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Abstract: Gamma-aminobutyric acid (GABA) is considered an important stress metabolite with regulatory functions in plants. Exogenous GABA can enhance the tolerance of different plant species to drought and salt stress. However, its effects on the functioning of stress-protective systems in triticale, an intergeneric wheat-rye hybrid with specific adaptation strategies to abiotic stresses, remain completely understudied. We examined the effects of exogenous GABA (0.5 mM) on the growth, antioxidant, and osmoprotective systems of triticale seedlings (cv. 'Raryet') under simulated drought (15% PEG 6000), salt stress (100 mM NaCl), or a combination of these two factors. GABA mitigated the growth-inhibitory effects of drought by approximately 15% and salt stress by approximately 23%. However, GABA's most noticeable impact was seen on biomass accumulation under combined stress: it decreased the biomass accumulation inhibition caused by a drought-salt stress combination by 67%; GABA also increased the water content in seedling tissues when subjected to each stressor separately and to the combination of the two. GABA considerably reduced drought- and salt-triggered enhancement of ROS generation and lipid peroxidation. GABA increased the activities of antioxidant enzymes, such as superoxide dismutase, catalase, and guaiacol peroxidase, under physiologically normal conditions under the influence of PEG 6000 or NaCl. Subjected to a drought-salt stress combination, GABA prevented a reduction in the activities of these enzymes. Under normal and stressful conditions, exogenous GABA also modulated the contents of proline, sugars, phenolic compounds, and anthocyanins in the seedlings. We concluded that the stress-protective systems of triticale were highly susceptible to GABA and that it was possible to use GABA to enhance the tolerance of this species to osmotic stresses, especially under a combined impact of drought and salinity.

Key words: × *Triticosecale*, gamma-aminobutyric acid, drought, salt stress, antioxidant system, osmolytes

1. Introduction

Ideas about the potential functions of gamma-aminobutyric acid (GABA) for plant adaptation to stress factors have recently emerged. Traditionally, GABA has been considered an important component of the free amino acid storage system in plants, supporting carbon and nitrogen metabolism, which is necessary for plant growth (Ansari et al., 2014; Goharrizi et al., 2021). The direct protective effect of GABA is associated with its involvement in the maintenance of reducing agent pools and its role in the tricarboxylic acid cycle through GABA shunt reactions (Che-Othman et al., 2020). Under plant tissue dehydration-related stresses, GABA and proline can significantly contribute to increased osmotic pressure in cells (Goharrizi et al., 2021).

However, GABA's effects are not limited to its direct protective impact as a stress metabolite. Many studies have demonstrated that GABA can support the functioning of the photosynthetic apparatus under stressful conditions (Ramzan et al., 2023), increase the activities of antioxidant enzymes (Wu et al., 2020; Ramzan et al., 2023), induce stomatal closure (Gahlowt et al., 2024), and affect the accumulation of ions in cells (Ullah et al., 2023). Researchers assumed that these effects were related to GABA's involvement in the signaling pathways of plant cells, including the regulation of the formation and detoxification of reactive oxygen species (ROS) (Jin et al., 2019) and the state of ion channels (Ramesh et al., 2017).

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Drought and salinity are the most common stressors affecting plants worldwide (Bakhroum et al., 2023; Sadak and Dawood, 2023). In rural areas, irrigation water reserves constantly decline—with an increase in soil salinization happening concurrently—because of the use of brackish groundwater (Abobatta, 2020).

Drought and salinity, especially when combined, cause a severe water deficit in plants, inhibit photosynthesis, disrupt energy metabolism, and, consequently, intensify the ROS formation and development of oxidative stress (Goharrizi et al., 2021; Sadak, 2023). The toxic impact of ions on biomacromolecules enhances the negative effects of drought (Ibrahim et al., 2019; Sadak et al., 2023).

GABA accumulation and conversion in GABA shunt reactions are known to be enhanced in many plant species under salt stress (Jalil and Ansari, 2020) and drought (Li et al., 2021). These processes help stabilize intracellular pH and carbon-nitrogen and energy metabolisms (Ramzan et al., 2023). GABA shunt has been shown to play a vital role in respiratory metabolism under salt stress, providing an alternative to the carbon supply to the tricarboxylic acid cycle through succinate synthesis due to bypassing the salt-susceptible mitochondrial pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes (Che-Othman et al., 2020).

To date, a large amount of phenomenological data has already been obtained on the exogenous GABA-induced increase in the tolerance of domestic plants to drought and salt stress (Seifikalhor et al., 2019; Sheteiwiy et al., 2019; Shomali et al., 2021; Kolupaev et al., 2023a; Ullar et al., 2023; Zhao et al., 2023). However, adaptation strategies to the same stresses can significantly differ between plant species and cultivars. The contributions of individual components of the antistress systems (in particular, antioxidant and osmoprotective) significantly depend on species characteristics (Kolupaev et al., 2022) and cultivar features (Seifikalhor et al., 2022). This does not allow for direct extrapolation of results obtained for one species to another species.

Triticale is a hybrid species derived from crossing wheat and rye. It is both a food and forage crop. Having analyzed field trial data on different cultivars, Blum (2014) reported that triticale was more drought-resistant than most wheat species. Triticale is also considered a promising salt-tolerant forage species that can be grown on arid and degraded pastures (Kankarla et al., 2020; Atak et al., 2023). However, a drought-salinity combination inhibits triticale's growth and reduces the yield to a much greater extent than each of the stressors alone (Mohammadi Alagoz et al., 2023). In this regard, searching for ways to enhance triticale's tolerance to salinity and drought—especially to a combination of the two—is entirely appropriate.

The GABA effects on the tolerance of triticale to abiotic stress factors, including drought and salinity, remain completely unexplored. Therefore, this study aimed to investigate the effects of exogenous GABA on the tolerance of triticale seedlings to PEG 6000-simulated drought, sodium chloride salinity, and a combination of these stresses. We assessed the effect of GABA on the most important antistress systems—antioxidant and osmoprotective—under physiologically normal conditions and in plants exposed to these stressors.

