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ADAPTATION OF CEREAL SEEDLINGS TO OXIDATIVE STRESS INDUCED BY HYPERTHERMIA

SUMMARY

The response of etiolated seedlings of winter rye (*Secale cereale*), bread (*Triticum aestivum*) and durum (*T. durum*) wheats, as well as triticale *(*× *Triticosecale*) to the action of hyperthermia in relation to their resistance to oxidative stress was studied. Exposure of seedlings to 45°C for 4 hours led to a significant inhibition of the growth of *T. aestivum*, while the growth of *T. durum* and × *Triticosecale* seedlings was less sensitive to hyperthermia, and *S. cereale* seedlings showed the greatest resistance to heat stress. In bread wheat seedlings after heating, intensive development of oxidative stress. In durum wheat and triticale, such effects were less pronounced, and in rye, they were almost absent. In rye, triticale, and durum wheat seedlings, peroxidase activity increased under hyperthermia conditions, while in bread wheat, on the contrary, it decreased. In all four studied cereals, in response to the action of high temperature, the content of multifunctional stress metabolite proline increased, however, in rye, its absolute content significantly exceeded that in other species. The content of sugars during hyperthermia increased in *S. cereale* and *T. durum*, but did not change in the other two cereals. Triticale and especially rye have a high base content of anthocyanins and its increase in response to high temperature. A conclusion was made about the relationship between the ability of cereal seedlings to maintain growth under the action of hyperthermia and their resistance to oxidative stress, which is mainly due to the accumulation of metabolites with antioxidant activity.

Keywords: *Secale cereale, Triticum aestivum, T. durum,* × *Triticosecale, hyperthermia, oxidative stress*

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INTRODUCTION

Probability of extreme conditions with abnormally high temperatures, lack or excess of precipitation in recent decades has been constantly increasing (Konapala *et al.*, 2020). Climate change is already having major implications for stability of food security crop industry (El Bilali *et al*., 2020). High temperatures are among the factors that have a particularly strong and dramatic effect on plants, which is not limited to membrane barriers (Wahid *et al*., 2007).

Despite the relevance of the problem of cereals heat tolerance for crop production and breeding, researchers still state the lack of effective markers for assessing this property of plants (Li *et al*., 2019; Pržulj *et al.*, 2020). One of the main reasons for the damaging effect of high temperatures is fluidization of the lipid base of cell membranes, including chloroplast and mitochondrial membranes (Yoshioka, 2016; Choudhury *et al*., 2017). This effect, in turn, causes generation of reactive oxygen species (ROS), which can lead to oxidative damage to biomacromolecules and membrane lipids (Farooq *et al.*, 2011). In this regard, attempts are being made to use markers of development of oxidative stress and state of antioxidant system to screen for heat-resistant cereal genotypes (Gupta *et al.*, 2013). At the same time, strategies for plant adaptation to oxidative stress depend on their taxonomic affiliation (Kolupaev *et al.*, 2015; 2016; 2020). In this regard, a comparison of the functioning of stress-protective systems in cereals belonging to different genera and species and having different resistance is of interest both for a deeper understanding of specific characteristics of adaptation and for developing methods for assessing resistance of varieties and lines that can be used in breeding.

Studies dealing with role of antioxidant system in heat resistance of winter cereals is carried out mainly on adult green plants (Sairam *et al*., 2000; Hameed *et al.*, 2012). However, in many regions, in particular, in the South and East of Ukraine, hyperthermia and drought have a harmful effect on plants not only in summer, but also in early autumn. Thus, over the past two decades, in the Steppe part of Ukraine, the average temperature in August and September increased by 2.8 and 1.9°C, respectively (Romanenko *et al.*, 2018). It has been shown that the heat tolerance of etiolated wheat seedlings correlates with the field resistance of plants (Oboznyi *et al*., 2013). In this regard, information about the features of the functioning of stress-protective systems of cereal seedlings can partly be extrapolated to characterize their resistance at later developmental stages.

Despite the simplicity of using seedlings as a model object for screening heat resistance, comparative studies of a species characteristics of stress-protective systems at the early stage of cereal grains development have not yet been carried out durum wheat and triticale are especially poorly studied in this regard (Ahmadizadeh *et al*., 2001; Blum, 2014; Rampino *et al.*, 2018).

The aim of this work was a comparative study of the response of seedlings of winter rye, bread and durum wheat and triticale to the action of hyperthermia in relation to their resistance to oxidative stress.

