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RESEARCH PAPERS

Functional Interaction of ROS and Nitric Oxide during Induction of Heat Resistance of Wheat Seedlings by Hydrogen Sulfide Donor

Yu. V. Karpets[a], Yu. E. Kolupaev[a], *, A. A. Lugovaya[a], N. V. Shvidenko[a], M. A. Shkliarevskyi[a], and T. O. Yastreb[a]

[a] *Dokuchaev National Agrarian University, Kharkiv, 62483 Ukraine*

**e-mail: plant.biology.knau@gmail.com*

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Abstract—The participation of reactive oxygen species (ROS) and nitric oxide (NO), and also enzymatic systems generating them, in the development of heat resistance of wheat (*Triticum aestivum* L.) seedlings, induced by the hydrogen sulfide (H_2S) donor sodium hydrosulfide (NaHS), has been studied. It was found that 24-h pretreatment of seedlings with 0.1–1 mM NaHS increased their survival after the subsequent 10-min damaging heating at 45°C. The content of hydrogen peroxide and nitric oxide increased together with the nitrate reductase (NR) activity in the seedling roots within the first 4 h of their treatment with the H_2S donor. The rise in the NO level significantly suppressed by the inhibitor of NR sodium tungstate but not the inhibitor of NO synthase (N^G-nitro-L-arginine methyl ester, L-NAME). The hydrogen peroxide scavenger dimethylthiourea (DMTU) and the NADPH oxidase inhibitor imidazole abolished the increase in NR activity and NO content in the roots. However, the nitric oxide scavenger (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, PTIO) and the inhibitors of the NO-producing enzymes only weakly influenced the increase in H_2O_2 content caused by the root treatment with sodium hydrosulfide. The NaHS-induced rise in the seedling heat resistance was eliminated by both ROS antagonists (DMTU and imidazole) and NO antagonists (PTIO and tungstate). It is concluded that the boost in the wheat seedling heat resistance, which is caused by exogenous hydrogen sulfide, is mediated by the increased ROS generation, followed by NR activation, and resultant rise in the level of nitric oxide produced by this enzyme.

Keywords: Triticum aestivum, hydrogen sulfide, reactive oxygen species, nitric oxide, nitrate reductase, heat resistance

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INTRODUCTION

Hydrogen sulfide is now recognized as one of the key gasotransmitters fulfilling regulatory functions in plants and animals [1]. It participates in the implementation of physiological effects of phytohormones, the control of plant growth processes, the transduction of stress signals, and plants' adaptation to adverse factors [2–4]. The rise in endogenous H_2S level in plants subjected to such stressors as dehydration [5], salinization [6], extreme temperatures [7], or different toxicants, including heavy metal ions [8, 9], was found. Some studies report on the induction of plant tolerance to stressors by externally applied donors of hydrogen sulfide [7, 10, 11].

Nevertheless, the signaling effects of hydrogen sulfide are not comprehended enough to formulate a more or less complete concept of their mechanism [1, 4]. Protein sulfhydration (persulfidation) is found to be one of the direct biochemical mechanisms of H_2S action [12]. Signaling effects of hydrogen sulfide are also implicated in their functional interactions with other mediators, in particular, ROS. For example, the rise in barley plant resistance to UV-B caused by a donor of H_2S is accompanied by hydrogen peroxide accumulation in the leaves [13]. These effects are eliminated by a hydrogen peroxide scavenger dimethylthiourea (DMTU) and NADPH oxidase inhibitor diphenyleneiodonium. The induction of heat resistance of wheat coleoptiles by treatment with NaHS is associated with the increased generation of the superoxide anion radical and hydrogen peroxide and is also abolished by DMTU and NADPH oxidase inhibitor imidazole [14].

Another signal mediator to which hydrogen sulfide is closely related is nitric oxide (NO) [15]. It was shown that both gasotransmitters may interact with the same protein targets causing either persulfidation or nitrosylation [4]. At the same time, NO and H_2S also affect the intracellular concentration of each other. It is suggested that, depending on the nature of

Abbreviations: DMTU—dimethylthiourea; L-NAME—N^G-nitro-L-arginine methyl ester; NR—nitrate reductase; PTIO—2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide.

