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Involvement of Nitric Oxide in γ-Aminobutyric Acid-Induced Cellular Mechanisms of Wheat Seedling Adaptation to Water Deficit

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Abstract—γ-Aminobutyric acid (GABA) is one of the key stress metabolites involved in the functioning of the plant cell signaling network. However, its functional relationships with main signaling molecules, particularly nitric oxide (NO), are not well understood. This study aimed to determine NO's involvement in GABA's stress-protective effect on wheat seedlings (*Triticum aestivum* L., Etana cultivar) under model drought conditions (13% PEG 6000). Priming grains with a 0.5 mM GABA solution or a 0.1 mM solution of the NO donor sodium nitroprusside (SNP) increased germination energy, seed germination, and water content in seedlings under stressful conditions. Additionally, GABA and SNP treatment mitigated the inhibitory effect of drought on the accumulation of root and shoot biomass. Osmotic stress increased NO content in shoots, and priming with GABA and SNP enhanced this effect. Treatment with the nitric oxide scavenger methylene blue (MB, 0.1 mM) eliminated the increase in NO content caused by stress or GABA action. MB also negated the positive effects of GABA on growth processes. Under the action of GABA and SNP, total amylase activity in grains and soluble carbohydrate content in shoots increased under stressful conditions; MB eliminated GABA's effect on these parameters. Under the model drought, the content of oxidative stress markers (superoxide anion radical, hydrogen peroxide, and malondialdehyde) increased in shoots, but pretreatment of grains with GABA or SNP significantly reduced these effects. Osmotic stress caused an increase in activity of catalase and guaiacol peroxidase in shoots. Preliminary priming with GABA, SNP, and MB did not affect the nature of the changes in activity of these antioxidant enzymes caused by model drought. Meanwhile, superoxide dismutase activity remained unchanged following exposure to the model drought; however, pretreatment seeds with GABA, SNP, and MB increased the enzyme activity. Priming seeds with GABA and SNP increased the total content of phenolic compounds in shoots and preserved the anthocyanin pool under stressful conditions, while MB treatment eliminated these effects. It was concluded that the enhancement of grain germination and seedling growth induced by GABA priming under model drought conditions is largely due to NO-mediated modulation of carbohydrate and secondary compound metabolism.

Keywords: γ-aminobutyric acid, nitric oxide, oxidative stress, antioxidant system, osmolytes, amylase, drought resistance, *Triticum aestivum*

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INTRODUCTION

In recent years, substances that are well known in human and animal physiology as neurotransmitters involved in the transduction of nervous excitation are considered as a separate group of cellular regulators of plant functions: melatonin, serotonin, dopamine, acetylcholine, and γ-aminobutyric acid (GABA) (Akula and Mukherjee, 2020). Currently, GABA, along with melatonin, is one of the most actively studied plant neurotransmitters (Caspi et al., 2023; Lv et al., 2023; Wu et al., 2023). GABA is believed to combine the functions of a stress metabolite and a molecule involved in intercellular and intracellular signaling in plants (Bor and Turkan, 2019; Caspi et al., 2023; Yuan et al., 2023). The signaling functions of GABA have so far been studied in detail on the example of a limited range of physiological processes, primarily the regulation of the state of stomata and the acceleration of pollen tube growth (Bor and Turkan, 2019; Domingos et al., 2019). At the same time, GABA is considered as a metabolite that plays an important role in many adaptive reactions of plants. In particular, it has been shown that, as a signaling molecule, GABA participates in the regulation of plant resistance to abiotic stresses of various nature: extreme temperatures (Zeng et al., 2021; Zhou et al., 2022), hypoxia (Yuan et al., 2023), salinization (Ullah et al., 2023), and drought (Zhao et al., 2023; Jurkonienė et al., 2025).

In response to the action of various stress factors, the content of GABA in plant cells increases significantly. Thus, under drought conditions, an increase in the amount of endogenous GABA has been found in various plant species, in particular, tomatoes, soybeans, beans, sesame (Li et al., 2021). Information is also being intensively accumulated on the induction of plant resistance to stressors of various nature by exogenous GABA. The ability of GABA to influence such important plant defense systems as antioxidant and osmoprotective was established (Kolupaev et al., 2024a; Kozeko et al., 2024). It is likely that the participation of GABA in the regulation of these systems determines its ability to significantly increase the drought and salt tolerance of plants (Li et al., 2016; Badr et al., 2024). For example, it has been shown that, under the influence of GABA in clover seedlings under conditions of osmotic stress, the sugar content and osmotic potential increased (Zhou et al., 2021). GABA treatment increased the salt tolerance of *Agrostis capillaris* plants, which is also associated with increased osmotic regulation due to the accumulation of sugars (Li et al., 2020). Recently, GABA has been considered an important molecule not only for increasing the drought tolerance of plants but also for enhancing the ability of seeds to germinate under conditions of moisture deficiency (Zhou et al., 2021). There is evidence of a relationship between endogenous GABA content and seed germination (Samarah Nezar et al., 2023). Of particular interest for enhancing seed germination and plant growth under drought conditions at early stages of development is seed priming with GABA solutions (Sheteiwy et al., 2019; Zhou et al., 2021).

According to current hypotheses, the signaling effects of GABA are largely realized through its functional interaction with other signaling mediators, in particular, calcium, reactive oxygen species (ROS), and nitric oxide (NO). For example, it was found that the development of tolerance of *Cucumis melo* plants to sodium salinity, caused by the action of exogenous GABA, was accompanied by increased gene expression of one of the main forms of NADPH oxidase (*RBOHD*) and transient accumulation of hydrogen peroxide (Jin et al., 2019). Recently, we have shown that the induction of heat tolerance of wheat seedlings by exogenous GABA is mediated by a calcium-dependent increase in ROS generation (Kolupaev et al., 2024b).

The involvement of nitric oxide in the implementation of GABA effects has been studied to a lesser extent. There are only isolated data obtained by the method of inhibitory analysis, indicating the involvement of NO in the formation of adaptive responses induced by GABA. Thus, GABA treatment of *Agrostis stolonifera,* which increased resistance to osmotic stress, caused an increase in the formation of NO, dependent on nitrate reductase and an enzyme similar to NO synthase, as well as an increase in the activity of antioxidant enzymes dependent on NO homeostasis (Tang et al., 2020). The stress-protective effect of GABA on *Cucumis melo* plants under sodium salinity was accompanied by an increase in the content of nitric oxide, and simultaneous treatment of plants with the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) neutralized the effects of GABA (Xu et al., 2021). However, the question of the involvement of nitric oxide in the implementation of the effect of GABA on grain germination and plant growth at early stages of development under osmotic stress remains unexplored. At the same time, the ability of exogenous NO to enhance grain germination, improve osmoregulatory processes, and seedling growth under drought or salt stress has been established for quite some time using the example of plants of various species (Duan et al., 2007; Sepehri and Rouhi, 2016; Yemets et al., 2019).

In connection with the above, the aim of the work was to clarify the role of NO in the implementation of the stress-protective effect of GABA on wheat seedlings (*Triticum aestivum* L.) under conditions of model drought. To this end, the effect of seed treatment with GABA and with the nitrogen oxide scavenger methylene blue (MB) and its donor sodium nitroprusside (SNP) on the process of grain germination, seedling growth, the content of endogenous nitric oxide, and the functioning of the antioxidant and osmoprotective systems under the action of the impermeable osmotic agent PEG 6000 was investigated.

MATERIALS AND METHODS

The experiments used seeds of soft winter wheat of the Etana variety (applicant: Deutsche Saatveredelung AG, Germany), which is one of the most common foreign varieties grown in Ukraine (https://agroportal.ua/news/rastenievodstvo/nazvano-naypopulyarnishi-sorti-pshenici-v-ukrajini).

