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RESEARCH PAPERS

Stress Responses of Wheat Leaves to Dehydration: Participation of Endogenous NO and Effect of Sodium Nitroprusside

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Abstract—Dynamics of endogenous NO in the leaves of 7-day-old seedlings of spring wheat (*Triticum aestivum* L., cv. Debyut) and the effect of exogenous NO donor (sodium nitroprusside, SNP) on the development of oxidative stress and activity of antioxidant enzymes in the leaves under water deficiency were investigated. Quick and phasic accumulation of NO in the leaves was observed under growing dehydration (0–3 h) and subsequent rehydration (0–3 h), which points to identical response of NO signal system to opposite changes in the water status of plants. A decrease in relative turgidity of tissues brought about accumulation of H_2O_2 and MDA therein. Protective effect of NO donor infiltrated in the leaves was associated with an elevation of ascorbate peroxidase and catalase activities and suppression of lipid peroxidation upon dehydration. Pretreatment with SNP (50–250 μM) induced the elevation of NO in the leaves both before action of the stress agent and in the beginning (0–30 min) of dehydration. The obtained results suggest that brief increase in endogenous NO is necessary for triggering protective and adaptive responses in wheat plants during the development of water deficiency.

Key words: Triticum aestivum, dehydration, NO, sodium nitroprusside, infiltration, oxidative stress, antioxidant enzymes.

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INTRODUCTION

Investigations of biological role of nitric oxide (NO) in plants exposed to biotic and abiotic stresses started in the 1990s are currently under way [1]. These investigations became more active when universal regulatory function of NO in the cell metabolism was ascertained. Changes induced by NO in plant cells and tissues are often observed when specific donors of nitric oxide, in particular sodium nitroprusside (SNP), are used. For instance, in different plant species exposed to salinity [2], heavy metals [3], hypothermia [4], overheating [5], UV radiation, and osmotica [6], protective properties of exogenous donor of NO were shown.

However, the data concerning the participation of NO as a signal compound in plant response to water deficiency are scanty. At the same time, drought is one of the common adverse environmental factors considerably reducing crop productivity [7]. In one of the first works dealing with the role of NO in the regulation of plant water metabolism, it was shown that SNP induced an elevation of water-retaining capacity of wheat leaf tissues [8]. These effects were accounted for by the participation of NO in the regulation of the plant stomatal apparatus and activation of dehydrin gene expression, i.e., the genes of osmoprotective proteins. Together with the maintenance of the water status during drought simulated with polyethylene glycol (PEG), exogenous NO is capable of reducing the content of hydrogen peroxide and the products of lipid peroxidation (POL) [6]. Prevention of oxidative injury of plant cells involving NO is realized through the system of antioxidant protection [1]. It was shown that, when plants were treated with NO donors, antioxidant enzymes became more active and expression of respective genes and de novo synthesis of the enzymes were boosted [9, 10]. In some works, along with the elucidation of the influence of NO on the development of oxidative stress and changes in the activity of antioxidant enzymes in plants, dynamics of NO during PEG-induced drought was monitored [11]. At the same time, investigation of changes in the NO level in tissues after plant pretreatment with NO donor could contribute to understanding the development of protective and adaptive responses associated with dehydration.

In this relation, the aim of this investigation was to look into participation of endogenous nitric oxide in stress response of the leaves of spring wheat to growing dehydration and subsequent rehydration and also to elucidate the effect of NO donor on the development

Abbreviations: APO—ascorbate peroxidase, CAT—catalase, POL—peroxidation of lipids; RWC—relative water content, SNP—sodium nitroprusside.

of oxidative stress and changes in the activity of antioxidant enzymes in plant tissues under water deficit.

MATERIALS AND METHODS

Plant material. Investigation was conducted with the leaves of 7-day-old seedlings of spring wheat (*Triticum aestivum* L., cv. Debyut) grown on tap water at 25/18°C and a 12-h photoperiod. In the experiment, we used middle parts of leaf blades. In order to remove wound stress, they were preincubated in distilled water for 2 h. To investigate the effects of NO donor, the leaves vacuum infiltrated with 25–500 μM SNP (Sigma, United States) or distilled water for 15 min and incubated for 1 h in the same solutions in the dark. Then the leaves were twice washed in distilled water, blotted with filter paper, and dehydrated. Dehydration was achieved by means of drying (from 5 min to 3 h) the leaves spread on white paper in the Biotron-3 growth chamber at 26–28°C and an illuminance of 5 klx. When the exposure was over, some leaves were rehydrated in test tubes with distilled water.