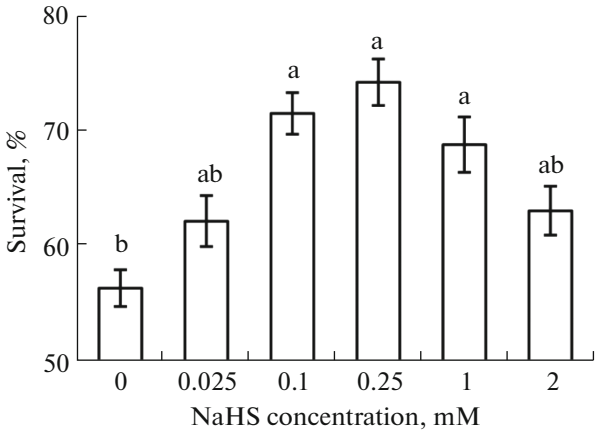

Fig. 1. Concentration dependence of effect of hydrogen sulfide donor on wheat seedling survival after damaging heating (10 min at 45°C). Equal letters designate means with insignificant (at $P \le 0.05$) differences.

a stressor and controlled processes, NO may act either upstream or downstream of hydrogen sulfide in signal transduction chains [4].

It was revealed that nitric oxide scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) eliminates a positive influence of the hydrogen sulfide donor NaHS on salt resistance and on gene expression of antioxidant enzymes in alfalfa plants [16]. Treatment of pea seedlings with sodium hydrosulfide increases their resistance to arsenic toxicity and elevates the level of endogenous nitric oxide [10]. The hydrogen sulfide donor alleviates the consequences of oxidative stress caused by 100 mM nitrate. Here, the H_2S donor elevates the content of endogenous NO in the roots, which is associated with the activation of nitrate reductase (NR) but not the animal NO synthase-like enzyme [17].

In general, the evidence is yet inadequate to clearly interpret the role of nitric oxide in transducing the hydrogen sulfide-related signals and in inducing plant resistance to stressors. In particular, the question is still open on NO involvement in the H_2S-induced plant resistance to heat stress. As already mentioned, the effects of H_2S on heat resistance are mediated by ROS [14]. It is known that tight functional relations exist between ROS and NO [18], including the case of the induction of plant heat resistance [19]. However, the functional interaction between hydrogen peroxide and nitric oxide as possible mediators in the realization of protective effects of hydrogen sulfide under the heat stress has almost not been studied.

The goal of the study was to reveal the sequence in which hydrogen peroxide and nitric oxide, as signal mediators, contribute to the processes of induction of the wheat seedling heat resistance afforded by the hydrogen sulfide donor NaHS. Another task was the ascertainment of NR's possible participation in the NO formation occurring under the influence of exogenous hydrogen sulfide.

MATERIALS AND METHODS

Four-day-old etiolated wheat (*Triticum aestivum* L.) seedlings of cv. Doskonala grown on purified tap water at 18–20°C were studied. The roots were incubated for 24 h in a medium to which the donor of H_2S (0.025–2 mM sodium hydrosulfide) was added. The optimal conditions of root exposure to this compound were preliminarily tested and chosen. In the experiments with the NO scavenger (0.1 mM PTIO), the NR inhibitor (5 mM Na_2WO_4), the inhibitor of NO synthase (5 mM N^G-nitro-L-arginine methyl ester, L-NAME), the antioxidant (0.15 mM DMTU), and the inhibitor of NADPH oxidase (0.01 mM imidazole), the roots were kept in these solutions for 26 h. The compounds were added to the incubation medium 2 h before NaHS if the NO or ROS antagonists should be combined with the H_2S donor. Concentrations at which the additions maximally modified the effects of hydrogen sulfide but were not toxic to the seedlings were preliminarily chosen.

To determine the heat resistance, the seedlings were subjected to 10-min damaging heating at 45.0 ± 0.1°C in a water ultrathermostat. After that, the seedlings of all samples were transferred to purified tap water, and their survival was estimated after 3 days [19].

The content of hydrogen peroxide and nitric oxide were analyzed in the roots since this organ is the most sensitive to exogenous substances [19]. The roots were homogenized in cold with 5% TCA followed by 10-min centrifugation at 8000 *g* and a temperature below 4°C in a MPW 350R centrifuge (MPW MedInstruments, Poland). The content of H_2O_2 was determined in the supernatant by a ferrothiocyanate method [20].