2. Materials and methods

2.1. Plant material and treatment

Caryopses of hexaploid winter triticale (\times *Triticosecale*) (cv. 'Rarytet' [originator: Yuriev Plant Production Institute of National Academy of Agrarian Sciences of Ukraine]) were surface sterilized with 3% NaClO for 20 min. Afterward, the seedlings were washed 8–10 times with distilled water and germinated in water-filled Petri dishes in a thermostat at 24 °C without light for 2 days. Seedlings of approximately equal length were then transferred to Petri dishes containing two layers of sterile filter paper moistened with 15% PEG 6000 or 100 mM NaCl or a combination of both. The control seedlings were transferred to the Petri dishes with filter paper moistened with distilled water. GABA (Acros Organics BV, China) was added to the final concentration of 0.5 mM. This GABA concentration, which exerts the most noticeable stress-protective effect (determined by an alleviation of the stress-induced growth inhibition), was chosen based on preliminary experiments with 0.1–2.5 mM GABA. After a two-day exposure of the seedlings to PEG 6000, NaCl, a combination of the two, and/or GABA, the biomass of the shoots and roots of the seedlings was measured, and biochemical analyses were performed.

2.2. Determination of total water content in seedlings

The total water content in the seedling tissues was determined gravimetrically by drying the samples at 103 °C to constant weight.

2.3. Superoxide anion radical generation

The superoxide anion radical generation by seedlings was estimated by nitroblue tetrazolium (NBT) reduction. Ten identical seedlings were incubated in a test tube with 5 mL of 0.1 M K,Na-phosphate buffer (pH 7.6) containing 0.05% NBT, 10 μ M EDTA, and 0.1% Triton X-100 for 1 h (Kolupaev et al., 2012). After the incubation, the seedlings were removed from the incubation solution and assessed for the intensity of O₂^{•-} generation. The incubation solution's absorbance was measured at 530 nm using an SF 46 spectrophotometer (LOMO, Russia). The results were expressed in units of absorbance \times 1000/g fresh weight (FW).

2.4. Evaluation of hydrogen peroxide content

To determine the H_2O_2 content, the shoots (300 mg) were homogenized in cold 5% trichloroacetic acid (TCA; 6 mL). The samples were centrifuged on an MPW 350R centrifuge (MPW MedInstruments, Poland) at 8000 g for 10 min at 2–4 °C. The hydrogen peroxide concentration in the supernatants was measured using the ferrothiocyanate method (Sagisaka, 1976) with slight modifications. Precisely 0.5 mL of 2.5 M ammonium thiocyanate (NH_4SCN), 0.5 mL of 50% TCA, 1.5 mL of supernatant, and 0.5 mL of 10 mM iron (II) ammonium sulfate ($(NH_4)_2Fe(SO_4)_2$) were added to the test tubes. After mixing, the samples were transferred to cuvettes, and the absorbance, at 480 nm, was determined. The results were expressed in nmol H_2O_2/g FW.

2.5. Evaluation of LPO product content

The lipid peroxidation (LPO) rate in the shoots was assessed by the reaction of its products (mainly malondialdehyde (MDA)) with 2-thiobarbituric acid (TBA) (Kolupaev et al., 2023b). The samples (300 mg) were homogenized in 6 mL of 0.1 M Tris-HCl buffer (pH 7.6). Exactly 1 mL of 0.5% TBA in 20% TCA was added to the homogenates. The mixture was heated in a boiling water bath for 30 min, then cooled and centrifuged at 8000 g for 10 min. The supernatant's absorbance was then measured at 532 nm. The nonspecific absorbance at 600 nm was also determined, with the value subtracted from the main result. The absorbance was measured against a blank sample (reagent mixture without TBA). The results were expressed in nmol MDA/g FW.

2.6. Measurements of the activities of antioxidant enzymes

The activities of antioxidant enzymes—superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and guaiacol peroxidase (GPX, EC 1.11.1.7)—were measured using the previously described protocols (Kolupaev et al., 2020). The seedling samples (300 mg) were homogenized in 10 mL of cold 0.15 M K,Na-phosphate buffer (pH 7.6) supplemented with EDTA (0.1 mM) and dithiothreitol (1 mM). The homogenates were centrifuged on an MPW 350R centrifuge (MedInstruments, Poland) at 8000 × g for 15 min at 2–4 °C to collect the supernatants for assays. The SOD activity was determined at pH 7.6. We used a method based on the enzyme's ability to compete with nitroblue tetrazolium for superoxide anions produced due to the aerobic interaction between NADH and phenazine methosulfate. The CAT activity was evaluated in terms of decomposed H_2O_2 amount. The GPX activity was analyzed at pH 6.2 (K,Na-phosphate buffer) using guaiacol as a hydrogen donor and hydrogen peroxide as a substrate. The activity of SOD was expressed in relative units/(min × g DW), CAT in mmol H_2O_2 /(min × g DW), and GPX in relative units/(min × g DW).

2.7. Evaluation of the proline content

Proline content in the shoots was determined using a modification of Bates et al.'s (1973) method. The absorbance of the color reaction product was determined at 520 nm using L-proline as a reference substance. The results were expressed in $\mu\text{mol/g}$ dry weight (DW).

2.8. Determination of the total soluble carbohydrate content

The total sugar content in the plants was determined by our modification of the Morris–Roe method based on the anthrone reagent (Yastreba et al., 2023). Sugars were extracted from the plants (300 mg) with distilled water (10 mL) by heating the samples in a boiling water bath for 10 min. The resulting extracts were clarified by adding equal volumes (0.3 mL) of 30% zinc sulfate and 15% yellow blood salt to the reaction test tubes. The samples were filtered through paper filters and, if necessary, several-fold diluted with distilled water before measurement. Each reaction test tube was filled with 3 mL of the anthrone reagent and 1 mL of filtrate. Instead of filtrate, distilled water was added to the control (blank) sample. After boiling them for 7 min in a water bath, the samples were cooled, and the absorbance (at 610 nm) was determined against the blank (control) solution. D-glucose was used as a reference substance. The results were expressed in mg/g DW.

2.9. Determination of the content of phenolic compounds and anthocyanin

To determine the total phenolic compounds and anthocyanins, the seedlings (300 mg) were homogenized in 6 mL of 80% ethanol. The extraction was conducted at room temperature for 20 min. The samples were centrifuged at 8000 × g for 15 min. To evaluate the content of the phenolic compounds, 0.5 mL of supernatant, 8 mL of distilled water, and 0.5 mL of Folin's reagent were added to the reaction test tubes and stirred; 1 mL of 10% sodium carbonate was added 3 min later. After 1 h, the reaction mixture absorbance was measured at 725 nm (Bobo-García et al., 2015). The content of the phenolic compounds was expressed as μmoles of gallic acid/g DW.