The grains were disinfected with a 5% NaClO solution for 15 min and washed eight times with sterile distilled water. Given that hydropriming itself increases the germination of cereal seeds (Kolupaev et al., 2024c), as a control, grains were kept for 3 h in a dark thermostat at 24°C in glasses with distilled water (1 : 4 volume ratio). Samples of the experimental variants were kept for 3 h in a 0.5 mM GABA solution, a 0.1 mM solution of the NO scavenger methylene blue (MB) (Zhang et al., 2006), or in a mixture of 0.5 mM GABA and 0.1 mM MB. In a separate variant, the grains were treated with a 0.1 mM solution of the NO donor SNP. The treatment regimen and optimal concentrations of the studied compounds were selected based on the results of previous experiments (Shakhov et al., 2024, 2025; Kolupaev et al., 2025a). After hydropriming (control variant) and treatment with the test substances, the seeds were dried for 24 h on a glass

surface in a dark thermostat at 24°C and a relative humidity of 40% to the initial humidity (11–12%).

Subsequently, 80 approximately identical grains were transferred to Petri dishes on double filters, which were moistened with 8 mL of distilled water (control) or 8 mL of 13% PEG 6000 (model drought). The seeds were germinated in a dark thermostat at 24°C for 3 days. After 2 and 3 days, germination energy and germination indices were evaluated, respectively. Germinated grains were considered to have shoots that exceeded the grain size in length or were at its level. Also, after 3 days of germination, the biomass of roots and shoots and the water content in the shoots were evaluated. For all biochemical analyses (except for determining amylase activity), only shoots of 3-day-old seedlings were used since it was impossible to wash the roots from osmotic residues without damage, which could affect the results of the analyses.

The water content in the shoots was determined by the gravimetric method, drying samples weighing at least 500 mg in a drying oven at 103°C for 1.5–2 h (until constant weight).

The content of nitric oxide in shoots was determined by the method described by Zhou et al. (2005) with some modifications. The method is based on the conversion of NO contained in plant material to nitrite and determination of the concentration of the latter in the Griess reaction. A portion of freshly cut plant material was homogenized on ice in 50 mM acetate buffer (pH 3.6) with the addition of 2% zinc acetate. The homogenate was centrifuged in an MPW 350R centrifuge (MPW MedInstruments, Poland) at 2–4°C at 8000 *g* for 15 min, then 250 mg of charcoal was added to 10 mL of the supernatant. The mixture was filtered through a paper filter, after which 2 mL of the filtrate was mixed with 1 mL of 1% Griess reagent in 12% acetic acid. After 30 min, the absorbance of the solution was determined on a UV-1280 spectrophotometer (Shimadzu, Japan) at a wavelength of 530 nm. Sodium nitrite solutions were used as a standard. The NO content was expressed in nmol/g of fresh weight.

Total amylase activity was determined in grains 48 h after the start of their germination using starch agar plates and ImageJ software (Yastreb et al., 2025). The analysis was performed 48 h after the start of seed soaking. The grains were cut with a lancet, the halves without embryos were placed cut side down in Petri dishes on plates consisting of 1% agar and 0.2% starch. The samples were incubated in a thermostat at 24°C for 3 h. After that, the gels were poured with 10 mL of diluted Lugol's solution (0.04% I_2 in 0.1% KI), the excess solution was removed after 5 min with an autopipette, and the images were photographed using a Samsung SM-N9750 horizontal camera on a glass covered with paper with bottom illumination. The obtained photographs were analyzed using ImageJ software (version 1.54 g). Color images were converted to single-channel halftone to remove variability caused by color information and analyze the difference in color intensity. On preprocessed images, masks of illuminated halos around the grains were created using the ImageJ selection tools, excluding the cut areas of the grains themselves (Yastreb et al., 2025). The pixel area of the selected areas was measured using ImageJ tools. The results were converted to mm^2. Enzyme activity was expressed in conventional units (mm^2) per hour.

To determine the proportion of β-amylase in the total amylase activity in a separate assay, 2 mM EDTA, which inhibits α-amylase, was added to starch-containing agar (Zhang et al., 2010).

The total content of soluble carbohydrates in the shoots of seedlings was determined by the Morris–Roe method (Kefu et al., 2003) with modifications. The plant material was homogenized in distilled water, the homogenate was boiled in a water bath for 10 min. To precipitate proteins, equal volumes of 30% $ZnSO_4$ and 15% $K_4[Fe(CN)_6]$ were added to the homogenate. The samples were mixed, filtered through paper filters, and diluted with distilled water to the appropriate concentration. Then, 1 mL of the diluted extract was mixed with 3 mL of anthrone reagent. The reference solution instead of the extract contained 1 mL of distilled water. The samples were boiled in a water bath for 7 min, cooled, and the absorbance at 610 nm was determined. D-glucose was used as a standard.

Proline content was determined by the method described by Bates et al. (1973) with minor modifications. Shoot portions were homogenized in distilled water, after which the homogenate was immediately boiled for 10 min in a water bath. The samples were cooled, and the extracts were filtered through paper filters. 1 mL of extract, glacial acetic acid, and ninhydrin reagent were mixed in reaction tubes. The tubes, closed with foil caps, were heated in a boiling water bath for 1 h. The absorbance was determined at a wavelength of 520 nm. L-proline served as the standard.

To determine the total content of phenolic compounds and anthocyanins, shoots were homogenized in 10 mL of 80% ethanol, extracted for 20 min at room temperature, and centrifuged at 8000*g* for 15 min. To analyze the content of phenolic compounds, 0.5 mL of supernatant, 8 mL of distilled water, and 0.5 mL of Folin's reagent were added to reaction tubes, mixed, and 1 mL of 10% sodium carbonate was added after 3 min. After 1 h, the absorbance of the reaction mixture was measured at 725 nm (Bobo-García et al., 2015). The content of phenolic compounds was expressed in mg-eq. of gallic acid per gram of dry weight.

Before determining the anthocyanin content, the supernatant was acidified with HCl to a final concentration of 1%. Absorbance was determined at 530 nm (Nogués and Baker, 2000). The results were expressed

in conventional units as absorbance per gram of dry weight.

When determining the activity of antioxidant enzymes, shoot aliquots were homogenized on ice in 0.15 M K, Na-phosphate buffer (pH 7.6) containing 0.1 mM EDTA and 1 mM dithiothreitol (Kolupaev et al., 2024b). The homogenate was centrifuged at 8000 *g* for 10 min at 2–4°C.

The total activity of superoxide dismutase (SOD) was determined at a pH of the reaction mixture of 7.6 using a method based on the ability of the enzyme to compete with nitroblue tetrazolium for superoxide anion radicals formed as a result of the aerobic interaction of NADH and phenazine methosulfate. Catalase activity was determined at a pH of the reaction mixture of 7.0 by the amount of hydrogen peroxide decomposed per unit time. Guaiacol peroxidase activity was analyzed using guaiacol as a hydrogen donor and H_2O_2 as a substrate. The pH of the reaction mixture was previously adjusted to 6.2 using K, Na-phosphate buffer. The absorbance of the reaction product was determined at a wavelength of 470 nm.

The protein content in the samples was determined according to Bradford (Bradford, 1976), using bovine serum albumin as a standard.