The content of nitric oxide was assayed with Griess reagent [21]; the method was modified by the authors [19]. The activity of NR (EC 1.7.1.1) was estimated in vitro by accumulation of the reaction product nitrite [22] according to the protocol reported earlier [23]. The enzymatic activity was expressed as nitrate nmol/(g fr wt/min).

The experiments were performed with four biological replications, which were independently repeated three times. Means and their SEs are reported in figures. Significance of differences was estimated by Student's *t*-test. The effects significant at $P \le 0.05$ are only considered unless otherwise stated.

RESULTS

Incubation of wheat seedlings in the solutions of hydrogen sulfide donor NaHS in concentrations of 0.1, 0.25 and 1 mM significantly increased their survival after the damaging heating (Fig. 1). There was no significant stimulation of the heat resistance at

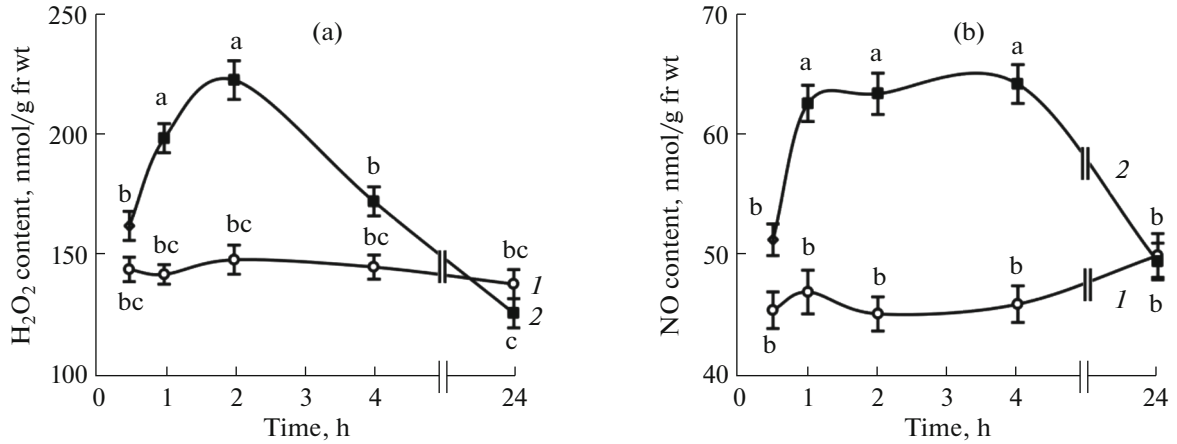

Fig. 2. Dynamics of content of hydrogen peroxide (a) and nitric oxide (b) in wheat seedling roots treated with hydrogen sulfide donor. (*1*) Untreated control; (*2*) treatment with 0.25 mM NaHS. Equal letters designate means with insignificant (at $P \le 0.05$) differences.