Before determining the anthocyanin content, the supernatants were acidified with HCl to a final concentration of 1%. The absorbance was determined at 530 nm (Nogués and Baker, 2000), and the results were expressed in units of absorbance/g DW.

2.10. Replication of experiments and statistical processing of data

The experiments had three biological replicates. When we determined the seedling biomass, each replicate consisted of at least 30 seedlings. In the biochemical assays, there were 12–15 seedlings per sample. Data were statistically processed using analysis of variance (ANOVA) and Fisher's least significant difference test. The Figures show

the mean values and the corresponding standard errors. Different letters denote values with significant differences at $p \leq 0.05$.

3. Results

3.1. Seedling biomass and water content under the influence of GABA, drought, and salt stress

Treatment with GABA slightly increased the weight of the triticale shoots and roots (Figure 1). Under PEG 6000-simulated drought, there was a twofold decrease in the weight of the shoots and roots. Salt stress had a slightly less significant effect on the growth of the seedlings' organs compared to drought (45% decrease in biomass compared to the control). The combined effect of drought and salt stress caused very severe inhibition of seedling growth. The weight of the shoots and roots decreased by approximately 77% compared to the control group (Figure 1).

Exogenous GABA significantly (by approximately 15%) alleviated the drought-induced inhibition of seedling growth (Figure 1). Under salt stress, GABA also notably enhanced the growth of both shoots and roots in a very similar manner (by 20–25%). GABA also significantly mitigated seedling biomass accumulation inhibition caused by the drought-salt stress combination

(Figure 1) and had a more noticeable impact on root growth. However, the total biomass of the GABA-treated seedlings and seedlings subjected to concurrent drought and salt stresses was significantly (67%) higher than that of untreated seedlings.

Treatment with GABA did not affect the water content in the seedling tissues under physiologically normal conditions (Figure 2). Drought reduced the water content by 9.3%. The effect of salt stress was much less pronounced: the water content decreased by 3.9%. A particularly dramatic drop in the water content in the seedlings (by 14.5%) was observed after initiating a drought-salt stress combination. GABA caused a significant ($p \leq 0.05$) increase in the water content in the seedling tissues exposed to both stressors or their combination (Figure 2).

3.2. GABA treatment alleviates the manifestations of oxidative stress in triticale seedlings under drought and salinity

Treatment with GABA caused a significant decline in the superoxide anion radical generation in the absence of stressors (Figure 3a). Under drought—especially under salt and combined stress—the $O_2^{\cdot -}$ generation by the seedlings increased significantly. GABA reduced the drought-enhanced superoxide anion radical generation to the

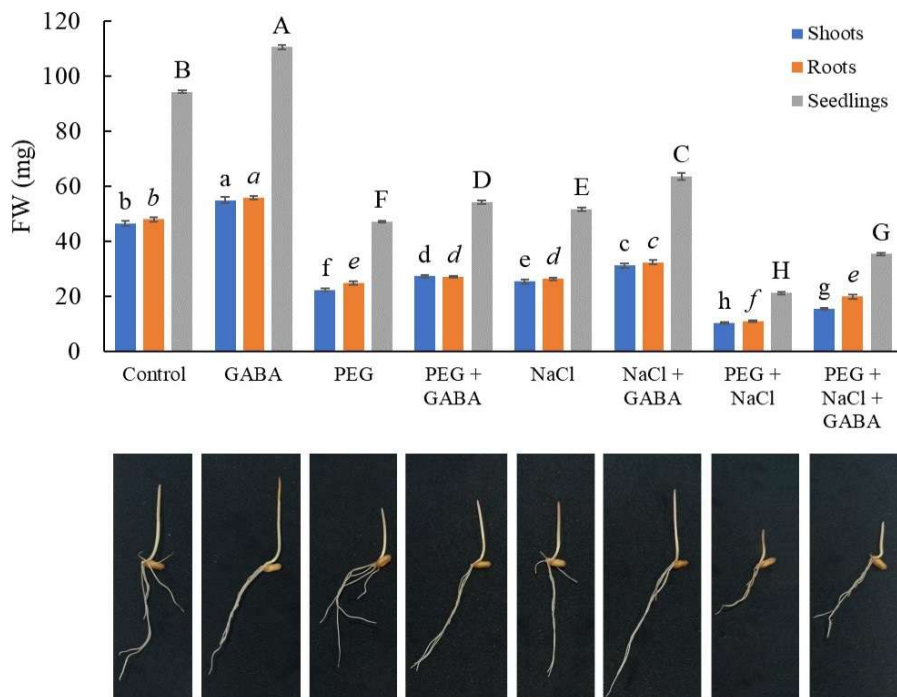


Figure 1. Fresh weight of organs of triticale seedlings and their phenotype under the influence of GABA, osmotic and salt stresses, and the combination of the two; 2-day-old seedlings were treated with GABA (0.5 mM), PEG 6000 (15%), NaCl (100 mM), or their combined effects; measurements were carried out 48 h after all treatments. Here and in Figures 2–6, identical letters above the columns indicate quantities whose differences are not significant at $p \leq 0.05$.

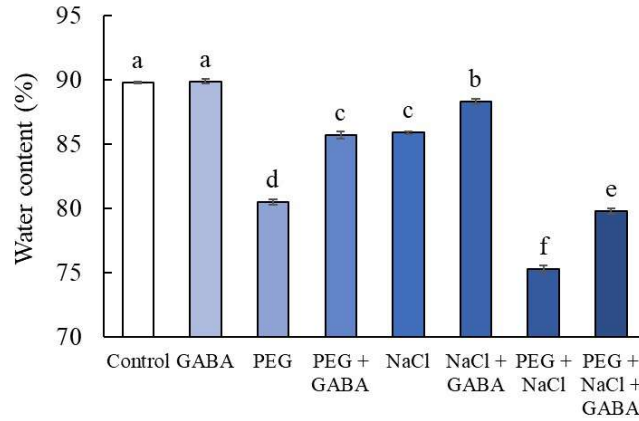


Figure 2. Water content (%) in triticale shoots under the influence of GABA, osmotic and salt stresses, and their combination; 2-day-old seedlings were treated with GABA (0.5 mM), PEG 6000 (15%), NaCl (100 mM), or their combined effects; measurements were carried out 48 h after all treatments.