MATERIAL AND METHODS

Plant material and treatments. Seedlings of winter rye (*Secale cereale*, cv. Pamyat Khudoyerko), bread (*Triticum aestivum*, cv. Doskonala) and durum (*T. durum*, cv. Priazovskaya) wheats, as well as triticale (× *Triticosecale*, cv. Raritet) were used for research. The experiments were carried out at the Laboratory of Plant Biochemistry of the Yuriev Plant Production Institute. Seed samples were provided by the National Center for Genetic Resources of Plants of Ukraine. Seeds of 2020 and 2021 years of reproduction were used for the research.

Grains were disinfected by immersion in 1% sodium hypochlorite solution for 15 min, after they were thoroughly washed with distilled water and germinated at 24°C for 3 days in Petri dishes on two layers of filter paper moistened with distilled water. Heat resistance of cereal seedlings was assessed by growth response to high temperature using the method proposed by Zhuk and Grygoryuk (Pat. 45879 UA, 2002) with our modifications. Three-day-old seedlings of experimental variants were placed in open Petri dishes in a thermostat with a temperature of 45±1°C and air humidity of 40-45% (exposure 4 hours). To prevent drying of the roots, the filter paper in the cups was moistened every hour with the same amount of distilled water. After the end of the exposure, one part of the seedlings was used for biochemical analyzes, and the other part was placed in a thermostat at a temperature of 24°C to assess the growth response. The seedlings of the control variants were kept in a thermostat at a temperature of 24°C throughout the experiment. 24 hours after heat stress, the inhibition of seedling growth was evaluated using the formula:

$$I = \frac{(C_2 - C_1) - (E_2 - E_1)}{C_2 - C_1} \cdot 100\%,$$

where I is growth inhibition (%); C_1 and C_2, E_1 and E_2, respectively, the initial and final values of the fresh weight of seedlings (without grains) in the control and experimental (heat stress) variants.

The water content in the plant material was determined by the gravimetric method by drying at 103°C to constant weight.

Evaluation of LPO products content. Analysis of the amount of lipid peroxidation (LPO) products reacting with 2-thiobarbituric acid (mainly malonic dialdehyde, MDA) in the shoots was carried out according to the protocol described earlier (Kolupaev *et al.*, 2015).

Generation of superoxide anion radicals by the shoots was estimated by reduction of nitroblue tetrazolium (NBT). Ten shoots of the same size were placed in bottles for 1 h with 5 ml of 0.1 M K, Na-phosphate buffer (pH 7.6) containing 0.05% NBT, 10 μM EDTA, and 0.1% Triton X-100 (Kolupaev *et al.*, 2013). At the end of the exposure, the shoots were carefully removed from the incubation solution and optical density of the incubation solution was measured at a wavelength of 530 nm. The increase in generation of superoxide anion radical under heat stress was determined by the ratio (%) of optical density in the experimental and control variants.

Evaluation of hydrogen peroxide content. To determine H_2O_2 content, the shoots were homogenized in cold with 5% trichloroacetic acid. The samples were centrifuged at 8000 g for 10 min at 2–4°C; concentration of hydrogen peroxide was determined in supernatant using the ferrothiocyanate method (Sagisaka, 1976).

Measurement of antioxidant enzymes activity. To assess the activity of antioxidant enzymes, shoots (200 mg) were homogenized in 10 ml of 0.15 M K, Na-phosphate buffer (pH 7.6) with the addition of EDTA (0.1 mM) and dithiothreitol (1 mM) on ice (Kolupaev *et al.*, 2016). Activity of catalase (EC 1.11.1.6) was analyzed at pH 7.0 of the reaction mixture, estimating the amount of hydrogen peroxide that decomposed per unit time. Peroxidase activity (EC 1.11.1.7) was analyzed with guaiacol used as a hydrogen donor and hydrogen peroxide as a substrate. The pH of the mixture was adjusted to 6.2 by K, Na-phosphate buffer.

Content of low molecular protective compounds. Proline content in the shoots was determined according to the modified method of Bates *et al*. (1973).

Total sugar content of the plant material was determined by the modified Morris-Roe method based on anthrone reagent (Zhao *et al.*, 2003).

For determination of anthocyanins, samples of plant material were homogenized in 1% HCl solution in 80% methanol. After centrifugation of the homogenate at 8000 g for 15 min, optical density of the supernatant was determined at a wavelength of 530 nm (Nogues and Baker, 2000).