The generation of superoxide anion radical by seedling shoots was determined by a method based on the interaction of $O_2^{\bullet-}$ with nitroblue tetrazolium with the formation of formazan (Karpets et al., 2012). Twelve shoots were placed for 1 h in jars with 5 mL of 0.1 M K, Na-phosphate buffer (pH 7.6) containing 0.05% nitroblue tetrazolium, 10 μM EDTA, and 0.1% Triton X-100. After the end of the exposure, the shoots were carefully removed from the jars and the light absorption of the incubation solution was measured at a wavelength of 530 nm. The $O_2^{\bullet-}$ generation index was expressed in conventional units (A_{530} ×1000/fresh weight of shoots).

To determine the hydrogen peroxide content, aliquots of shoots were homogenized on ice in 5% trichloroacetic acid (TCA) solution. The samples were centrifuged at 8000 *g* for 10 min at 2–4°C and the H_2O_2 concentration in the supernatant was determined using the ferrithiocyanate method (Sagisaka, 1976).

To analyze the content of lipid peroxidation products (LPO) (mainly malondialdehyde (MDA)), portions of plant material were homogenized in a solution of 0.25% thiobarbituric acid in 10% TCA (test sample) or in a solution of only 10% TCA (control). The mixtures were boiled in test tubes, closed with foil caps, in a water bath for 30 min. After that, they were cooled and centrifuged for 15 min at 10000 *g*. The absorbance of the supernatant was measured at wavelengths of 532 nm (main signal) and 600 nm (nonspecific light absorbance, the magnitude of which was subtracted from the main result $A_{532)}$ (Kolupaev et al., 2024d). The MDA content was calculated using the molar extinction coefficient of 1.55×10^5 M^{-1} cm^{-1} and expressed as nmol/g of fresh weight.

When determining the effect of GABA, MB, and SNP treatments on seed germination and seedling biomass, each replicate consisted of 80 grains or seedlings, and each experimental variant had at least three replicates. When conducting biochemical analyses (except for determining amylase activity), each sample consisted of at least 12 shoots, and the analyses were performed in three replicates. Amylase activity was determined in five replicates, each in a separate Petri dish containing all experimental variants, each with four grains. To determine the significance of differences between variants in terms of the studied parameters, a one-way analysis of variance (ANOVA) was used with subsequent multiple comparisons using the Tukey method.

RESULTS AND DISCUSSION

Model drought caused a significant decrease in the germination energy and germination of wheat seeds (Fig. 1a*)*. Priming of grains with GABA significantly increased these indicators. Treatment with the NO scavenger MB did not significantly affect the germination process of seeds; however, when combined with GABA, it completely eliminated the positive effect of the latter on the germination energy and germination of grains (Fig. 1a). Treatment of grains with the NO donor SNP increased their germination indicators, and the magnitude of the effects of SNP was practically the same as with priming of grains with GABA.

During the germination of grains on a medium with the addition of 13% PEG 6000, a multiple decrease in shoot biomass was observed compared to the control (Figs. 1b, 1c). GABA priming likely increased shoot biomass under stress conditions. Treatment of grains with MB did not affect the amount of shoot biomass under model drought conditions; however, it completely eliminated the positive effect of GABA on biomass accumulation under stress. When seeds were treated with the NO donor SNP, the inhibitory influence of osmotic stress was somewhat mitigated, but this effect was not significant at $p \le 0.05$ (Fig. 1b).

The mass of seedling roots under the influence of model drought decreased more than twofold (Fig. 1b). GABA treatment significantly mitigated the effect of root growth inhibition. Under the influence of MB, seedling root growth under stress conditions did not change, and the positive effect of GABA on root growth was not manifested with combined treatment with GABA and MB. Under the influence of the nitric oxide donor, an increase in root growth was noted against the background of stress; however, this effect was smaller compared to the effect of GABA priming (Figs. 1b, 1c).

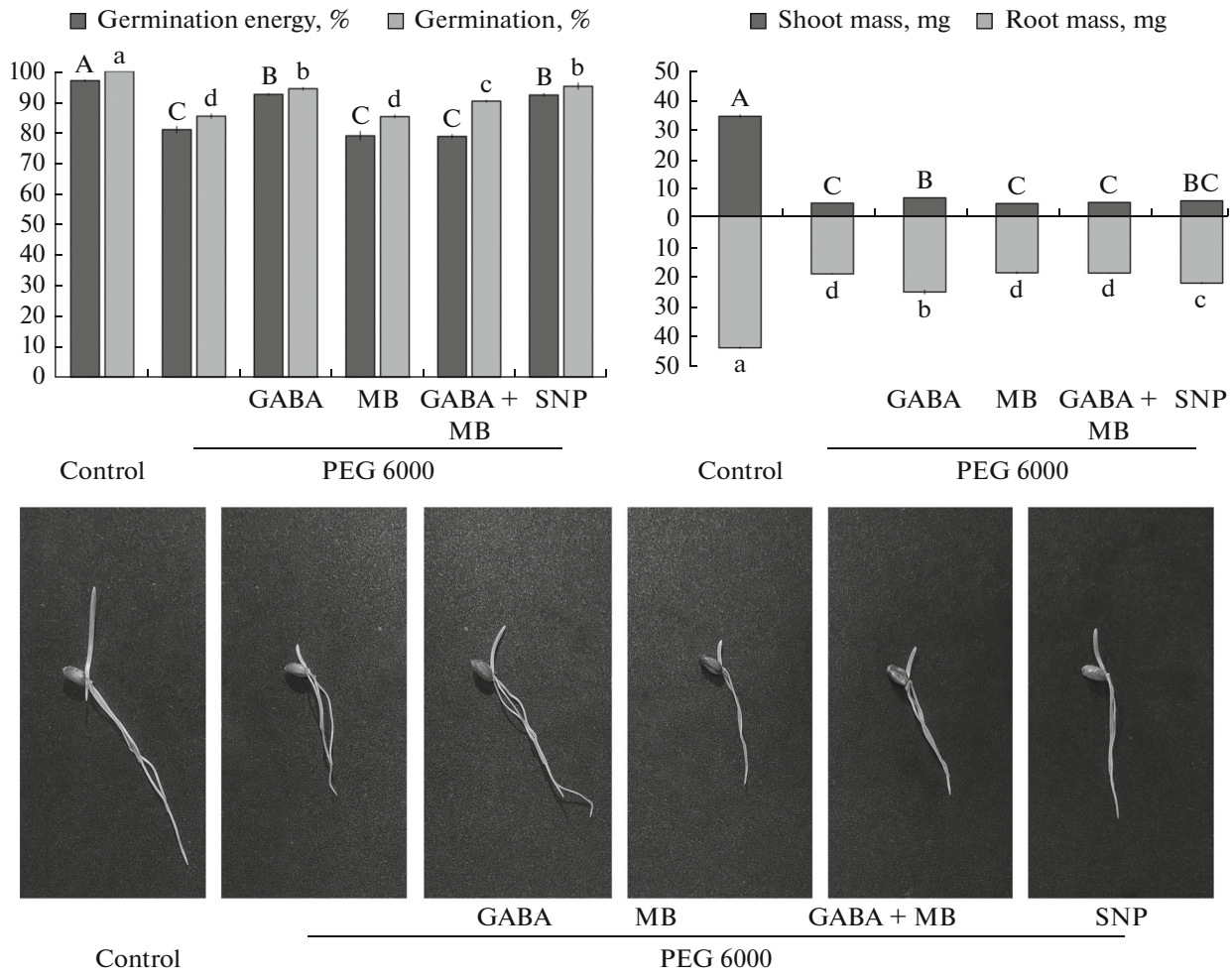

Fig. 1. (a) Germination energy, germination of wheat grains, (b) mass of seedling organs, and (c) photos of their typical samples under the influence of model drought (13% PEG 6000), GABA (0.5 mM), MB (0.1 mM), and SNP (0.1 mM). The same Latin letters indicate values between which there is no difference, significant at $p \le 0.05$.