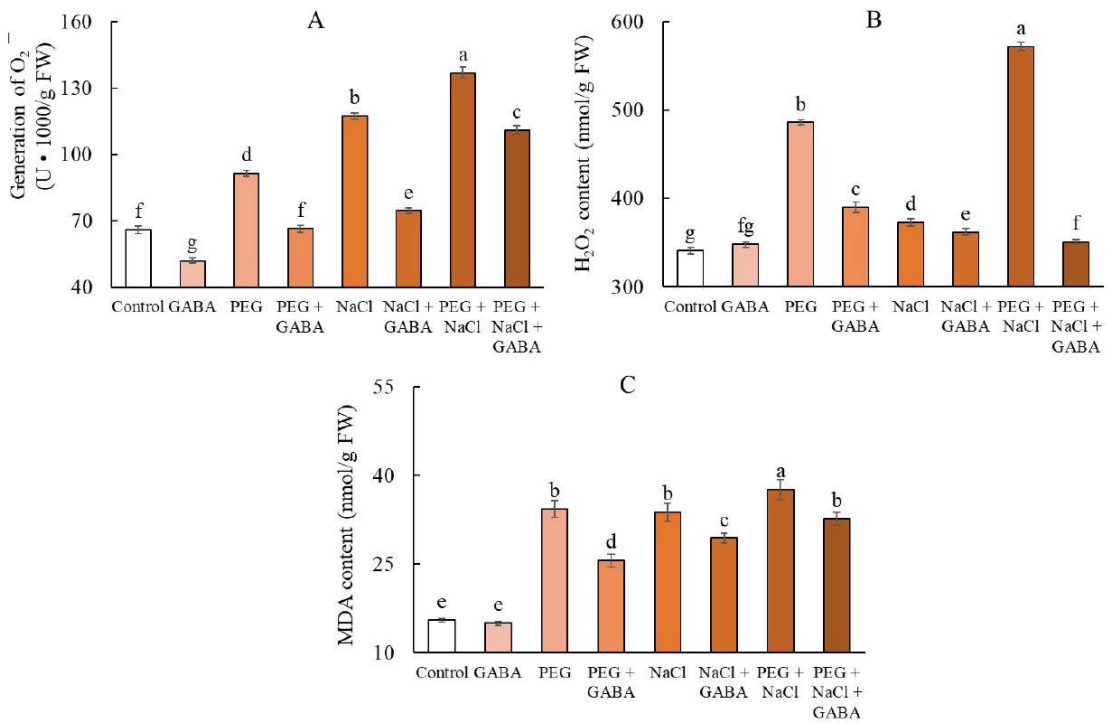


Figure 3. Superoxide anion radical generation (A), contents of hydrogen peroxide (B), and malondialdehyde (MDA) (C) in triticale shoots under the influence of GABA, osmotic and salt stresses, and their combination; 2-day-old seedlings were treated with GABA (0.5 mM), PEG 6000 (15%), NaCl (100 mM), or their combined effects; measurements were carried out 48 h after all treatments.

control level (Figure 3a). In addition, GABA considerably reduced the O₂⁻ generation in seedlings exposed to salt stress. When drought and salt stress were combined, the effect of GABA on the amount of superoxide anion radical was less pronounced, though still significant ($p \leq 0.05$).

The hydrogen peroxide content in the seedlings did not change significantly when they were treated with GABA under physiologically normal conditions (Figure 3b). Drought increased the H₂O₂ content in the shoots by approximately 43%. The effect of salt stress was

less pronounced, although significant ($p \leq 0.05$). A particularly drastic rise in the hydrogen peroxide content (68% compared to the control) in the triticale shoots was observed following exposure to a drought-salinity combination. GABA significantly mitigated oxidative stress manifestations, reducing the hydrogen peroxide content in shoots exposed to the stressors or their combination to the control level (Figure 3b).

The content of a major end product of LPO or MDA did not change the GABA-treated seedlings in the absence of stressors (Figure 3c). Drought and salt stress caused a more than twofold increase in the MDA content in the shoot tissues. This indicator of oxidative stress rose to an even greater extent under the combined action of the two abiotic stresses (Figure 3c). Treatment with GABA significantly mitigated oxidative stress manifestations under drought, salt stress, and their combination, reducing the MDA content in the triticale shoots. This GABA-related effect was especially pronounced after the drought experiment.

3.3. GABA modulation of the activities of antioxidant enzymes in triticale shoots under normal conditions, drought, and salt stress

Treatment of seedlings with GABA increased SOD activity in the absence of stressors (Figure 4a). Under drought,

the enzyme activity also increased, but salt stress and the drought-salinity combination caused no significant changes in SOD activity. Under drought, the GABA treatment resulted in an additional increase in SOD activity in the shoots (Figure 4a). GABA also significantly increased the enzyme activity under salt stress and a combination of the two stressors.

GABA treatment increased CAT activity in the absence of stressors (Figure 4b). Drought decreased the enzyme activity in the triticale shoots, while salt stress did not significantly affect this parameter. The combination of the two stressors significantly reduced CAT activity in the shoots. Treatment with GABA prevented a drought-induced decrease in CAT activity in the shoots. Under salt stress, enzyme activity was increased in the GABA-treated seedlings (Figure 4b). GABA also mitigated a potent inhibition of CAT activity caused by the combined action of drought and salinity on the seedlings.