Replication of experiments and statistical processing of results. The experiments had 3 biological replicates. Data for each parameter were statistically analyzed using analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test. Different letters denote values, the differences of which are significant at $P \leq 0.05$.

RESULTS

Growth reaction of seedlings and their water content under heat stress. After a 4-hour exposure to 45°C, inhibition of the growth of rye seedlings was insignificant (Figure 1). The high-temperature effect inhibited the growth of triticale and durum wheat more significantly and approximately to the same extent (by ≈ 36%). The strongest growth inhibitory effect of heat stress was observed in bread wheat seedlings (more than 60%).

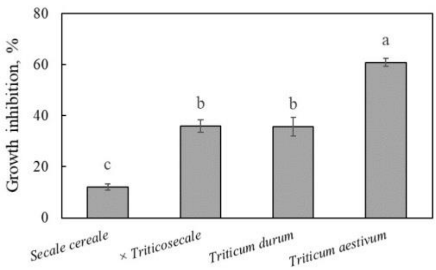

Figure 1. Growth inhibition (%) of cereal seedlings after heat stress (45°C, 4 h)

Water content in the roots of seedlings of all four species was 89-90% and did not change significantly after heat stress (results not shown), since the filter paper and the root system were moistened several times with distilled water during the heat treatment period (see the Material and Methods section). At the same time, water content in the shoots decreased in all species, except for rye (Figure 2). The most significant decrease in the water content in the shoots was in bread wheat (almost 4%). For durum wheat and triticale, this effect was less pronounced.

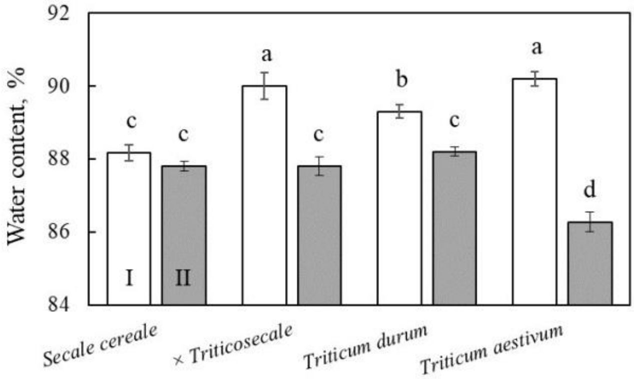

Figure 2. Water content (%) in cereal seedlings

Manifestation of the effect of oxidative stress in seedlings after high temperature exposure. In seedlings of all studied cereal species, after heat stress, an increase in the content of LPO product MDA was observed (Figure 3A). It was most noticeable in bread wheat, and least significant in rye. In rye seedlings, no significant increase in the generation of superoxide anion radical was registered after exposure to high temperature (Figure 3B). At the same time, in triticale and durum wheat, the $O_2^{\bullet-}$ formation increased by 27-28%. The most significant (2.2-fold) increase in the generation of this ROS under heat stress occurred in the shoots of bread wheat seedlings (Figure 3B).

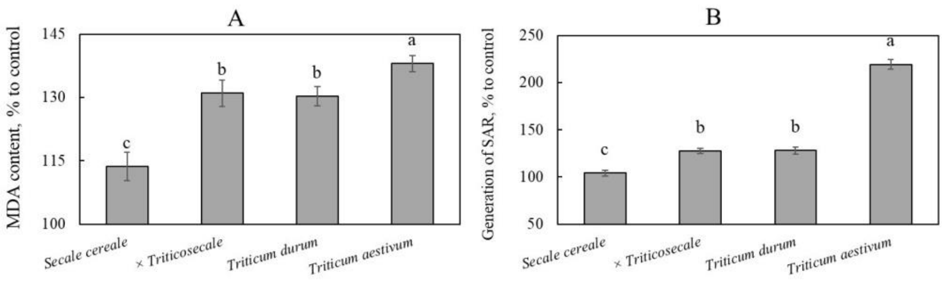

Figure 3. MDA content (A, % to control) and generation of superoxide anion radical (SAR) (B, % to control) by cereal seedlings after heat stress (45°C, 4 h)

Changes in the content of hydrogen peroxide under stress conditions in the shoots of cereal seedlings were comparable with the values observed for $O_2^{\bullet-}$.