Model drought led to a significant (almost 13%) decrease in water content in shoots (Fig. 2). Pretreatment of grains with GABA reduced this negative effect of drought. At the same time, when grains were treated with the NO scavenger MB, the water content in shoots did not change against the background of drought, but such treatment slightly reduced the effect of GABA on tissue hydration. The nitric oxide donor SNP, like GABA, reduced the negative effect of osmotic stress on water content in shoots (Fig. 2).

Thus, in general, priming of seeds with GABA promoted wheat grain germination and seedling growth under a model drought and also increased tissue hydration. All these effects of GABA were significantly reduced under the influence of the NO scavenger MB, indicating the involvement of nitric oxide as a mediator in the implementation of the stress-protective effect of GABA. It is noteworthy that priming of grains with the NO donor SNP had positive effects on grain germination, seedling biomass, and water content, which were similar to the effect of GABA (Figs. 1, 2).

The participation of nitric oxide in the adaptation of wheat seedlings to drought and the implementation of the effect of GABA on drought resistance is also indicated by the results of direct determination of the NO content in shoots (Fig. 3).

Osmotic stress caused a significant increase in NO content ($p \le 0.05$) in shoots (Fig. 3). However, in the variant with a combination of drought and GABA priming, this increase was significantly greater than in the variant with only osmotic stress. In the presence of the NO scavenger MB, the effect of drought and its combination with GABA priming on the content of nitric oxide was not manifested (Fig. 3). However, seed treatment with the NO donor SNP caused an increase in the amount of NO in shoots.

Given the important role of amylase in the hydrolysis of reserve starch and the entry of soluble carbohydrates into the organs of seedlings during seed germi-

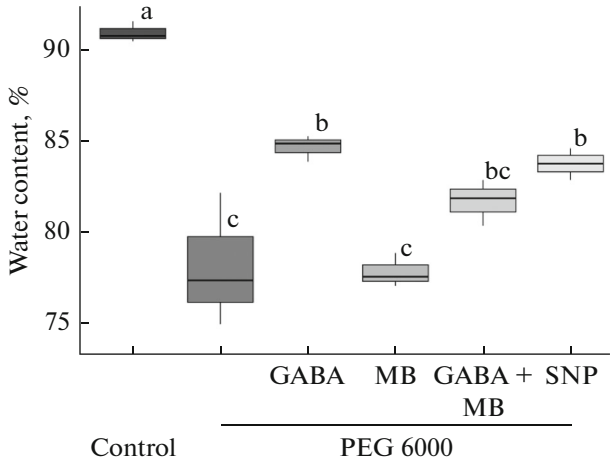

Fig. 2. Water content in shoots of 72-h wheat seedlings under the influence of model drought (13% PEG 6000), GABA (0.5 mM), MB (0.1 mM), and SNP (0.1 mM). The same Latin letters above the Tukey boxes indicate values between which there is no difference, significant at $p \leq 0.05$.

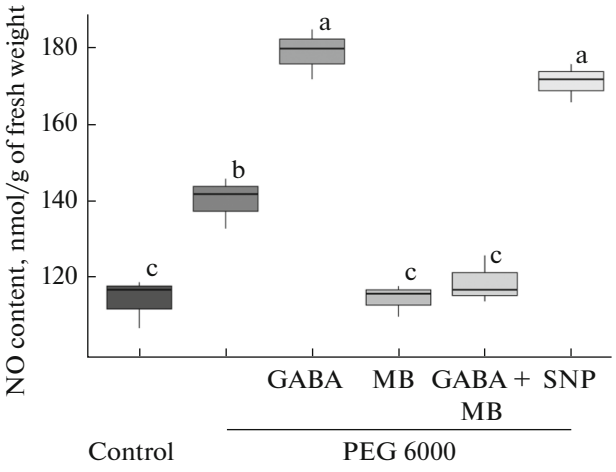

Fig. 3. Nitric oxide content in shoots of 72-h wheat seedlings under the influence of model drought (13% PEG 6000), GABA (0.5 mM), MB (0.1 mM), and SNP (0.1 mM). The same Latin letters above the Tukey boxes indicate values between which there is no difference, significant at $p \leq 0.05$.

nation, its activity was assessed on sections of grains 48 h after the start of their germination (Fig. 4).

Drought had hardly any effect on amylase activity in grains (Fig. 4). Pretreatment of grains with GABA resulted in a significant increase in enzyme activity. However, this effect of GABA was completely eliminated by the action of the NO scavenger MB. However, priming of grains with 0.1 mM NO donor SNP led to a significant increase in total amylase activity and its quantitative effect significantly exceeded the effect of GABA (Fig. 3). All this indicates the possible participation of NO as a mediator in the stress-protective action of GABA. It should be noted that the β-amylase activity identified in wheat grain slices in the presence of an α-amylase inhibitor (2 mM EDTA) was no more than 10–12% of the total amylase activity. Given this, quantitative measurement of β-amylase was not performed, considering that the main share in measuring the total enzyme activity in grains is α-amylase activity.

The increase in amylase activity in grains under the influence of GABA could be at least one of the reasons for the increase in the sugar content in shoots (Fig. 5a). Drought caused a decrease in the sugar content in shoots compared to the control, but the treatment of grains with GABA not only eliminated this effect of drought but also caused an increase in the amount of soluble carbohydrates to values higher than the control variant. Treatment with the scavenger of nitric oxide MB partially neutralized the effect of GABA on the sugar content in shoots of seedlings. At the same time, under the influence of the NO donor SNP, the sugar content in shoots increased significantly under model drought conditions (Fig. 5a).

Along with sugars (Kiriziy et al., 2024), one of the main osmolytes of plant cells necessary for adaptation to drought is considered to be proline (Ghosh et al., 2022). In our experiments, the proline content in shoots under the influence of model drought increased more than threefold. However, the treatment of grains with GABA, MB, their combination, and SNP did not significantly affect the proline content under stress conditions (Fig. 5b).

In addition to sugars and proline, important multifunctional stress metabolites that perform antioxidant, osmoregulatory, and membrane-protective functions are secondary metabolites (Babenko et al., 2019; Yadav et al., 2021; Kolupaev et al., 2023a).

Under the influence of model drought, the total content of phenolic compounds in the shoots of seedlings did not change significantly (Fig. 6a). Priming of GABA grains before their germination under drought conditions caused a noticeable increase in the content of phenolic compounds. MB seed treatment itself had a weak effect on the content of phenolic compounds but eliminated their accumulation under the influence of GABA. SNP treatment, like the action of GABA, significantly increased the total content of phenolic compounds in the shoots of wheat seedlings.

Under drought conditions, the content of anthocyanins in the shoots of seedlings sharply decreased (Fig. 6b), which may be associated with their intensive oxidation under stress conditions. Pretreatment of grains with GABA stabilized the content of anthocyanins in the shoots to the control level. Treatment with the NO scavenger MB almost completely eliminated this effect of GABA. Under the action of the NO donor SNP, the content of anthocyanins under drought conditions increased significantly.

The influence of model drought and the studied compounds on the activity of key antioxidant enzymes in the shoots of seedlings was ambiguous. Thus, drought did not significantly affect SOD activity (Fig. 7a). Pretreatment of grains with GABA caused an increase in SOD activity under drought conditions. A significant increase in enzyme activity was also observed in the variant with MB grain treatment as well as in its combination with GABA. Higher values of SOD activity than in the control and drought variant were also observed in the SNP grain treatment.