In the absence of stress factors, the treatment of seedlings with GABA led to an increase in guaiacol peroxidase (GPX) activity (Figure 4c). Drought slightly decreased enzyme activity, while salt stress—especially combined stress—led to a noticeable rise in GPX activity. Under drought, GABA caused a small though significant ($p \leq 0.05$) change in GPX activity, increasing it to the

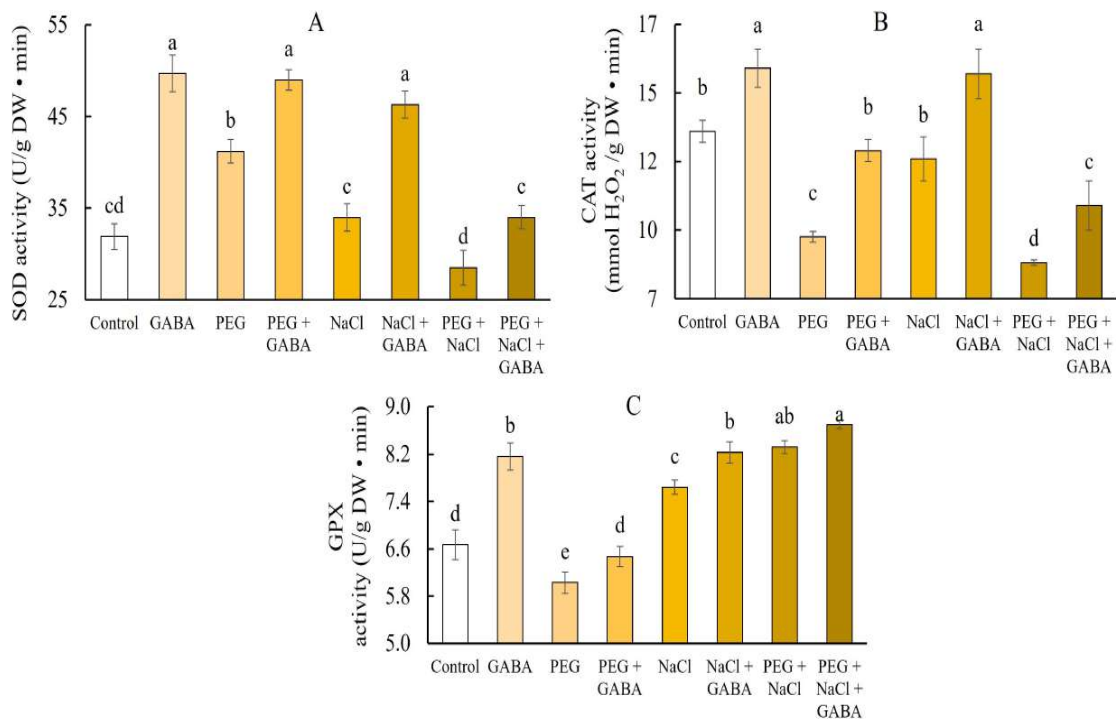


Figure 4. Activities of superoxide dismutase (SOD) (A), catalase (CAT) (B), and guaiacol peroxidase (GPX) (C) in triticale shoots under the influence of GABA, osmotic and salt stresses, and their combination; 2-day-old seedlings were treated with GABA (0.5 mM), PEG 6000 (15%), NaCl (100 mM), or their combined effects; measurements were carried out 48 h after all treatments.

control level (Figure 4c). Under salt stress in the presence of GABA, enzyme activity also increased. The combined effect of the two stressors was manifested as a trend.

3.4. GABA treatment increases the contents of compatible osmolytes in triticale shoots under normal conditions, drought, and salt stress

In the absence of stress factors, treatment of triticale seedlings with GABA resulted in a 28% increase in the proline content compared to the control (Figure 5a). Under drought and salt stress, there was a 2.62- and 2.47-fold increase in the proline content in the shoots, respectively. We observed an even more pronounced (2.75-fold) increase in the proline content in shoots exposed to the drought-salt stress combination (Figure 5a). GABA also increased the proline content under drought and salt stress. However, this effect was negligible under the combined action of the two stressors (Figure 5a).

Treatment with GABA increased the total content of soluble carbohydrates by almost 20% in triticale shoots in the absence of stressors (Figure 5b). There was a downward trend (the difference was insignificant: $p > 0.05$) in the sugar content under drought. Under salt stress, however, the content of soluble carbohydrates in the shoots increased, albeit the difference was relatively small. Under the combined action of the two stress factors, the sugar content in the shoots decreased compared to the control group. Exposure to GABA increased the content of soluble carbohydrates in shoot tissues to varying degrees under stress conditions (Figure 5b). GABA exerted the most pronounced effect under salt stress: the sugar content was 1.5 times as high as in the control group. At the same time, under the combined action of drought and salt stress, GABA only stabilized the sugar content at the control level.

3.5. GABA modulation of the contents of phenolic compounds and anthocyanins in triticale shoots under normal and stressful conditions

In the absence of stress factors, GABA increased the total content of phenolic compounds by approximately 30% (Figure 6a). Drought and the drought-salt stress combination caused no significant changes in the phenol content, but this parameter increased by 20% in response to salt stress alone compared to the control. Treatment with GABA under both stresses separately and their combination led to a 1.4 to 1.6-fold increase in the content of phenolic compounds in triticale shoots compared to the control group (Figure 6a).

Under physiologically normal conditions, treatment of seedlings with GABA resulted in an almost 25% increase in the anthocyanin content (Figure 6b). Drought led to a more than 2-fold drop in the anthocyanin content in the shoots. Salt stress had a similar but less pronounced effect. Simultaneous exposure to drought and salinity caused the most conspicuous decline in the anthocyanin content: the anthocyanin pool decreased by 73% (Figure 6b).

Under drought, treatment with GABA mitigated the reduction in the anthocyanin content, maintaining this parameter almost at the control level (Figure 6b). Under salt stress, GABA considerably increased the anthocyanin content; in this experiment, the anthocyanin content significantly exceeded the control value. Under the combined action of the two stressors, GABA also stabilized the anthocyanin content; it was almost twice as high as in the experiments with stress exposures without GABA, although it remained lower than in the control group (Figure 6b).

4. Discussion

The results indicate that exogenous GABA enhanced the tolerance of triticale seedlings to drought, salt stress,