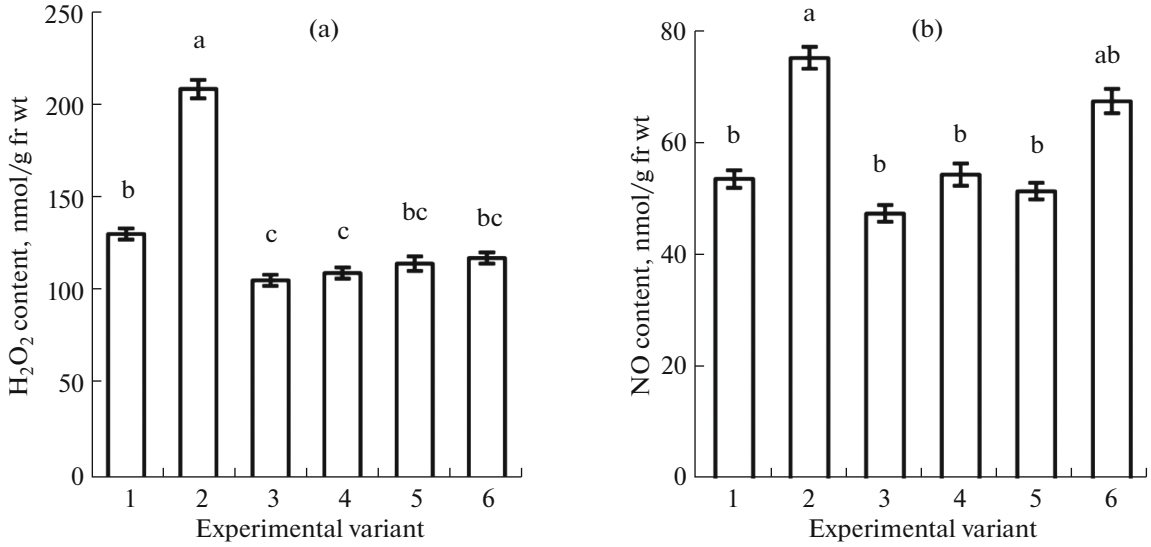

Fig. 3. Content of hydrogen peroxide (a) and nitric oxide (b) in wheat seedling roots treated with hydrogen sulfide donor combined (or not) with antagonists of ROS or NO. (a): (*1*) Untreated control; (*2*) 0.25 mM NaHS; (*3*) 0.15 mM DMTU; (*4*) 0.25 mM NaHS + 0.15 mM DMTU; (*5*) 0.01 mM imidazole; (*6*) 0.25 mM NaHS + 0.01 mM imidazole; (b) (*1*) Untreated control; (*2*) 0.25 mM NaHS; (*3*) 2 mM Na_2WO_4; (*4*) 0.25 mM NaHS + 2 mM Na_2WO_4; (*5*) 2 mM L-NAME; (*6*) 0.25 mM NaHS + 2 mM L-NAME. Equal letters designate means with insignificant (at $P \le 0.05$) differences. The analyses were performed 2 h after the start of the seedling treatment with NaHS and/or 4 h after the start of treatment with other indicated reagents.

0.025 or 2 mM NaHS. In the subsequent experiments, sodium hydrosulfide was applied at 0.25 mM, since this concentration was the most heat-protective.

The content of endogenous hydrogen peroxide significantly increased in the seedling roots in 1 h after the start of their treatment with the hydrogen sulfide donor (Fig. 2a). In 2 h, the index attained the maximal value, which was 1.5 times higher than the control. Then it progressively decreased and reached the control level by 24 h.

The content of nitric oxide also manifested an increase in the NaHS-treated roots that was also transient (Fig. 2b). The augmentation above the control was the most pronounced within 1–4 h of the treatment and disappeared by 24 h.

Seedling treatment with the scavenger of hydrogen peroxide (DMTU) slightly decreased the peroxide content in the control roots (Fig. 3a). Furthermore, DMTU abolished the rise in the H_2O_2 content initiated by the H_2S donor. As well, the NADPH oxidase inhibitor (imidazole) completely prevented the NaHS-stimulated accumulation of H_2O_2 pointing to the involvement of this enzyme in the H_2S-induced ROS generation.

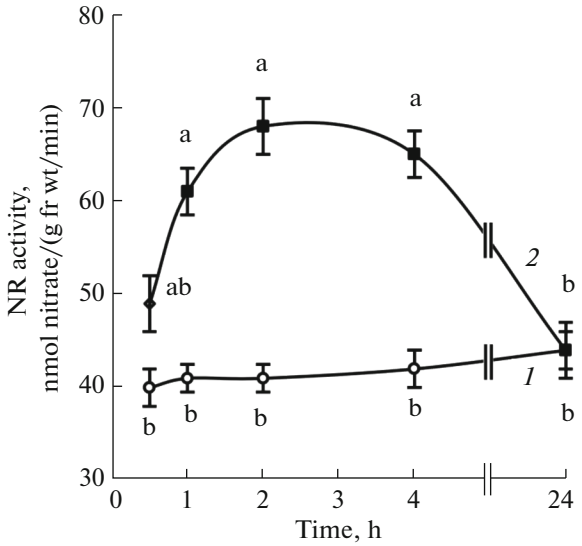

Fig. 4. Effect of hydrogen sulfide donor on dynamics of NR activity in wheat seedling roots. (*1*) Untreated control; (*2*) treatment with 0.25 mM NaHS. Equal letters designate means with insignificant (at $P \le 0.05$) differences.