Relative water content (RWC) in the leaves was determined according to the formula: RWC = FW – DW)/(TW – DW) × 100%, where TW—fresh weight of turgescent leaves, FW—fresh weight of withered leaves, DW—dry weight of the leaves determined after their drying at 105°C. The state of turgor was attained by placing detached leaves for 2 h in water.

The content of NO in the leaves was determined with Griss reagent [9]. The sample of leaves (0.25 g) was ground with 5 ml of cooled 50 mM acetate buffer (pH 3.6) containing 4% $Zn(CH_3COO)_2 \times 2H_2O$. After centrifugation for 15 min at 8000 rpm and 4°C, the supernatant was supplemented with 0.1 g of charcoal, stirred, and passed through a paper filter. The obtained filtrate (1 ml) was mixed with 1 ml of Griss reagent, kept at room temperature for 30 min, and optical density was measured at 540 nm with water as a blank solution. The level of NO was calculated using calibration solutions of $NaNO_2$ in acetate buffer and expressed in μmol/g dry wt of the leaf. Estimation of the NO level by the content of nitrite anion showed a good comparability with other methods for determination of nitric oxide in plants (hemoglobin and arginine-dependent synthesis of NO with the formation of citrulline) [12].

H_2O_2 in the leaves was determined according to Gay and Gebicki [13]. The sample of leaves (0.25 g) was ground in ice-cold 50 mM borate buffer (pH 8.4): supplemented with 12.5 mM $Na_2B_4O_7 \times 10H_2O$. The homogenate was centrifuged for 10 min at 8000 rpm. The reaction mixture containing the supernatant, 25 mM $FeSO_4$, 25 mM $(NH_4)_2SO_4$, 25 mM H_2SO_4, 100 mM sorbitol, and 125 mM Xylenol Orange (Sigma) was incubated for 15–30 min at room temperature, and optical density was measured spectrophotometrically at 560 nm. The concentration of hydrogen peroxide was determined using calibration solutions of H_2O_2 and expressed in μmol/g dry wt.

POL determination. A sample of leaves (0.2 g) was ground with 4 ml of 0.1% TCA. The homogenate was centrifuged for 10 min at 8000 rpm. The supernatant (1 ml) was mixed with 4 ml of 20% TCA containing 0.5% 2-thiobarbituric acid. The mixture was heated for 30 min on a water bath to 95°C, cooled on ice, and centrifuged again. Optical density of the supernatant was measured at 532 and 600 nm. The content of MDA (μmol/g dry wt) was calculated using a coefficient of molar extinction (ε) equal to 155/(mM cm) after subtraction of nonspecific absorption at 600 nm [14].

Assays of antioxidant enzyme activities. A sample of leaves (0.2 g) was ground at 4°C in 4 ml of 50 mM Tris–HCl buffer (pH 7.8) containing 0.1 mM EDTA, 5% polyvinylpyrrolidone (PVP), and 10% sorbitol. The extract was filtered through two layers of capron tissue and centrifuged for 30 min at 8000 rpm. The content of protein in the enzyme extracts was assessed according to Lowry method modified by Hartree [15]. The activity of ascorbate peroxidase (APO, EC 1.11.1.11) was determined by a reduction in light absorption ($\lambda = 290$ nm) by the reaction mixture during oxidation of 1 mM ascorbate for 1 min [16] and expressed in μmol of oxidized ascorbic acid/(mg protein min), $\varepsilon = 2.8$/(mM cm). Activity of catalase (CAT, EC 1.11.1.6) was determined by a reduction in light absorption by reaction mixture for 2 min at 240 nm induced by the breakdown of 50 mM H_2O_2 and expressed in μmol H_2O_2/(mg protein min), $\varepsilon = 39.4$/(mM cm) [17].

Statistics. The obtained results were treated statistically. Figures show the means and their standard errors. Significance of differences between the means was estimated by Student's *t*-test at $P \le 0.05$.

RESULTS

When the excised wheat leaves were slightly dried, RWC therein progressively decreased (Fig. 1). The quickest decrease in relative turgidity (by 26.2%) occurred during the first hour of dehydration. After 3-h-long dehydration, the leaves lost about one half of their initial fresh weight, and RWC decreased to 51.6% (Fig. 1a). Upon leaf rehydration, RWC rapidly restored its initial level, which points to restoration of normal water status by the leaf tissues.