CAT activity in seedling shoots increased in response to the effect of model drought (Fig. 7b). However, seed treatment with any of the studied compounds did not modify the effects of drought. A similar picture was observed with GPO activity: it increased under the influence of drought, but treatment with GABA, MB, their combination, and SNP did not cause additional changes in enzyme activity (Fig. 7c).

Despite the ambiguous effect of GABA and the NO donor and scavenger on the activity of the studied antioxidant enzymes, under the action of GABA and SNP, a decrease in the development of oxidative stress in the shoots of seedlings was noted. Thus, drought caused an increase in the generation of superoxide anion radical by the shoots of seedlings (Fig. 8a). GABA treatment significantly mitigated the effect of drought on the generation of $O_2^{\bullet-}$. In contrast, under the action of the NO scavenger MB, an increase in the formation of superoxide anion radical and partial elimination of the effect of GABA on this process were noted. The NO donor SNP significantly reduced the generation of superoxide anion radical by the shoots of seedlings under conditions of model drought (Fig. 8a).

Under the influence of drought, the content of hydrogen peroxide in the shoots of seedlings increased (Fig. 8b). GABA priming reduced the manifestation of this effect of oxidative stress, while MB pretreatment, on the contrary, increased the content of H_2O_2 and completely eliminated the decrease in its amount caused by the action of GABA. Priming with the NO donor SNP led to a significant decrease in the content of hydrogen peroxide in shoots under drought conditions.

The content of the final product of LPO (MDA), as well as other markers of oxidative stress, increased significantly under the influence of model drought (Fig. 8c). Treatment of grains with GABA mitigated this negative effect of drought. Treatment with MB, on the contrary, enhanced the drought-induced accumulation of MDA in shoots of wheat seedlings and largely eliminated the decrease in this indicator under the influence of GABA. Priming of wheat grains with SNP significantly reduced the drought-induced accumulation of MDA in shoots of seedlings (Fig. 8c).

Thus, the obtained results indicate that treatment of wheat grains with GABA significantly enhanced

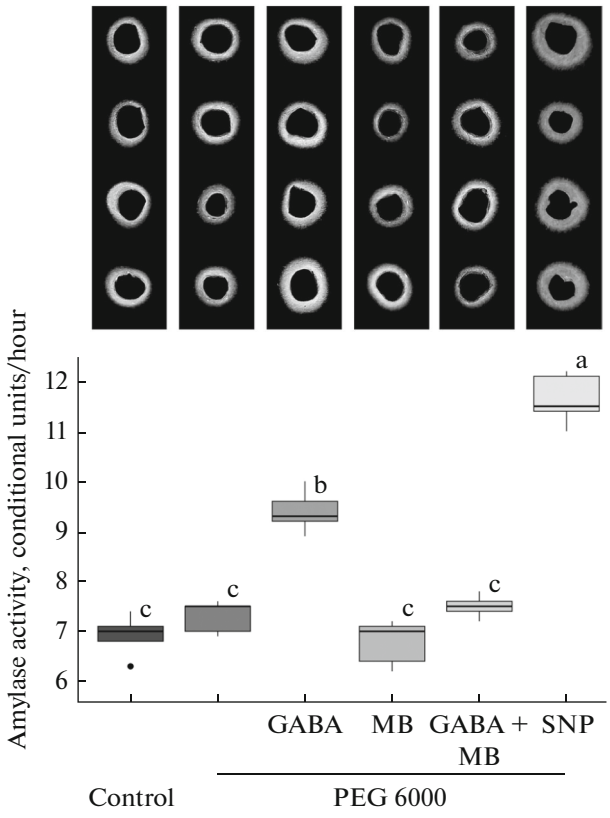

Fig. 4. Total amylase activity and a typical example of its visualization under the influence of model drought (13% PEG 6000), GABA (0.5 mM), MB (0.1 mM), and SNP (0.1 mM). The same Latin letters above the Tukey boxes indicate values between which there is no difference, significant at $p \le 0.05$.

their germination under osmotic stress and increased the ability of seedlings to accumulate biomass and maintain tissue hydration under the action of a model drought. Over the past few years, various scientific teams have shown an increase in drought and salt tolerance of cereal grains by exogenous GABA (Sheteiwy et al., 2019; Ashraf et al., 2024; Kolupaev et al., 2024d; Al Ghafri et al., 2025; Shakhov et al., 2025). A new aspect of this work is the experimental confirmation of the participation of NO in inducing wheat seed germination and the formation of drought tolerance of seedlings during priming of GABA grains. In this case, the role of nitric oxide was proven by a combination of three experimental approaches: using the NO scavenger MB, direct determination of the NO content in the shoots of seedlings, and reproduction of a number of stress-protective effects of GABA by treating wheat seeds with the NO donor SNP. We are aware of only one work performed on wheat plants in which using the NO scavenger PTIO has shown the involvement of nitric oxide in inducing salt tolerance by the action of GABA (Khanna et al., 2021). However, this work used foliar GABA treatment of 30-day-old plants; i.e., its

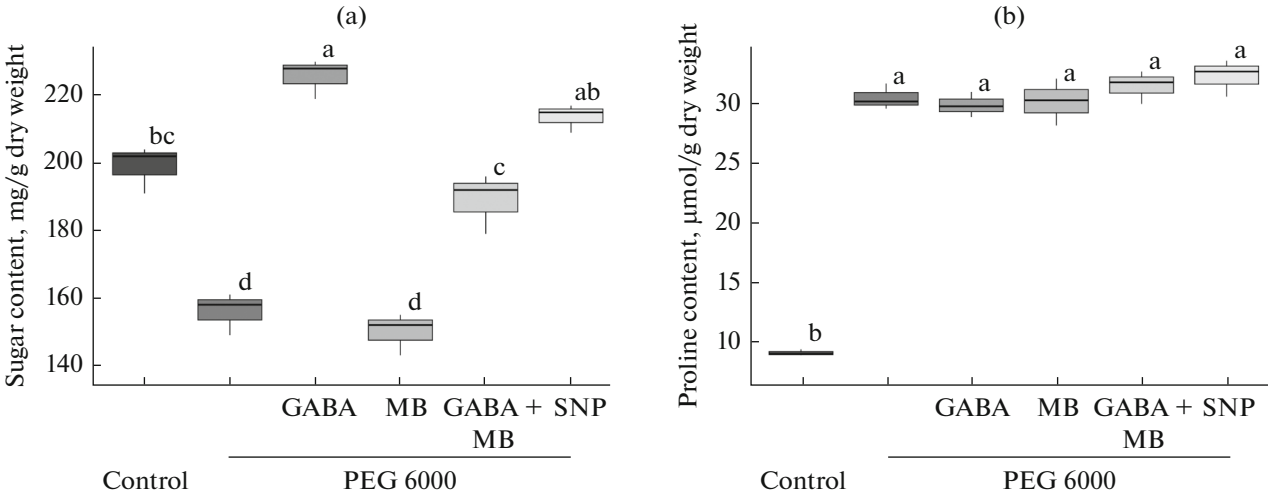

Fig. 5. (a) Sugar content and (b) proline in shoots of wheat seedlings under the influence of model drought (13% PEG 6000), GABA (0.5 mM), MB (0.1 mM), and SNP (0.1 mM). The same Latin letters above the Tukey boxes indicate values between which there is no difference, significant at $p \le 0.05$.