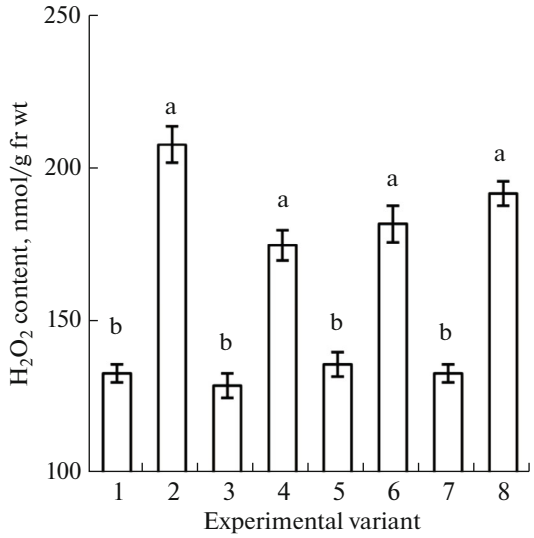

Fig. 5. Effect of hydrogen sulfide donor combined (or not) with antagonists of NO on content of hydrogen peroxide in wheat seedling roots. (*1*) Untreated control; (*2*) 0.25 mM NaHS; (*3*) 0.1 mM PTIO; (*4*) 0.25 mM NaHS + 0.1 mM PTIO; (*5*) 2 mM Na_2WO_4; (*6*) 0.25 mM NaHS + 2 mM Na_2WO_4; (*7*) 2 mM L-NAME; (*8*) 0.25 mM NaHS + 2 mM L-NAME. Equal letters designate means with insignificant (at $P \le 0.05$) differences. The analyses were performed 2 h after the start of the seedling treatment with NaHS and/or 4 h after the start of treatment with other indicated reagents.

Treatment with the NR inhibitor (sodium tungstate) resulted in a tendency (significant at $P \le 0.1$) to decrease in the nitric oxide level in the roots (Fig. 3b). This parameter was almost unaffected by the inhibitor of NO synthase (L-NAME). The increase in the NO content caused by the H_2S donor was markedly suppressed by the inhibitor of NR (Fig. 3b), whereas the inhibitor of NO synthase did not significantly (at $P \le 0.5$) change this effect. The results suggest an essential role of NR in the elevated formation of nitric oxide caused by the hydrogen sulfide donor.

The direct analysis of the NR activity in the seedling roots revealed its transient stimulation by the H_2S donor (Fig. 4). This effect was significant after 1–4 h of root treatment with NaHS, and the activity did not differ from the control value after 24 h.

To unravel the cause-effect relationships between the H_2O_2 and NO levels in the wheat seedling roots treated with the H_2S donor, the influence of the NO scavenger (PTIO), the inhibitors of NR (Na_2WO_4) and NO synthase (L-NAME) on the content of H_2O_2 was estimated. The nitric oxide antagonists themselves did not affect the hydrogen peroxide level in the roots (Fig. 5). The treatment with PTIO briefly attenuated the NaHS-induced enhancement in this index. Sodium tungstate caused a similar but insignificant (at $P \le 0.05$) effect. L-NAME was almost ineffective toward the hydrogen peroxide content in the roots of the NaHS-treated seedlings. Therefore, different antagonists of NO did not eliminate the accumulation of hydrogen peroxide in the roots stimulated by the hydrogen sulfide donor.

The next experimental series was aimed at the effects of antioxidant (DMTU) and NADPH oxidase inhibitor (imidazole) on the NR activity and NO content in the wheat seedling roots subjected to the H_2S donor. Treatment with DMTU alone was nearly indifferent against the NR activity and NO content, but it abolished the augmentation of both parameters caused by the hydrogen sulfide donor (Fig. 6). The NR activity and NO content were not significantly affected by imidazole, but their NaHS-induced increase was fully prevented by this inhibitor of NADPH oxidase (Fig. 6). The results show that the induction mechanism of the NR-dependent NO accumulation primary involves the accumulation of hydrogen peroxide in the seedlings subjected to hydrogen sulfide donor.