In the course of dehydration, we observed a short-term rise in the NO level in wheat leaves (Fig. 1a). Subsequent rehydration of dehydrated leaves was also associated with a rise in the NO level. The greatest increase in the content of NO (up to 2 times) fell at the first and second hours of both dehydration and rehydration. The content of nitric oxide in the leaves rose as early as in 15 min of dehydration and occurred after a quick decrease of RWC in the leaves (Fig. 1b).

In dehydrated leaves, we observed accumulation of H_2O_2 and MDA, which points to the development of

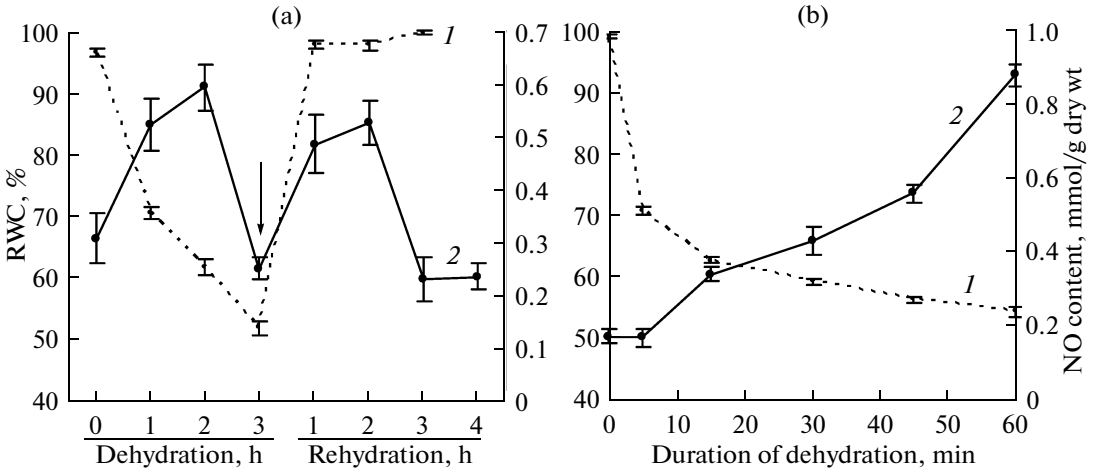

Fig. 1. Dynamics of RWC (*1*) and NO (*2*) in wheat leaves during dehydration and rehydration (a) and within the first hour of dehydration (b).
Arrow shows the start of rehydration.

oxidative stress and POL intensification (Fig. 2). After 3 h of dehydration, the contents of H_2O_2 and MDA in the leaf tissues reached the peak values exceeding their initial levels by 2.70 and 1.25 times, respectively (Fig. 2). After rehydration during 1 h, the content of H_2O_2 decreased to the control level, whereas the content of MDA did not change. There was a strong negative correlation between changes in RWC and H_2O_2 in the leaves ($r = -0.91$).

Leaf infiltration with SNP brought about a considerable decrease in MDA accumulation during subsequent dehydration. In the leaves infiltrated with water, MDA level after 3-h-long dehydration increased by 37%, whereas SNP (25–250 μM) brought about an increase by only 11–24% over control level (Fig. 3). The greatest suppressing effect on POL was exerted by 50 μM SNP that inhibited accumulation of MDA by

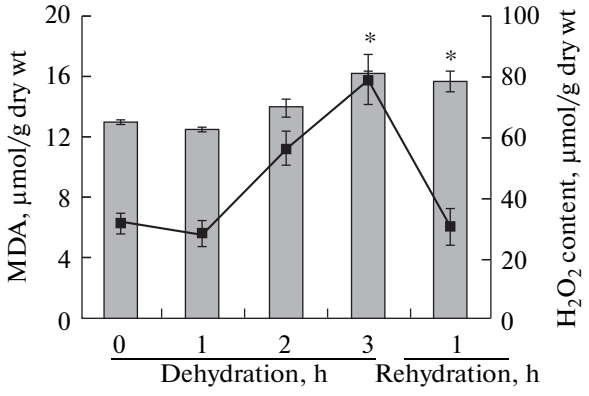

Fig. 2. Dynamics of H_2O_2 (line) and MDA (columns) in wheat leaves during dehydration and rehydration.
Asterisks show differences relative to control level (0 h) reliable at $P \le 0.05$.