Rye seedlings had a lower base content of hydrogen peroxide compared to other cereals, while heat stress did not affect its amount (Figure 4). In triticale, the constitutive content of hydrogen peroxide was slightly higher than in rye and increased by 22% after heating. Durum wheat seedlings were characterized by a higher content of hydrogen peroxide compared to other cereals, and heating caused an increase in its amount by 23%. In bread wheat, with a relatively low basic content of hydrogen peroxide, its more than twofold increase was noted after heat stress (Figure 4).

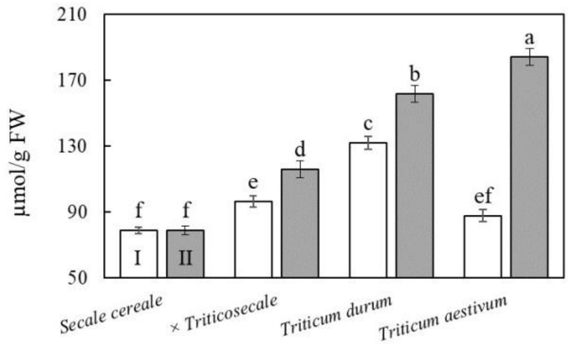

Figure 4. The content of hydrogen peroxide (µmol/g FW) in cereal seedlings. I – control; II – stress (45°C, 4 h)

Activity of antioxidant enzymes in cereal seedlings under heat stress. The basal level of catalase activity in rye seedlings was somewhat lower than in triticale and two wheat species (Figure 5A). Heat stress caused some decrease in the enzyme activity in rye and triticale. In durum and bread wheat, the activity of the enzyme practically did not change.

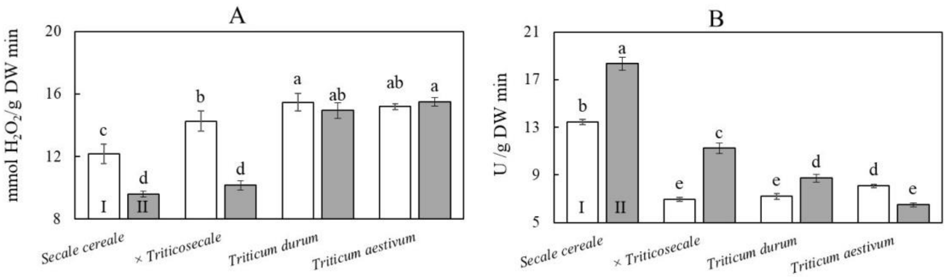

Figure 5. Activity of catalase (A, mmol H_2O_2/g DW min) and guaiacol peroxidase (B, U/g DW min) in wheat seedlings. I – control; II – stress (45°C, 4 h)

The constitutive activity of peroxidase in rye significantly exceeded that in other species (Figure 5B). In bread wheat, the enzyme activity was slightly higher than in durum wheat and triticale. Under the influence of heating, there was a significant increase in activity of the enzyme in rye and triticale and a slight increase in durum wheat. At the same time, a decrease in peroxidase activity was observed in bread wheat after heat stress (Figure 5B).

Content of multifunctional stress metabolites in cereal seedlings. The highest basic content of proline was observed in rye and durum wheat seedlings (Figure 6A). In bread wheat seedlings and especially triticale, it was significantly lower. Effected by heat stress, the content of proline in cereals of all types increased proportionally (approximately 2.5 times compared with the initial values). Where in the absolute values of rye exceeded those of other cereals. At the same time, the content of proline in triticale after stress exposure was noticeably lower than in other cereals (Figure 6A).