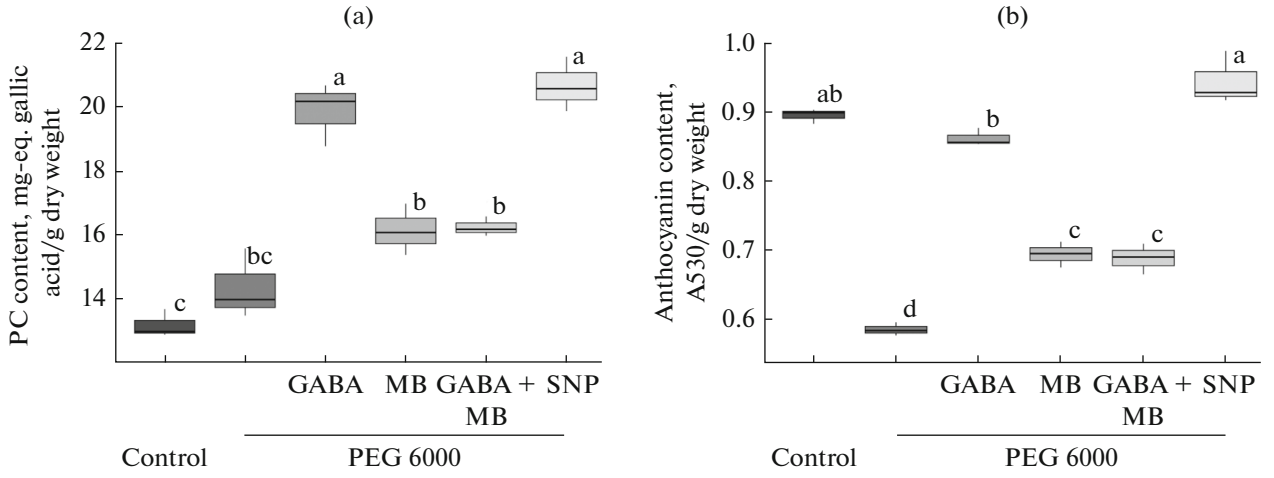

Fig. 6. (a) Total content of phenolic compounds (PC) and (b) the number of anthocyanins in shoots of wheat seedlings under the influence of model drought (13% PEG 6000), GABA (0.5 mM), MB (0.1 mM), and SNP (0.1 mM). The same Latin letters above the Tukey boxes indicate values between which there is no difference, significant at $p \le 0.05$.

effect on grain germination and plant resistance at the early stages of development was not investigated.

In our study, the role of nitric oxide as a signaling mediator in the stress-protective action of GABA is indicated by the nearly complete elimination of the positive effect of GABA seed priming on grain germination and seedling growth under stress conditions, as well as on their water content, upon simultaneous treatment with the NO scavenger MB (Figs. 1, 2). An increase in endogenous NO content was also detected in shoots of 3-day-old seedlings under the action of GABA and the absence of such an effect in the presence of MB (Fig. 3). At the same time, treatment of grains with SNP led to an increase in the content of endogenous NO in shoots and reproduced most of the physiological effects caused by the GABA action.

Probably one of the earliest effects of GABA seed priming is the increase in amylase activity in the kernels under its influence (Fig. 4). Similar effects of GABA have been recorded in some other plant species. Thus, under the influence of GABA, a dose-dependent increase in α-amylase activity, increased expression of its gene, and a decrease in starch content in barley kernels were shown (Sheng et al., 2018). The same work showed a relationship between the increase in endogenous GABA content in grains and their amylase activity. It was also found that GABA treatment of white clover seeds (*Trifolium repens*) under salt stress conditions caused an increase in starch hydroly-

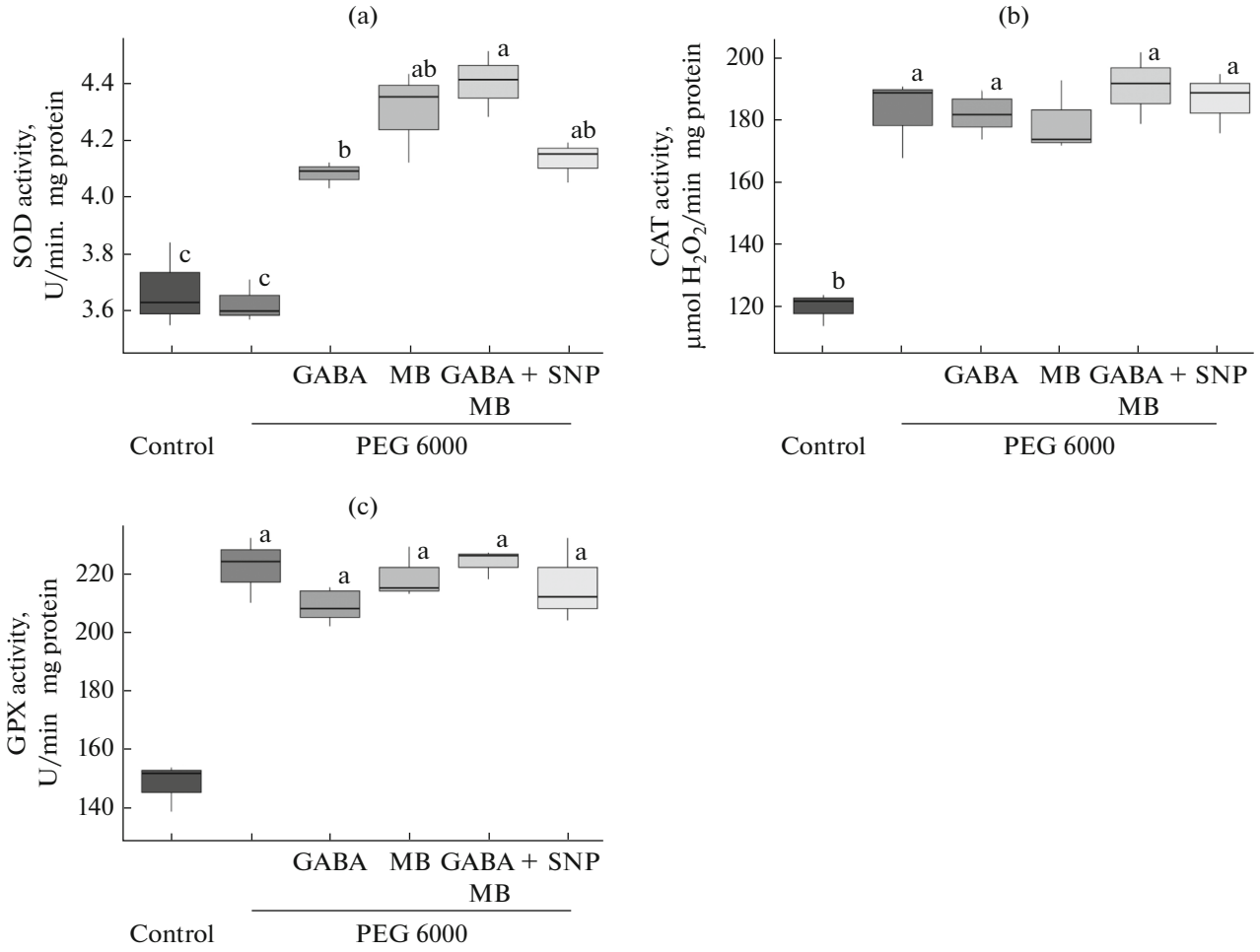

Fig. 7. (a) SOD, (b) CAT, and (c) GPO activity in wheat seedlings shoots under the influence of model drought (13% PEG 6000), GABA (0.5 mM), MB (0.1 mM), and SNP (0.1 mM). The same Latin letters above the Tukey boxes indicate values between which there is no difference, significant at $p \leq 0.05$.

sis, due to an increase in amylase activity (Sheteiwy et al., 2019). Our experiments show for the first time the involvement of NO by the inhibitory method in increasing amylase activity under the action of GABA on grains (Fig. 4). The absence of an increase in enzyme activity in the variant with simultaneous treatment of grains with GABA and the NO scavenger MB indicates the role of nitric oxide as a mediator in the implementation of this effect of GABA. It is important that exogenous NO also caused an increase in amylase activity in grains that was greater in magnitude compared to the action of GABA. The mechanisms of NO-mediated increase in amylase activity under the action of GABA remain unclear. However, there is data in the literature on the ability of NO to increase amylase activity in grains. In particular, it has been shown quite recently that amylase activity in wheat grains was increased by SNP under salt stress (Zheng et al., 2009). The authors consider this effect as one of the mechanisms of increasing the salt tolerance of seedlings. The ability of the NO donor SNP to increase the activity of the enzyme in corn kernels has also been shown (Patel et al., 2017). However, the specific mechanisms of the effect of nitric oxide on amylase activity remain unknown.