To check for possible involvement of hydrogen peroxide and nitric oxide in the processes of inducting of heat resistance of wheat seedlings by exogenous hydrogen sulfide, the effects of antagonists of ROS and NO on the NaHS-afforded heat protection effect were studied. Treatment of the wheat seedlings with DMTU somewhat increased their survival after the heat stress (Fig. 7). By contrast, this reagent markedly suppressed the positive effect of the hydrogen sulfide donor on the survival. The NADPH oxidase inhibitor imidazole alone did not influence the heat resistance but abolished the enhancement of this capability brought about by NaHS (Fig. 7). These results imply the role of ROS generated by NADPH oxidase in the accomplishment of the heat stress-protective action of the hydrogen sulfide donor on wheat seedlings.

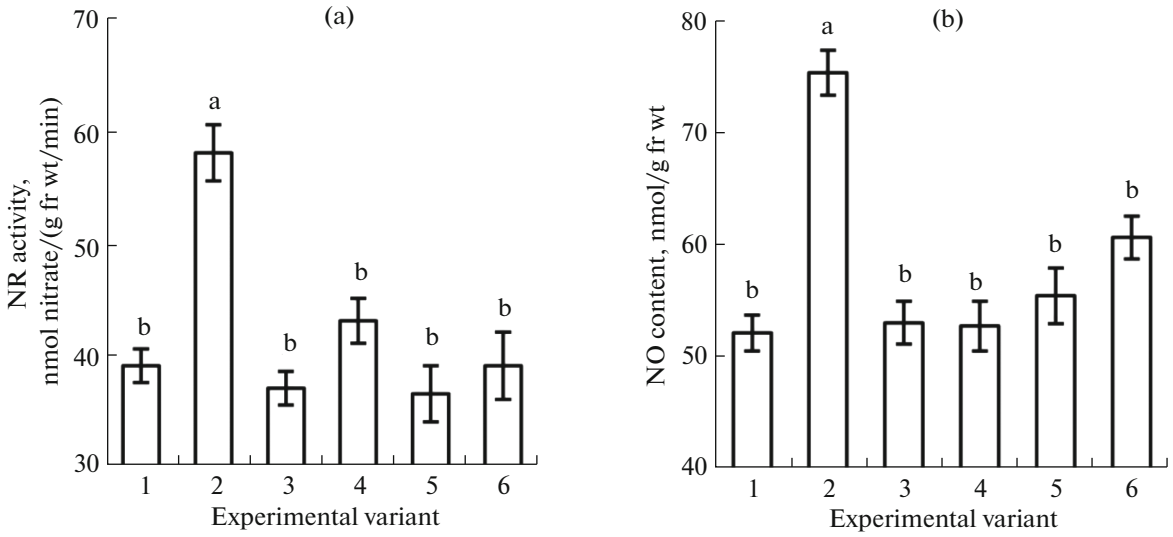

Fig. 6. Effect of hydrogen sulfide donor combined (or not) with antagonists of ROS on NR activity (a) and NO content (b) in wheat seedling roots. (*1*) Untreated control; (*2*) 0.25 mM NaHS; (*3*) 0.15 mM DMTU; (*4*) 0.25 mM NaHS + 0.15 mM DMTU; (*5*) 0.01 mM imidazole; (*6*) 0.25 mM NaHS + 0.01 mM imidazole. Equal letters designate means with insignificant (at $P \leq 0.05$) differences. The analyses were performed 2 h after the start of the seedling treatment with NaHS and/or 4 h after the start of treatment with other indicated reagents.

Treatments of the seedlings with the nitric oxide scavenger PTIO or the NO synthase inhibitor L-NAME increased their heat resistance to some extent but insignificantly at $P \leq 0.05$ (Fig. 7). The NR inhibitor sodium tungstate alone was also indifferent towards the seedling heat resistance. Meanwhile, both PTIO and L-NAME significantly reduced the positive influence of the H_2S donor on this indicator (Fig. 7). Some tendency of this effect was also observed in the combined treatment with NaHS and L-NAME, but it was not significant at $P \leq 0.05$.

