.33 μmol of H_2O_2 per g wet weight, and SOD and peroxidase activities were 160 and 2530 arbitrary units/min per mg protein, respectively.

Enzymes implicated in H_2O_2 production in apoplast are cell wall peroxidases, amine oxidases, germin-like oxalate oxidases, and SOD [37].

The protein pattern obtained by electrophoretic separation of weakly bound cell wall proteins found in apoplast shows more than 20 components (Fig. 3a). Visualization of SOD using nitroblue tetrazolium revealed a single isoform with high electrophoretic mobility $m = 0.87$ (Fig. 3b). It is known that sensitivity of SOD to classical inhibitors depends on the metal atom in its catalytic center [38]. Mn-containing SODs are sensitive to azide, Fe-containing SODs to azide and hydrogen peroxide, and Cu/Zn-containing SODs to cyanide and hydrogen peroxide.

Preincubation of gels with SOD inhibitors, 3-mM H_2O_2 or 3-mM KCN, for 30 min led to complete inhibition of SOD activity in the gel, whereas 3-mM NaN_3 had no visible effect (Fig. 3b). Besides, incubation of apoplastic fluid with SOD inhibitors, 10-mM H_2O_2 or 10-mM KCN, for 60 min resulted in inhibition of SOD activity by 90 and 80%, respectively, and addition of 10-mM NaN_3 did not change the activity. These data suggest the main contribution of Cu,Zn-containing SOD to overall superoxide dismutase activity in the fraction of weakly bound cell wall proteins of apoplast. Earlier, extracellular Cu,Zn-SOD was isolated from spinach [39], pea [40], pine [41], and cotton [42].

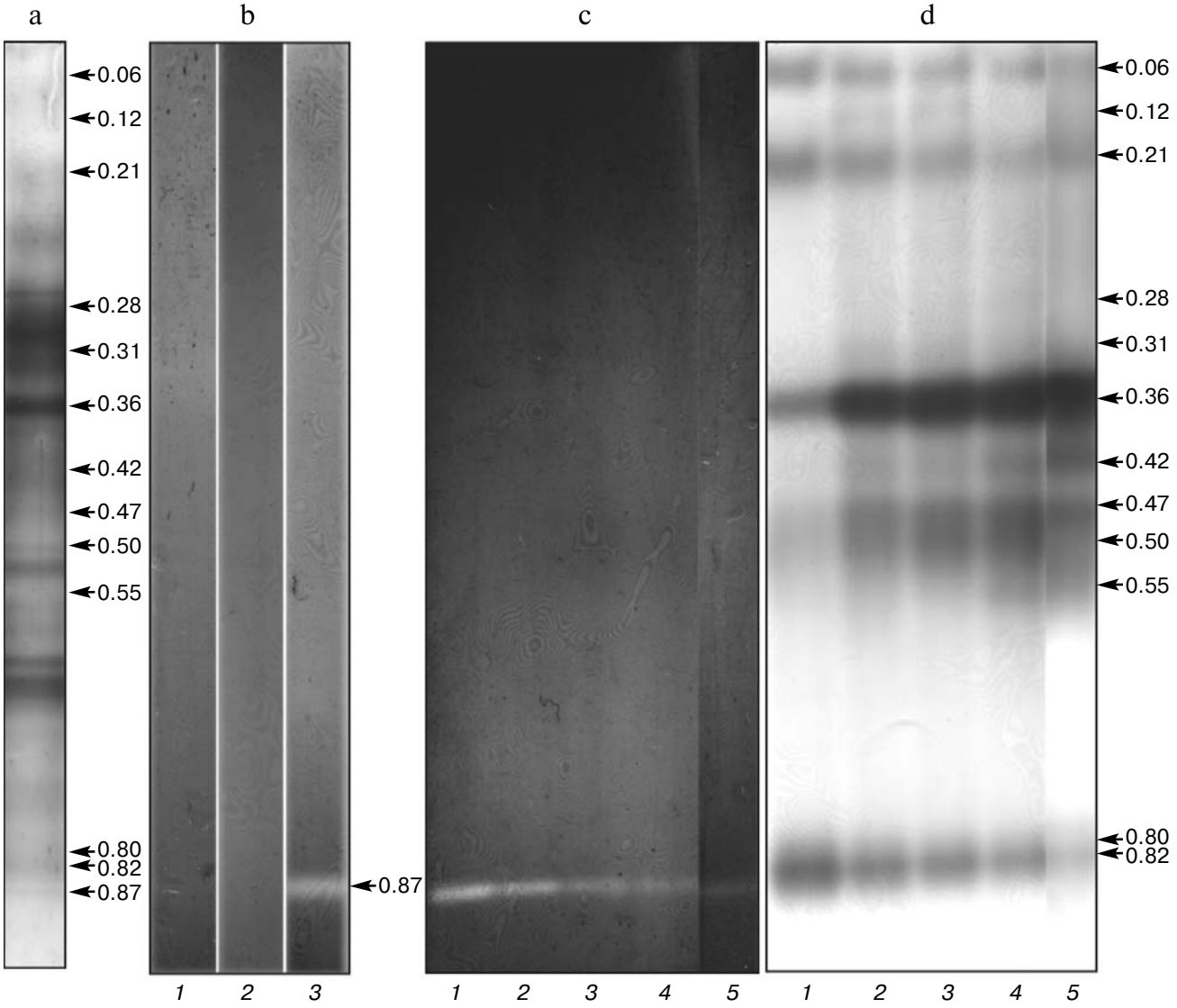

Fig. 3. Electrophoregrams of apoplastic proteins. a) Extracellular proteins stained with silver nitrate. b) Effect of inhibitors on activity of extracellular SOD. Lanes: *1*) H_2O_2; *2*) KCN; *3*) NaN_3. c, d) Isoenzymes of extracellular SOD and extracellular peroxidase, respectively. Lanes: *1*) H_2O; *2*) KNO_3 (50 mM); *3*) $Ca(NO_3)_2$ (25 mM); *4*) sodium nitroprusside (5 mM); *5*) KNO_2 (50 mM). Arrows indicate enzyme isoforms differing in electrophoretic mobility.