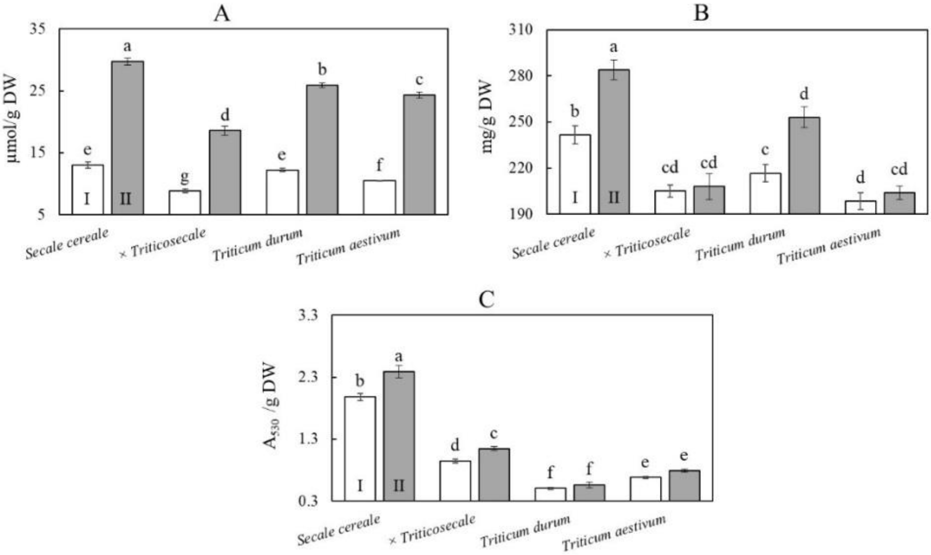

Figure 6. Content of proline (A, µmol/g DW) and sugars (B, mg/g DW) and anthocyanins (C, A_{530} /g DW) in cereal seedlings. I - control; II – stress (45°C, 4 h)

The constitutive sugar content in rye and durum wheat was higher than that in the other two cereals (Figure 6B). In those species it also increased under heat stress. At the same time, the content of sugars in triticale and bread wheat seedlings did not change significantly.

A high base content of anthocyanins was characteristic of rye. In triticale, it was two times lower than in rye, and in two types of wheat, anthocyanin content was very low (Figure 6B). In response to heat stress, the content of anthocyanins in rye seedlings and triticale further increased. In the two types of wheat, a significant increase in the content of anthocyanins was not observed.

DISCUSSION

The results obtained allow us to speak about the dependence of the growth intensity after exposure to hyperthermia on the ability of seedlings to maintain pro-/antioxidant balance. Thus, rye seedlings were characterized by a lower basic content of hydrogen peroxide and the absence of a significant increase in its amount, as well as the $O_2^{\bullet-}$ generation after heat stress (Figures 3,4). They also showed less accumulation of lipid peroxidation products compared to other cereals after heating (Figure 3A). In durum wheat and triticale seedlings, the manifestation of such oxidative stress signs was more significant, but moderate. Finally, in bread wheat, which growth of seedlings was significantly slowed down, the generation of superoxide anion radical and the accumulation of hydrogen peroxide increased very strongly, in addition, the LPO products content increased more significantly than in other species (Figures 3, 4).

The higher resistance of rye to hyperthermia compared to other types of cereals may be due to high resistance to oxidative stress. Thus, it has been shown that the basic resistance of rye seedlings to direct agents of oxidative stress (hydrogen peroxide and iron(II) sulfate) was significantly higher than that of bread wheat (Kolupaev *et al.*, 2016). It is well known that rye is more frost resistant than most other cereals (Fu *et al.*, 1998; Kolupaev *et al.*, 2015). Fu *et al.* (1998) associated rye resistance to both low and high temperatures with a significant accumulation of various carbohydrates, including fructans. Our work showed that rye seedlings differed from other cereals in their higher total soluble carbohydrate content and its increase after heat stress (Figure 6B), which indicates their possible role in protection against hyperthermia. There is reason to believe that soluble carbohydrates are directly involved in antioxidant defense of cells. Moreover, their effects can be due to both direct interaction with radical ROS (Morelli *et al.*, 2003) and involvement in complex regulatory processes (Khanna *et al.*, 2022).

At the same time, rye seedlings were also distinguished by a high constitutive content of other stress metabolites, in particular, proline and anthocyanins (Figure 6). Proline, along with the osmoprotective and membrane-protective functions, can play the role of antioxidant (Liang *et al.*, 2013). Flavonoid compounds, in particular anthocyanins, have a very powerful antioxidant effect (Gould and Lister, 2006); their basic content in rye was several times higher than in other cereals (Figure 6C).

It is possible that accumulation of various low-molecular-weight compounds contributes to general colligative properties of intracellular solution of rye seedlings. Livingston (2006) stated that rye had a significantly higher amount of sugars than oats and noted their contribution to resistance to dehydration caused by various influences. It is noteworthy that, under the conditions of our experiments, it was rye seedlings that were distinguished by the ability to retain the amount of water in tissues under conditions of heat stress, which is characteristic of physiologically normal conditions (Figure 2). This property may be due to the high content of various osmotically active compounds, such as sugars, proline, anthocyanins (Figure 6) and, probably, many other compounds, including secondary metabolites (Kolupaev *et al.*, 2016). In addition to low-molecular-weight compounds, defense processes in rye seem to involve antioxidant enzymes, in particular, peroxidase (Figure 5B). Its activity in rye was significantly higher than in other cereals under normal conditions and increased after heat stress.