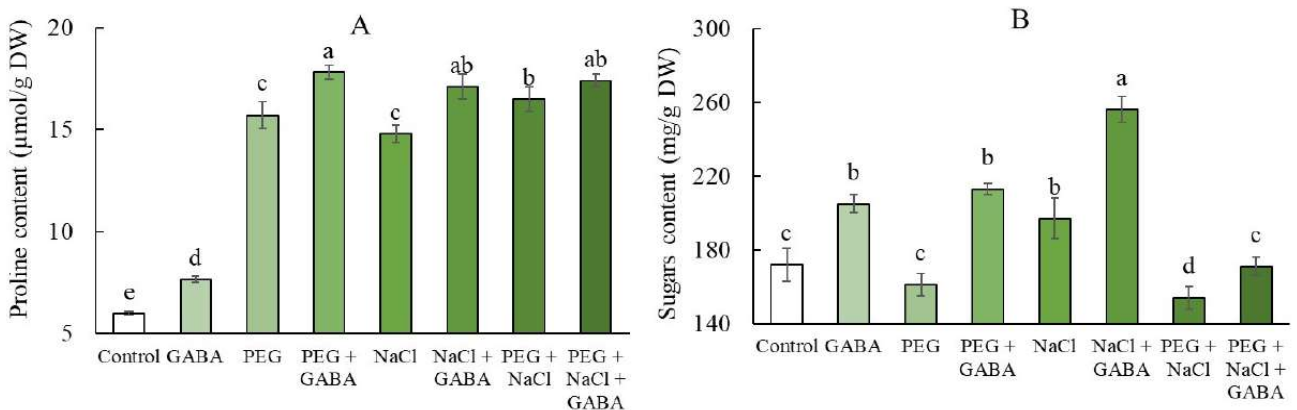


Figure 5. Contents of proline (A) and sugar (B) in triticale shoots under the influence of GABA, osmotic and salt stresses, and their combination; 2-day-old seedlings were treated with GABA (0.5 mM), PEG 6000 (15%), NaCl (100 mM), or combined effects; measurements were carried out 48 h after all treatments.

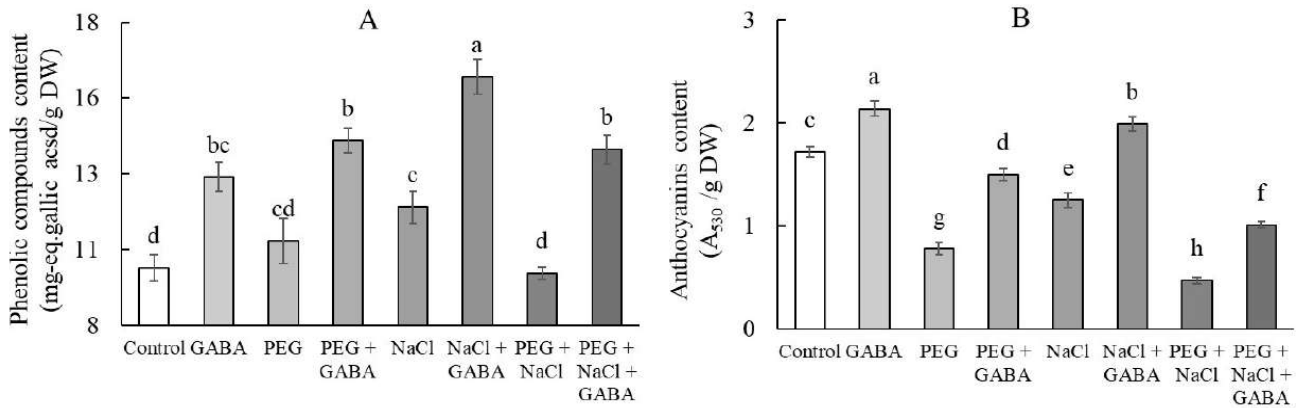


Figure 6. Contents of phenolic compounds (A) and anthocyanins (B) in triticale shoots under the influence of GABA, osmotic and salt stresses, and their combination; 2-day-old seedlings were treated with GABA (0.5 mM), PEG 6000 (15%), NaCl (100 mM), or their combined effects; measurements were carried out 48 h after all treatments.

and the combination of these two stressors. GABA had a pronounced effect on the most integral indicator—the growth of the seedlings under stressful conditions (Figures 1 and 7). It should be noted that GABA significantly ($p \leq 0.05$) affected seedling growth under physiologically normal conditions, as well; however, this stimulating effect was much less pronounced. Under salt stress, the impact of GABA on seedling biomass was more conspicuous than under drought, but under the drought-salt stress combination, the effect of GABA on seedling growth was especially pronounced (Figure 1).

Growth normalization by exogenous GABA under stressful conditions was demonstrated on different plant taxons. Treatment of drought-exposed young bean plants increased their wet weight and leaf area (Abd El-Gawad et al., 2021). GABA-treated *Vigna radiata* plants showed enhanced growth and biomass accumulation under salt stress (Ullah et al., 2023). A significant GABA-induced reduction in the germination time of *Lactuca sativa* seeds exposed to salinity was also reported (Kalhor et al., 2018). As already noted, we also observed a slightly enhanced accumulation of the biomass of triticale roots and shoots under the influence of GABA in the absence of stress (Figure 1). We assume that such phenomena may be related to the stimulating effect of GABA on cell division and elongation (Zarbakhsh and Shahsavari, 2023).

The growth effects of GABA are also associated with its protective action on the photosynthetic apparatus (Vijayakumari and Puthur, 2016; Kalhor et al., 2018; Shomali et al., 2021). However, the stress-protective effect of GABA in our study was demonstrated on etiolated triticale seedlings. Previously, we reported on GABA-caused mitigation of drought-induced inhibition of the growth of etiolated wheat seedlings (Kolupaev et al., 2023a). Thus, the protective effects of GABA are not limited to green plants with a developed photosynthetic apparatus; they can also be

seen in etiolated seedlings. This may be partly attributed to GABA-ensured stabilization of plant respiration and energy metabolism (Che-Othman et al., 2020).

It should be noted that in our experiments, 0.5 mM GABA (a relatively high concentration) was the most effective treatment for the seedlings. Some studies demonstrated a GABA-induced stress-protective effect at lower concentrations. For example, in experiments on chickpea plants, the stress-protective effect of GABA under drought was seen at concentrations of 25 and 50 μ M (Seifikalhor et al., 2022). However, plants in those experiments were treated with GABA several times by adding it to growth medium. On the other hand, in experiments on melon plants exposed to salinity-alkalinity stress, the stress-protective effect of GABA was manifested at a much higher concentration (50 mM) (Jin et al., 2019). A high concentration of GABA (2 mM) was required to register its protective effect on *Piper nigrum* plants exposed to PEG 6000-caused osmotic stress (Vijayakumari and Puthur, 2016). Finally, to enhance the heat tolerance of etiolated wheat seedlings (Kolupaev et al., 2024) and young green wheat plants (Wang et al., 2022), the greatest effect was achieved when 0.5 mM GABA was used. Apparently, optimal concentrations of GABA can differ significantly depending on plant species, methods of treatment, and, in general, the experiment design.