30% as compared with the leaves infiltrated with distilled water.

In the leaves infiltrated with water,, APO activity decreased under the effect of dehydration, whereas in the leaves pretreated with SNP, the enzyme activity increased (at 50 μM SNP) or did not change (at 100 and 250 μM SNP) (Fig. 4a). Activity of another antioxidant enzyme (CAT) changed similarly with a slight decrease in response to dehydration and increase by 35, 25, and 42% after leaf treatment with 50,100, and 250 μM SNP, respectively (Fig. 4b).

Infiltration of wheat leaves with SNP solutions (50, 100, and 250 μM) induced therein an increase in the content of NO as compared with control leaves (Fig. 5). After 1-h-long dehydration, we did not find any reliable change in the level of NO in the leaves of wheat of all the types of treatment (Fig. 5). When the time-course of NO content in control leaves was investigated, we found a slight increase in NO level in 30 min and a considerable rise in 3 h of dehydration (Fig. 6). Pretreatment of leaves with 50 μM SNP reliably increased both the initial level of NO (1.3 times) and NO content in 15 and 30 min of dehydration (2.1 and 1.4 times, respectively). Thus, exogenous donor of nitric oxide induced a brief rise in the level of NO in the leaf tissues before the effect of the stress agent and in the course of dehydration.

DISCUSSION

It was found that wheat leaves responded to dehydration and rehydration by a short-term phasic accumulation of endogenous NO (Fig. 1a). The elevation in the content of NO in the leaves detected as early as after 15 min of dehydration occurred after a sharp decrease in RWC during the first 5 min of wilting; a subsequent decrease in relative turgidity was slower

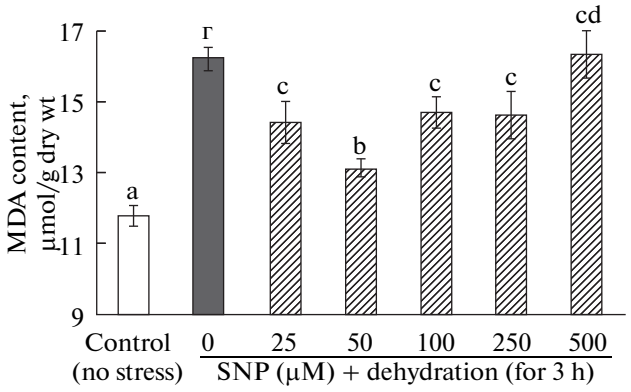

Fig. 3. Effect of SNP on changes in POL in wheat leaves after dehydration (for 3 h).
Different letters designate reliable differences between the treatments ($P \le 0.05$).

(Fig. 1b). As a result, during 3 h of withering in the air, the leaves lost about one half of the initial RWC, which points to deep dehydration (Fig. 1a). A decrease in RWC in plant tissues is one of the main indications of drought [18]. However, restoration of the initial level of RWC in the course of subsequent rehydration points to preservation of viability of plant tissues and cells.

We believe that reduction in RWC preceding the rise in NO level indicates that the loss of leaf turgor can cause an increase in the NO content therein. The obtained results point to a signal role of NO in the leaf cells associated with growing dehydration. Quick transient accumulation of endogenous NO has been earlier shown in the cells of tobacco suspension culture treated with 250 mM sorbitol [19] and in the leaves of maize under the effect of 10% PEG [11]. At the same time, under the influence of hyperthermia and salinity, the elevation of NO was steady: its level increased and remained high during the entire period of exposure to the stress agent [19]. In the maize leaves, the accumulation of NO under the effect of PEG occurred in the mesophyll cells [10, 11]. It was found that NO production of under the osmotic stress depended on the activity of two main NO-producing enzymes: NO-synthase and nitrate reductase [11]. Apparently, in all these cases endogenous NO acts as a signal molecule (mediator of protective and adaptive physiological and metabolic plant responses). It is known that one of these responses may be NO-dependent stomatal closure [20]. In our case, a reduction in the rate of water loss by the leaves in 15 min of dehydration also indirectly points to stomatal response to water deficit (Fig. 1b). It is interesting that changes in the NO content in the leaves are identical in response to opposite processes (loss and uptake of water by the leaves) (Fig. 1a). The obtained results suggest that dehydration and rehydration of the leaves are associated with NO-dependent triggering the mechanisms of adaptation to sharply changing water content in the tissues.