The increase in amylase activity in grains under the influence of GABA may be one of the reasons for the increase in the content of soluble carbohydrates in the shoots of seedlings (Fig. 5a). It is worth noting that the effect of the model drought itself caused a decrease in the content of sugars in the shoots in the absence of significant changes in amylase activity. One of the probable reasons for this effect may be the increased consumption of soluble carbohydrates as a substrate for the processes of respiration and the synthesis of other metabolites necessary for adaptation to dehydration, in particular, proline. The effects of increased respiration intensity and the accumulation of stress metabolites of a noncarbohydrate nature under conditions of dehydration, have been described using the example of various plant species, including wheat (Zagdanska, 1995; Farooq et al., 2024).

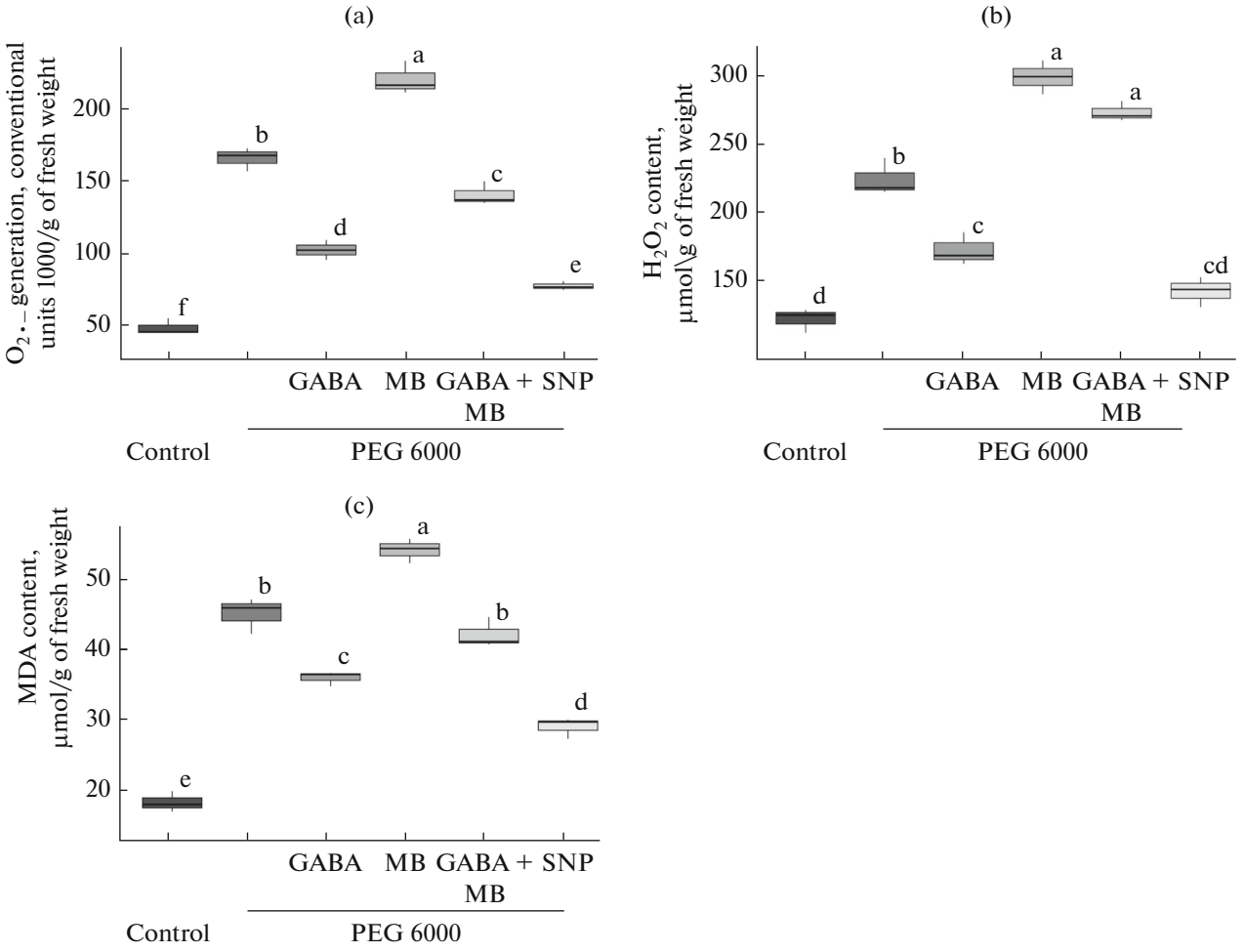

Fig. 8. (a) Generation of superoxide radical by seedling shoots, (b) their hydrogen peroxide content, and (c) MDA under the influence of model drought (13% PEG 6000), GABA (0.5 mM), MB (0.1 mM), and SNP (0.1 mM). The same Latin letters above the Tukey boxes indicate the values between which there is no difference, significant at $p \leq 0.05$.

The increase in the content of soluble carbohydrates in shoots caused by GABA treatment, as well as the increase in amylase activity, depended on the NO status of plant cells, although MB treatment only partially eliminated the GABA-induced increase in the content of sugars in shoots (Fig. 5a). It is possible that the content of sugars under the influence of GABA is regulated by a complex of processes, including processes dependent and independent of NO. In general, it can be safely assumed that the increase in the content of soluble carbohydrates in tissues is one of the important components of the effect of GABA on the resistance of plants to osmotic stresses. Similar effects were observed with different methods of GABA treatment (input through the roots or seed priming) under conditions of model drought (effect of PEG 6000) and/or salinity in triticale and wheat seedlings (Kolupaev et al., 2024d; Shakhov et al., 2025). GABA priming of white clover seeds also induced an increase in sugar content under salt stress (Cheng et al., 2018; Sheteiwy et al., 2019). The involvement of NO in GABA-induced changes in sugar content was not specifically investigated in these works. At the same time, it was shown that, under salt stress, priming wheat seeds with the NO donor SNP caused a decrease in starch content and an increase in sugar content (Zheng et al., 2009). Our experiments also revealed an increase in the sugar content in the shoots of wheat seedlings under conditions of osmotic stress under the influence of SNP seed priming (Fig. 5a).