Treating triticale seedlings with GABA maintained the water levels in tissues exposed to drought and salt stress (Figures 2 and 7). Similar phenomena were observed in other plant species. For example, drought-stressed and GABA-treated *Trifolium repens* had a higher relative water content in leaves (Yong et al., 2017). Treatment of 4-day-old rice seedlings with GABA increased their water content under heat stress (Nayyar et al., 2014). In one of our previous studies, we detected an increase in the relative water content in drought-stressed and GABA-treated etiolated wheat seedlings (Kolupaev et al., 2023a).

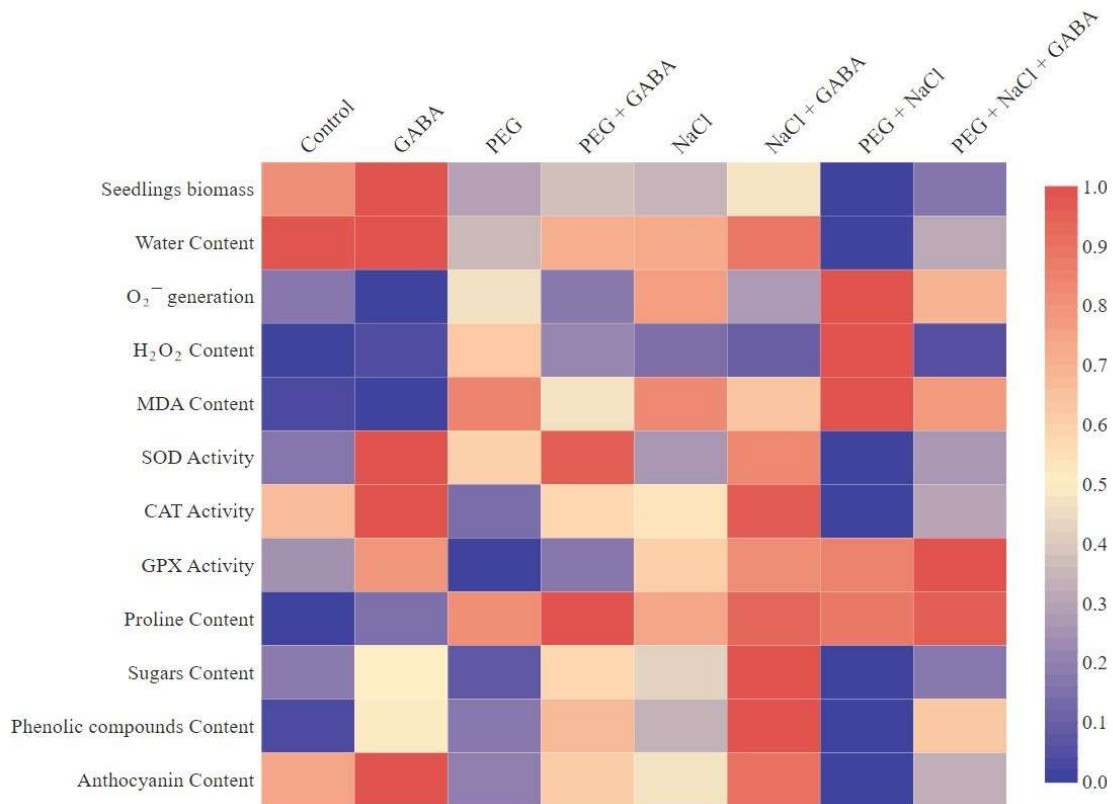


Figure 7. Heat map of changes in the parameters of the antioxidant and osmoprotective systems of triticale seedlings under simulated drought or/and salt stress and their modulation by GABA treatment. When constructing the map, each parameter was normalized from 0 to 1.

The beneficial effects of GABA on water balance may be related to intensified osmolyte synthesis. We recorded the enhanced accumulation of one of the most important multifunctional osmolytes, proline, in unstressed triticale seedlings and seedlings exposed to drought, salt stress, and their combination (Figures 5a and 7). Proline accumulation is associated with the GABA-enhanced tolerance of *Agrostis stolonifera* to osmotic stress (Li et al., 2020a). However, as exemplified when used on *Cicer arietinum*, GABA treatment under moderate drought increased the proline content in a susceptible cultivar, but it offset the drought-induced increase in the proline amount in leaves of a tolerant cultivar (Seifikalhor et al., 2022). Similar effects were also observed when the GABA effects on the proline content were compared between wheat cultivars that differed in drought tolerance: GABA enhanced the osmotic stress-triggered accumulation of proline in seedlings of a susceptible cultivar but counter-balanced an increase in the proline content in a tolerant cultivar (Kolupaev et al., 2023a). Therefore, the stress-protective effects of GABA can be specific and depend on the genotype's tolerance; exogenous GABA can activate protective mechanisms that are barely active without such stimulus (Seifikalhor et al., 2022).

GABA-enhanced accumulation of various soluble carbohydrates appears to be another component of the regulation of osmotic properties of cells. GABA treatment increased the total sugar content in triticale shoots under normal conditions and drought and salt stress (Figures 5b and 7). GABA increased the sugar content in salinity-stressed *Agrostis stolonifera* (Li et al., 2020b). GABA priming of *Trifolium repens* seeds promoted their germination under water deficit; this phenomenon was previously associated with a considerable increase in soluble carbohydrates (Zhou et al., 2021). GABA treatment of *Lens culinaris* plants enhanced sucrose synthesis under heat stress (Bhardwaj et al., 2021).

Along with the growth response and water content in tissues, the intensity of oxidative stress is considered an integral indicator of plant tolerance to abiotic factors (Li et al., 2019; Ragaey et al., 2022; Sadak et al., 2022). In our experiments, treatment with GABA significantly abated the drought-, salinity-, and drought-salinity-intensified generation of superoxide anion radical by the seedlings and the stress-induced accumulation of hydrogen peroxide and MDA (Figures 3 and 7). Thus, we can unequivocally state that preventing the development of oxidative damage

in triticale seedlings is a component of the protective effect of GABA under osmotic stress. We previously reported on GABA's similar effects in etiolated wheat seedlings subjected to osmotic stress (Kolupaev et al., 2023a). The contents of hydrogen peroxide and MDA in *Vigna radiata* plants decreased due to GABA treatment under salt-stress conditions (Ullah et al., 2023). In corn plants exposed to cadmium toxicity, major markers of oxidative stress—superoxide anion radical generation, H_2O_2 , and MDA contents—were attenuated when corn was treated with GABA (Seifikalhor et al., 2020).