In durum wheat seedlings under the conditions of our experiments, the content of proline and sugars was found to be significantly higher than in bread wheat, especially under stress, which may be one of the reasons for the higher heat resistance of this species (Figure 6). Rascio *et al.* (1994) noted a relationship between proline content and drought tolerance in durum wheat varieties. The maintenance of redox homeostasis in sufficiently heat-resistant durum wheat probably also occurred with the participation of enzymatic antioxidants. Thus, in this type of cereals under heat stress, a slight increase in peroxidase activity was noted, while maintaining the basic level of catalase activity (Figure 5). At the same time, less resistant to heat stress, bread wheat demonstrated decrease in peroxidase activity after heat stress in absence of change of activity of catalase.

Thus, the highest resistance of rye to heat-induced oxidative stress among the studied cereals is probably due to the high content of multifunctional low-molecular-weight protective compounds – proline, sugars, and anthocyanins, as well as the high activity of peroxidase (Figures 5, 6). The moderately high resistance of durum wheat seedlings can also be associated with a rather high content of proline and sugars at relatively low values of other studied parameters of stress-protective systems.

It is more difficult to talk about the adaptive strategy of triticale. The triticale cv. Raritet seedlings used in our experiments did not have high basic content of proline and sugars, although the amount of proline significantly increased after heat stress (Figure 6). It is possible that the relative resistance of triticale to oxidative stress is due to the increased base content of anthocyanins compared to the two types of wheat, which further increased after stress exposure (Fig. 6C). Another feature of the reaction of the triticale antioxidant system may be a significant increase in peroxidase activity after heating (Figure 5B). It is possible that this was the reason for the relatively low content of hydrogen peroxide in triticale seedlings under heat stress (Figure 4).

As already noted, durum wheat varieties are generally considered to have some advantage in terms of heat tolerance compared to bread wheat varieties (Kavita *et al.*, 2016). The variety of bread wheat used in our experiments was created in conditions of the Forest-Steppe and does not differ in high heat and drought resistance. Along with a strong growth inhibition (Figure 1), bread wheat seedlings showed a significant loss of water during hyperthermia (Figure 2) and a very large increase in ROS generation (Figures 3, 4). At the same time, there was practically no increase in the functioning of the studied stress-protective systems in bread wheat seedlings. The only significant reaction was an increase in proline content in response to heat stress (Figure 6).

Of course, not all known components of the antioxidant system have been studied in this work. In particular, functioning of ascorbate-glutathione system, whose contribution to plant resistance to drought and hyperthermia can be quite high, has not been studied (Lou *et al.*, 2018). Nevertheless, nature of changes in the integral indicators of oxidative stress (generation of superoxide anion radical, content of hydrogen peroxide and MDA) allows us to state the existence of a relationship between the ability of seedlings of various types of cereals to grow after exposure to hyperthermia and their resistance to oxidative stress.

It is quite natural that resistance of cereal species to hyperthermia may also depend on their varietal characteristics. At the same time, it is possible that in some indicators varietal differences may exceed species differences. However, further study of species characteristics will make it possible to compile a general picture of the strategies of adaptive reactions that are characteristic of certain taxonomic groups of cereals. Knowledge of such strategies is important not only for a fundamental understanding of adaptation mechanisms of plants of different taxonomic affiliations, but also for the selection of a set of heat resistance markers that is optimal for screening this property in each specific cereal species.

CONCLUSIONS

At the stage of etiolated seedlings, significant differences were found in the heat resistance of the four studied types of cereals. According to this indicator, they were arranged in a row: *Secale cereale* > *Triticum durum* ≥ × *Triticosecale* > *T. aestivum*. After exposure to 45°C, the manifestation of the oxidative stress effect was most pronounced in *T. aestivum*, and the least pronounced in *S. cereale*. The obtained results indicate the relationship between the heat resistance of seedlings of various types of cereals and their resistance to oxidative stress. At the same time, strategies for protection against the oxidative stress development depend on taxonomic affiliation. The most resistant rye is characterized by a high content of proline, sugars, anthocyanins, as well as high peroxidase activity under heat stress conditions. *T. durum* is characterized by a relatively high content of sugars and proline, while × *Triticosecale* is characterized by increased content of anthocyanins and increase in peroxidase activity under stress conditions. In *T. aestivum*, the studied indicators of the functioning of defense systems had low values, and all (except for the content of proline) decreased or did not change after exposure to stress.

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