As already noted, along with sugars, proline is considered one of the important osmolytes of plant cells (Mansour and Salama, 2020; Dubrovna et al., 2022a; 2022b; Ghosh et al., 2022). Proline and GABA are closely related metabolically and functionally. Both compounds are synthesized from the same substrate: glutamate (Pál et al., 2018). It is known that exogenous GABA is able to increase the content of endogenous GABA due to the activation of the GABA shunt (Li et al., 2021). At the same time, an increase in the GABA and glutamate pool can also cause increased proline synthesis (Kolupaev et al., 2025b). However,

data on the effect of exogenous GABA on proline content in plants under stress conditions are quite contradictory. Thus, it has been shown that long-term (for 18 h) priming of wheat seeds with 1 μM GABA caused an increase in proline content in plant leaves under drought conditions (Al Ghafri et al., 2025). GABA treatment of wheat seedlings by its entry through the roots increased the proline content in a drought-sensitive variety under osmotic stress but decreased it in a drought-resistant variety (Kolupaev et al., 2023b). A decrease in proline content upon GABA seed treatment has also been recorded in white clover seedlings under salt stress (Cheng et al., 2018). However, in this work, we were unable to detect changes in proline content in shoots of wheat seedlings exposed to model drought. Osmotic stress itself caused a significant increase in proline content in shoots (Fig. 5b). At the same time, treatment with neither GABA nor other studied compounds caused significant modulations of proline content. It can be assumed that, at least under such experimental conditions, proline accumulation does not have a critical dependence on the amount of GABA and NO in cells.

The regulation of secondary metabolite synthesis by GABA and nitric oxide was much more pronounced in the model we studied (Fig. 6a). The GABA-induced increase in the total content of phenolic compounds was not detected in the presence of the NO scavenger MB, while SNP treatment also caused an increase in their amount under stress conditions. The literature reports positive relationships between endogenous GABA content and the content of phenolic compounds in germinating wheat grains (Kim et al., 2018). It was also found that priming of rice grains with GABA caused the activation of phenolic metabolism enzymes and the accumulation of phenolic compounds under osmotic and salt stress conditions (Sheteiwy et al., 2019). In general, the ability of GABA to enhance the synthesis of secondary metabolites has been recorded in plants of different taxonomic affiliation under osmotic stress conditions. It is assumed that GABA, among stress bioregulators, plays a specific role in regulating the content of phenolic compounds in plants (Dabravolski and Isayenkov, 2023). Among the variety of secondary metabolites, polyphenolic compounds, in particular, anthocyanins, are considered to be particularly important for antioxidant protection (Neill and Gould, 2003). Our data indicate that priming wheat grains with GABA prevented the depletion of the anthocyanin pool caused by the action of a model drought (Fig. 6b). However, this effect of GABA was eliminated by the action of the NO scavenger MB. On the other hand, treatment with the NO donor, like GABA, contributed to the preservation of the anthocyanin pool under the action of osmotic stress. It should be noted that, when inducing germination of aged wheat and triticale seeds by SNP priming, the effects of increasing the total content of phenolic compounds and anthocyanins were detected (Kolupaev et al., 2025a).

However, we do not know the data on the participation of nitric oxide as a mediator in the regulation of the content of phenolic compounds under the action of GABA in cereals. At the same time, it was shown that, in soybean plants, the increase in the synthesis of total phenolic compounds caused by GABA treatment under salt stress was reduced in the presence of both the inhibitor of NO synthesis via the oxidative pathway L-NAME (N(ω)-nitro-L-arginine methyl ester) and the nitrate reductase inhibitor sodium tungstate, which indicates the role of two main pathways of nitric oxide synthesis in the manifestation of the stress-protective effect of GABA (Xie et al., 2021). Therefore, it is likely that the involvement of GABA in the regulation of the synthesis of secondary metabolites in plants is mediated by nitric oxide.

The effect of priming of grains with GABA and NO donor on the activity of antioxidant enzymes under model drought conditions was less pronounced than its effect on the synthesis of stress metabolites. In particular, only an increase in SOD activity was detected when treated with these compounds; however, the activity of CAT and GPO increased under the influence of osmotic stress, but priming with GABA or SNP did not cause any significant additional modulations of the activity of these enzymes (Fig. 7). An unusual phenomenon is the increase in SOD activity under stress conditions when treated with the NO scavenger MB. In general, a large amount of data has been accumulated in the literature on the enhancement of the enzymatic antioxidant system of plants under the influence of nitric oxide (Correa-Aragunde et al., 2013; Arora et al., 2016; Mukherjee and Corpas, 2023). Such effects can be due to both posttranslational modifications of enzymatic proteins and the effect of NO on the expression of genes of these enzymes. However, isolated phenomena of increased antioxidant enzyme activity under the action of nitric oxide scavengers or inhibitors of its synthesis have been described. In particular, with regard to SOD, it has been shown that treatment of bean plants with 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) enhanced the increase in enzyme activity caused by the toxic effect of arsenic (Talukdar, 2013). The effect of increased SOD activity in wheat roots was found when they were treated with both nitric oxide antagonists (PTIO and L-NAME) and its donor SNP (Karpets et al., 2015). These phenomena may be due to probable, but not yet specifically studied, posttranslational modifications of enzymatic proteins (Arora et al., 2016). Such modifications of antioxidant enzyme molecules by nitric oxide can lead to both an increase and a decrease in activity, depending on the experimental conditions, in particular, the local concentration of NO. At the same time, direct modulation of the activity of antioxidant enzymes by nitric oxide can lead to changes in redox homeostasis and, accordingly, to the formation of sig-

nals that cause shifts in the expression of antioxidant enzyme genes (Mukherjee and Corpas, 2023; Kolupaev et al., 2023c). Thus, it is quite likely that, under certain experimental conditions, activation of antioxidant enzymes by both exogenous NO and its scavengers can be observed as can the absence of noticeable changes in the activity of these enzymes. To clarify the mechanisms of such phenomena, special studies on other models are necessary, including the use of methods for determining posttranslational modification of target proteins.

Overall, the obtained results indicate that priming wheat grains with GABA significantly reduced the development of oxidative stress induced by the model drought (Fig. 8). At the same time, the reduction in ROS generation and the decrease in LPO intensity under the influence of GABA were eliminated by the action of the NO scavenger MB and, therefore, were probably NO-mediated. This is also indicated by a significant reduction in all studied oxidative stress indicators upon seed treatment with the nitric oxide donor SNP (Fig. 8). Such results are fully consistent with the numerical data on the reduction of oxidative stress manifestations in plants under drought conditions under the influence of exogenous GABA (Yong et al., 2017; Tang et al., 2020; Zhao et al., 2023) and nitric oxide donors (Tian and Lei, 2006; Majeed et al., 2018).

Finally, it is worth noting that there is no evidence in the literature to date of the participation of NO in the protective reactions of plants induced by the action of GABA, which would be obtained by molecular genetic methods (Kolupaev et al., 2024a). However, recently obtained data from metabolomic analysis indicate that NO and GABA, causing stress-protective effects, can activate both common and different metabolic pathways (Kabała and Janicka, 2024). At the same time, the possible interaction between nitric oxide and other key signaling mediators, primarily ROS and calcium ions, in the formation of GABA-induced NO-mediated adaptive responses of plants remains completely unexplored.

Thus, our work shows for the first time that the induction of seed germination and growth of wheat seedlings by GABA priming under osmotic stress is mediated by nitric oxide. The main components of this effect of GABA are changes in carbohydrate metabolism and synthesis of secondary metabolites, namely, an increase in amylase activity in grains and accumulation of sugars in shoots of seedlings under its influence, an increase in the total content of phenolic compounds, and stabilization of the content of anthocyanins, which are characterized by particularly high antioxidant activity, under stress conditions. The metabolic changes caused by GABA caused a decrease in the manifestation of oxidative stress (ROS generation and accumulation of LPO products) in wheat seedlings under drought conditions. None of the listed effects of GABA were manifested in the presence of the NO scavenger MB. At the same time, under the action of GABA, there was an increase in the endogenous content of NO in the shoots, and exogenous treatment of seeds with the nitric oxide donor SNP led to physiological effects similar to those caused by the action of GABA.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any research using humans or animals as subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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