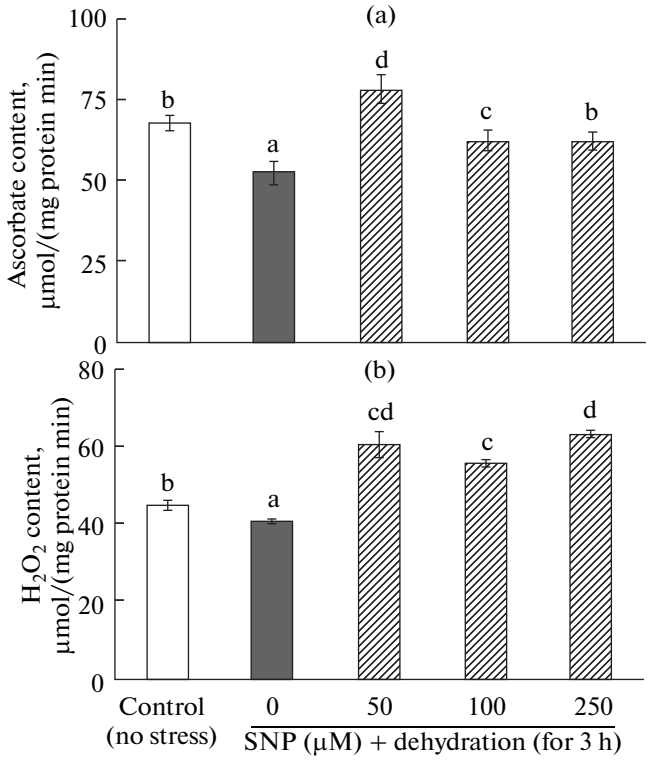

Fig. 4. Effect of NO donor on activity of APO (a) and CATt (b) in the wheat leaves after dehydration (for 3 h).
Different letters designate reliable differences between the types of treatment ($P \le 0.05$).

One of the universal nonspecific responses of plant organism to dehydration is ROS accumulation in the cells [21]. In our experiments, the accumulation of H_2O_2in dehydrated wheat leaves also points to the development of oxidative stress (Fig. 2). Detected strong correlation between RWC and H_2O_2 in the leaves points to the accumulation of H_2O_2 in plant cells induced by dehydration. It is worth noting that, in addition to adverse effects, the accumulation of hydrogen peroxide also acted as a signal mediating adaptive responses of the leaves to increasing water deficit. It is known that H_2O_2 as a signal molecule participates in the regulation of stomatal movements and expression of defense genes [1, 20]. In our experiments, we found dissimilar dynamics of H_2O_2 and NO in the leaves during dehydration (Figs. 1, 2). Sang et al. [11] also showed that gradual accumulation of H_2O_2 in maize leaves under drought did not correspond to phasic changes in NO level therein. The effect of other stress factors may cause a steady elevation of NO in the tissues [19].

Along with H_2O_2, MDA is one of the main markers of oxidative stress [18, 21]. It has been shown earlier that dehydration of excised wheat leaves is accompanied by activation of electrolyte leakage, which is a consequence of oxidative damage of the membranes [8]. MDA accumulation was shown in the course of

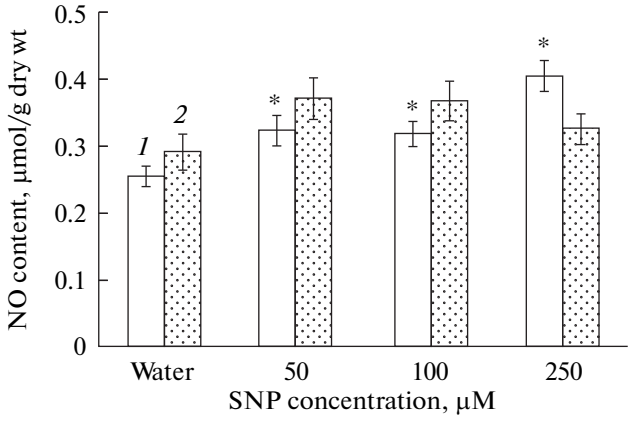

Fig. 5. Effect of SNP on the NO content in the wheat leaves before (*1*) and after (*2*) dehydration (for 1 h).
Asterisks show differences relative to control level (water) reliable at $P \le 0.05$.