Introduction of nitrates and sodium nitrite and nitroprusside into the intercellular space resulted in inhibition of SOD activity in apoplastic fluid (Fig. 2b) with the most prominent effect of potassium nitrite and sodium nitroprusside. The tint of Cu,Zn-SOD stain in gel also decreased (Fig. 3c). Elevation of ROS level in cells can cause an increase in probability of oxidative modification of some proteins, including antioxidant enzymes. Data have been reported on inhibition of SOD activity by hydrogen peroxide [43, 44] and peroxynitrite [45]. According to some authors' conclusion, the elevated level of H_2O_2 leads to protein carbonylation, thus resulting in Cu,Zn-SOD inactivation [46].

High level of H_2O_2 in conjunction with low activity of SOD that is observed after introduction of NO sources into apoplast might be mediated by activity of other proteins generating or eliminating hydrogen peroxide. In particular, nitrite causes a significant decrease in catalase activity *in vivo* [47]. Inhibition of catalase might result from nitrosation of the enzyme due to protonation of nitrite anions – either in compartments with lowered pH or within the enzyme itself with participation of proton-donating amino acid residues [48]. Data exist indicating that nitrosonium cations appear in catalase molecules contacting with nitrite at high concentration of the latter (10^{-2}-10^{-3} M) [49]. Inhibition of catalase activity was also found in tissues treated with exogenous NO [50].

Peroxidases, which are commonly looked upon as antioxidants protecting plant cells affected by various stress factors against devastating effect of H_2O_2, are also implicated in maintenance of hydrogen peroxide balance in the cell. Peroxidase can serve as a source of ROS, thus demonstrating oxidase activity [51]. Any of the tested solutions, when introduced into leaf apoplast, led to elevation of peroxidase activity (Fig. 2c). The elevation of enzymatic activity was most prominent under the action of nitrite. Since plasmatic contamination was less than 0.3%, we suppose that changes in the level of extracellular peroxidase activities are not due to egress of the enzymes from cells during extraction of apoplastic fluid. The drastic increase in their activity seems to be purposive for elimination of H_2O_2 excess. Moreover, nitric oxide can serve as a cofactor substrate for peroxidase [52].

Peroxidases are characterized by a variety of molecular forms differing in physical and chemical features, localization, etc. Many authors believe that peroxidase is a family of genetically dissimilar enzymes differing in function *in vivo*. The pattern of extracellular peroxidases shows three zones of enzyme activity (Fig. 3d). The first had three components with low electrophoretic mobility, the second had seven components with moderate electrophoretic mobility, and the most mobile third zone had two components. Isoforms with $m = 0.06$, 0.21, 0.80, and 0.82 were characterized by rather high peroxidase activity in low contents.

Infiltration of leaves with nitrates did not lead to change in the extracellular peroxidase pattern composition, but it resulted in significant increase in activity of four isoforms with $m = 0.36$, 0.42, 0.47, 0.50 and decrease in activity of four isoforms with $m = 0.06$, 0.21, 0.80, 0.82 (Fig. 3d). Sodium nitroprusside and potassium nitrite caused additional increase in activity of four isoforms with $m = 0.36$, 0.42, 0.47, 0.50, and 0.55. Isoforms with $m = 0.36$, 0.47, and 0.50 the most substantially contributed to increase in overall peroxidase activity following the introduction of the examined substances into apoplast.

Free-radical processes proceeding in the intercellular space can alter pH of the medium, thus causing changes in activity of enzymes in apoplast [51]. It should be taken into account that one possible mechanism of nitrate action is shifting of extracellular pH due to reduction of nitrate anion [53]. Change in pH determines a balance between peroxidase and oxidase activities of peroxidases. For instance, cell wall peroxidase producing hydrogen peroxide at neutral pH restored its peroxidase function when surrounding pH is lowered [54]. Alkalinization of apoplast was necessary for drastic increase in H_2O_2 production by peroxidase in suspension-cultured cells of French bean (*Phaseolus vulgaris*) [55]. Data were also reported on inactivation of SOD even at slight (to 6.0) decrease in pH [56].

Thus, our data suggest accumulation of hydrogen peroxide and change in activity of antioxidant enzymes in apoplast following introduction of nitrates, sodium nitrite, or NO donor sodium nitroprusside into the intercellular space of the leaf. A considerable enhancement of NO production following the introduction of nitrite into apoplast was demonstrated by the ESR assay. Nonetheless, it is worth noting that the results of these experiments cannot exclude the possibility of NO production from nitrates.

Mechanisms of separate and joint action of nitric oxide and hydrogen peroxide in the plant cell are not yet completely understood. However, coincidence of specific cellular responses to these signaling molecules implies interference between NO and ROS. This view is supported by presence of identical intracellular targets, namely proteins containing accessible heme, thiol, and iron-sulfur (Fe-S) groups. It is likely that NO and ROS directly influence transcription factors via oxidation (H_2O_2) or *S*-nitrosation (NO) of cysteine residues. Accessibility of a group for corresponding modification depends on levels of competing ROS and NO that influence regulatory systems mediated by G-proteins, cGMP, and MAP-kinases.

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