DISCUSSION

Our experiments demonstrated the increase in the heat resistance of wheat seedlings (Fig. 1), which was elicited by the hydrogen sulfide donor NaHS and was preceded by transient increase in the hydrogen peroxide content in the roots (Fig. 2a). This temporary burst of the H_2O_2 level was prevented by seedling treatment with imidazol inhibiting NADPH oxidase (Fig. 3a). This inhibitor also eliminated the NaHS-induced rise in heat resistance of the seedlings (Fig. 7). It indicates the role of NADPH oxidase in the formation of ROS that are involved in signaling processes during the realization of the stress-protective effect of exogenous H_2S. Similarly, the role of this enzyme, as a ROS source, in the H_2S-induced barley plant resistance to UV-B was shown using another NADPH oxidase inhibitor diphenyleneiodonium [13].

The content of not only hydrogen peroxide but also nitric oxide transiently increased in the wheat seedling roots treated with the H_2S donor (Fig. 2b). Other authors also reported similar effects of exogenous hydrogen sulfide on the NO level in the plant cells. For example, in pea seedlings, hydrogen sulfide donor NaHS alleviates the arsenic phytotoxicity that is associated with a boost in the NO content [10]. The H_2S donor also enhances both salt resistance of tomato plants and the nitric oxide content in their roots [24].

Our results allow for the assumption that NR is an H_2S-stimulated enzymatic source of NO. This is evidenced by its transient activation in the roots occurring simultaneously with the rise in the NO content in them (Figs. 2b, 4). The NO accumulation in the roots treated with the donor of hydrogen sulfide was eliminated by the NR inhibitor tungstate but not the NO synthase inhibitor L-NAME (Fig. 3b). The rise in the NR activity caused by exogenous hydrogen sulfide is also known in other objects. For example, pretreatment of maize roots with NaHS increases the NR activity and prevents its decrease in response to the toxic action of lead [25]. In pea roots, the hydrogen sulfide donor elevates the NO level, which is accompanied by an increase in the NR activity [10]. Our study is the first that has shown the role of NR as an NO source in the establishment of the heat resistance induced by the H_2S donor in wheat seedlings. The NR inhibitor tungstate, as well as the NO scavenger PTIO, prevented the positive effect of NaHS on the seedling heat resistance (Fig. 7). In contrast, the NO synthase inhibitor L-NAME only weakly reduced the protective action of the hydrogen sulfide donor.

As is known, views of mechanisms of nitric oxide synthesis in plants are debatable so far [26]. The nitrate/nitrite-dependent and L-arginine-dependent

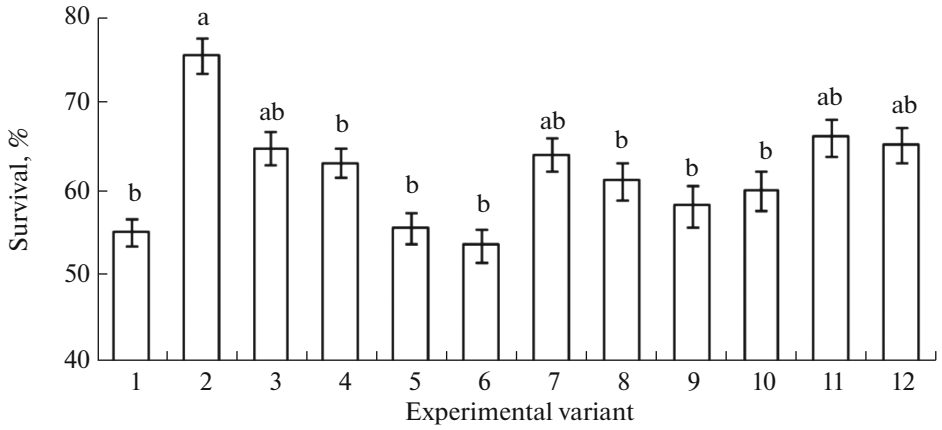

Fig. 7. Survival of wheat seedlings pretreated with hydrogen sulfide donor combined (or not) with antagonists of ROS or NO after damaging heating (10 min at 45°C). (*1*) Untreated control; (*2*) 0.25 mM NaHS; (*3*) 0.15 mM DMTU; (*4*) 0.25 mM NaHS + 0.15 mM DMTU; (*5*) 0.01 mM imidazole; (*6*) 0.25 mM NaHS + 0.01 mM imidazole; (*7*) 0.1 mM PTIO; (*8*) 0.25 mM NaHS + 0.1 mM PTIO; (*9*) 2 mM Na_2WO_4; (*10*) 0.25 mM NaHS + 2 mM Na_2WO_4; (*11*) 2 mM L-NAME; (*12*) 0.25 mM NaHS + 2 mM L-NAME. Equal letters designate means with insignificant (at $P \leq 0.05$) differences.