The mechanisms of GABA-ensured mitigation of oxidative stress are not completely clear (Seifikalhor et al., 2022). Several causes of this effect have been discussed. In particular, Bor and Turkan (2019) assumed that the GABA shunt might regulate ROS production by mitochondria. In this case, GABA prevents the development of oxidative stress as a supplier of NADH in mitochondria where the tricarboxylic acid cycle is inhibited, respiration is impaired, and ROS are accumulated. Another mechanism may be related to the involvement of GABA in ROS signaling, which induces the enzymatic antioxidant system. Thus, as exemplified in melon plants, exogenous GABA induced the expression of genes for one of the forms of the catalytic subunit of NADPH oxidase (RBOHD) and the generation of ROS under physiologically normal conditions, simultaneously mitigating the development of oxidative stress under salt stress (Jin et al., 2019). Recently, the role of calcium-dependent enhancement of ROS generation in the induction of heat tolerance of wheat GABA-treated seedlings was demonstrated (Kolupaev et al., 2024).

In our study, GABA increased the activities of the key antioxidant enzymes—SOD, CAT, and GPX—even in seedlings not experiencing stress factors (Figures 4 and 7). Under stressful conditions, the SOD and CAT activities were also higher in the GABA-treated seedlings than in untreated ones in all cases. Previously, we showed that treatment of wheat seedlings with GABA stabilized the SOD activity in both drought-resistant and susceptible wheat cultivars under osmotic stress (Kolupaev et al., 2023a). Li et al. (2020a) reported an increase in the activities of antioxidant enzymes attributed to GABA treatment in *Cynodon dactylon* grass under salt stress.

GABA-induced activation of secondary metabolism may be another important component of the antistress effect of this compound. Triticale considerably exceeds wheat in the basic contents of secondary metabolites, particularly anthocyanins (Kolupaev et al., 2022). Due to this phenomenon, we consider triticale a rather advantageous model species for studying the effect of GABA on major characteristics of secondary metabolite syntheses. In our experiments, treatment with GABA in the absence of stress significantly increased the total

content of phenolic compounds and anthocyanins (Figure 6), polyphenolic compounds with very high antioxidant activity (Neill and Gould, 2003). Under salt and osmotic stresses, the total content of phenolic compounds in triticale seedlings increased, and treatment with GABA significantly enhanced this effect. Under the two-stressor combination, which potently inhibited growth, the content of phenolic compounds in the seedlings did not increase; however, treatment with GABA in this experiment caused an almost 1.5-fold increase in phenolic compounds. The stress factors, especially their combination, significantly reduced the anthocyanin content, which appeared to be oxidized during the development of oxidative stress caused by drought and salinity. GABA treatment also eliminated or mitigated the adverse effects of drought and salt stress (Figure 6b).

Data on etiolated wheat seedlings subjected to drought stress are consistent with results indicating the involvement of GABA in regulating the contents of secondary metabolites in triticale (Zhao et al., 2023). This study found a GABA-induced increase in the activities of major enzymes responsible for the syntheses of secondary metabolites (phenylalanine ammonia lyase, cinnamic acid 4-hydroxylase (C4H), and 4-coumarate CoA ligase) and in the content of phenolic compounds in tissues.

Exogenous GABA-intensified functioning of the antioxidant system is probably associated not only with an increase in the activities of antioxidant enzymes and activation of the syntheses of secondary antioxidant metabolites but also with the accumulation of multifunctional low-molecular protectors, in particular proline (Figure 7). Proline is involved in osmoregulation, as discussed above, and in antioxidant protection (Sadak, 2022; Kolupaev et al., 2023b); it can detoxify various ROS, including the most aggressive hydroxyl radicals (Signorelli et al., 2014; Abd Elhamid et al., 2021). Moreover, in addition to its antioxidant effect, proline can act as a chaperone that stabilizes protein molecules and reduces the impact of ions on them (Rjychoudhury et al., 2015). This is especially important during salt stress and cell dehydration (Sadak, Ramadan, 2021).

Sugars, the levels of which were either elevated or stabilized in stressed triticale seedlings in the presence of GABA (Figure 6b), are also now considered a new class of antioxidant molecules (Gangola et al. 2018). With classical antioxidants (ascorbate, glutathione, and polyphenolic compounds), disaccharides and fructans are believed to form a cytoplasmic antioxidant network (Bolouri-Moghaddam et al. 2010). In this case, increased levels of sucrose can act as a signal that specifically triggers the expression of genes for the key enzyme in the synthesis of secondary metabolites, phenylalanine ammonia-lyase, and several subsequent enzymes for the synthesis of

anthocyanins (Solfanelli et al., 2006). In our study, high correlation coefficients between sugars and phenolic compounds ($r = 0.82$, $p \leq 0.05$), as well as between sugars and anthocyanins ($r = 0.82$, $p \leq 0.05$), suggest a possible functional relationship between sugars and secondary metabolites.

5. Conclusion

This study has proved that it is possible to enhance the drought and salt tolerance of triticale seedlings by treating them with GABA. GABA substantially mitigated the growth-inhibitory effects of these two stressors, especially their combination; it also significantly increased tissue hydration and reduced the generation of ROS and the accumulation of the LPO product, MDA, under stressful conditions. The protective effect of GABA was manifested in the increased activities of antioxidant enzymes under both physiologically normal and stressful conditions. It also enhanced the accumulation of proline, stabilized the sugar pool, and led to a noticeable increase in the contents of phenolic compounds and anthocyanins.

To the best of our knowledge, this is the first study focusing on the GABA effects on the stress tolerance of triticale seedlings. The activation of a wide range of adaptive reactions caused by GABA in triticale seedlings under osmotic and salt stress, especially when combined,

indicates the probable systemic action of GABA as a molecule involved in the functioning of the signaling network of plant cells. However, its functional interaction with main signaling mediators requires specialized studies. GABA activation of a wide range of adaptive reactions in triticale seedlings under osmotic and salt stresses—especially under their combination—indicates a probable systemic action, which shows that GABA is a molecule involved in the functioning of signaling pathways in plant cells. However, specialized studies are needed to elucidate the functional interactions between GABA and major signaling mediators. These results allow us to consider GABA to be a promising bioregulator of the adaptive responses of plants (including in the early phases of development) to dehydration-associated stresses.

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