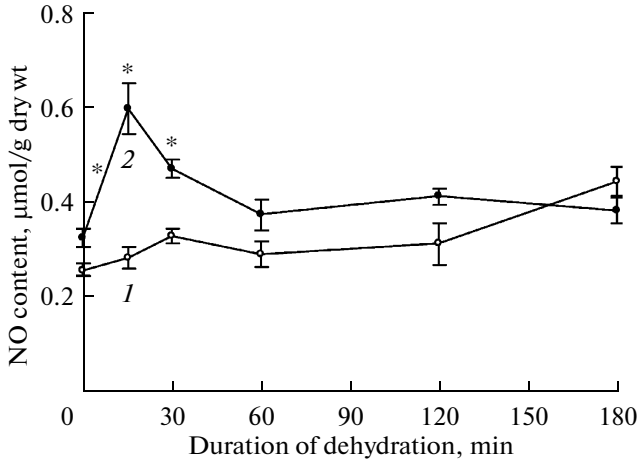

Fig. 6. Dynamics of NO in wheat leaves infiltrated with water (*1*) and 50 μM SNP (*2*) in the course of dehydration (for 3 h).
Asterisks show differences relative to control level (water) reliable at $P \le 0.05$.

drying the leaves of moss *Tortula ruralls* in the air [22]. According to our results, POL occurred simultaneously with the accumulation of H_2O_2 in the leaves (Fig. 2). Suppression of MDA accumulation in wheat leaves pretreated with SNP points to a protective effect of NO donor on plant tissues under water deficit (Fig. 3). Garcia-Mata and Lamattina [8] have also shown an antioxidant effect of SNP on wheat leaves upon dehydration. In another work, exogenous SNP reduced POL intensity in wheat shoots exposed to PEG or PEG combined with UV-B radiation [6]. In addition to drought, antioxidant effect of nitric oxide donors was observed upon the exposure of plants to other abiotic stresses: heavy metals [3], salinity [2], and overheating [5, 23].

It is known that one of the mechanisms of NO donor protective effect is activation of antioxidant enzymes [1]. In our experiments, exogenous SNP simultaneously with POL suppression upon dehydration activated APO and CAT responsible for H_2O_2 utilization in the cell (Figs. 3, 4). Literature data suggest that NO is capable of efficient stimulating the activities of the major antioxidant enzymes, e.g., APO, CAT, and SOD under normal conditions [9] and upon the effect of stress agents [3, 11]. Therefore, protective effect of NO on cell membranes may depend on activation of antioxidant enzymes.

Physiological effects of exogenous NO donors on plants as a rule are accompanied by an elevation in the NO content in plant tissues [2, 24]. According to our results, wheat leaves infiltrated with SNP have much higher NO levels both under normal conditions and in the initial stages of dehydration (during 30 min) (Figs. 5, 6), which points to a release of NO from SNP and is a response to the stress agent causing the loss of leaf turgor. After pretreatment with SNP, the content of NO in the leaves rapidly increased during the first 15 min of the effect of stress agent. In the leaves of control material, such a peak was lacking, although it could be observed somewhat earlier in response to quicker reduction in RWC in the leaves. Earlier, it has been found that the content of NO in the cells of tobacco plants increased 3 times as early as 2 min after the effect of osmoticum and decreased to the permanent level 10 min later [18]. The lack of reliable differences in the content of NO between the control and experimental leaves after first, second, and third hours of dehydration is probably caused by a brief existence and high reactivity of NO molecules, which ensures their quick binding to intracellular physiological targets. It was found that NO molecule interacting with reactive oxygen radicals disturbs peroxidation of membrane lipids [6, 25], which suggests that exogenous NO is consumed in the reactions with ROS ($O^{\bullet -}$). Along with activation of antioxidant enzymes, this may be an additional reason for POL suppression in the leaves during dehydration. The obtained data show that exogenous NO donor induced a brief elevation in the NO content in leaf tissues and this is related to switching on adaptive reactions therein.

The results of our experiments suggest that endogenous NO, as a component of signal transduction pathway, directly participates in stress response and activation of protective and adaptive mechanisms in the leaves of spring wheat during dehydration. One can think that protective effect of exogenous NO donor on wheat leaves depends on NO-induced elevation of antioxidant enzyme activity and ROS binding with the molecules of nitric oxide.

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