pathways are recognized to be the main sources of NO. The NR role in reduction of nitrate and nitrite to nitric oxide is well established, but the nature of enzyme(s) synthesizing NO from L-arginine remains obscure [26]. In our case, the discussion of a possible role of enzyme similar to the animal NO synthase in implementation of physiological effects of hydrogen sulfide is beyond the available experimental material in fact, that its inhibitor L-NAME was almost indifferent towards the effects of the H_2S donor on the NO content in the roots and on the induced heat resistance of the wheat seedlings (Figs. 3b, 7).

Nitric oxide and hydrogen peroxide as signal mediators are in close functional relationship. According to our data, both compounds are involved in accomplishment of the hydrogen sulfide physiological action leading to increase in heat resistance of wheat seedlings. Rise in levels of NO and H_2O_2 obeyed a similar dynamics, namely, their main effects took place 1–4 h after a start of plant treatment with the H_2S donor. This does not allow to identify a sequence of these mediators' action in a signaling chain. At the same time, we found that the antioxidant DMTU and NADPH oxidase inhibitor imidazol prevented the H_2S-induced boost in the NO content, while the NO scavenger PTIO and the inhibitors of NO-synthesizing enzymes did not significantly influence the H_2O_2 content in the roots (Figs. 5, 6b). This inhibitory analysis suggests that the burst of hydrogen peroxide is primary as compared with the changes in the NO pool. It should be emphasized that the NR activation afforded by the hydrogen sulfide donor was also eliminated by the H_2O_2 scavenger DMTU and NADPH oxidase inhibitor imidazole (Fig. 6a). In compliance with our results, the hydrogen peroxide-stimulated activation of NR was also reported [18].

The sequence of signaling events suggested here does not exclude different signaling interactions between hydrogen sulfide, hydrogen peroxide, and nitric oxide. For example, the heat resistance induced by the NO donor sodium nitroprusside in maize seedlings is accompanied by the activation of L-cysteine-desulfhydrase and the accumulation of H_2S [27]. Signaling from NO that induces tomato salt resistance is also supposed to be mediated by hydrogen sulfide [24]. However, the positive influence of the hydrogen sulfide donor on the salt resistance of alfalfa plants [16] and barley seedlings [28] is eliminated by the NO scavenger PTIO. In addition, it is shown that nitric oxide and hydrogen sulfide mediate the induction of heat resistance of maize seedlings [29]. The given examples imply that signaling and/or stress-protecting effects of exogenous compounds are specifically expressed depending to a large extent on the peculiarity of experimental models chosen. In a particular model, fragments of complicated signaling networks are usually studied, where the same mediators may function in different links of regulatory chains [4].

Induction processes of plant heat resistance initiated by hydrogen sulfide donors comprise functional interactions of not only ROS and NO but also different mediators; among them calcium appears to be obligatory. Thus, our previous publication reported that various calcium antagonists diminish the effects of hydrogen sulfide on heat resistance of wheat coleoptile cells, ROS generation, and the activity of antioxidant enzymes [14]. The importance of calcium in accomplishment of the stress-protective action of hydrogen sulfide was also shown on different objects [8].

In general, the results of the present work allow inferring that the induction of heat resistance of wheat seedlings by the hydrogen sulfide donor is mediated by ROS (generated by NADPH oxidase) and nitric oxide

(mainly produced by NR). Presumably, the NR activation involves ROS that take place upstream of NO in the signaling chain. The H_2S-induced signaling cascade may activate a complex of physiological reactions causing the increase of heat resistance. Among them, probably, activation of antioxidant enzymes and increase in expression of their genes might have special importance [10, 13, 26]. In addition, the contents of polyfunctional low-molecular weight protectors—proline, sucrose, betaine, and flavonoid compounds—also increase in plants subjected to exogenous hydrogen sulfide [11, 13, 30]. Exploration of mechanisms of the H_2S-induced protective reactions in plants provides a theoretical basis to employ hydrogen sulfide donors as components of stress-protective formulations to be used in plant growing.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any work conducted on animal or human participants.

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