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ORIGINAL ARTICLE

The Role of Superoxide Dismutase in Inducing of Wheat Seedlings Tolerance to Osmotic Shock

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Influence of short-term hardening osmotic exposure (immersion in 1 M sucrose solution with subsequent transferring to distilled water for 20 min) on the hydrogen peroxide generation and superoxide dismutase activity in wheat (*Triticum aestivum* L., cv. Elegiya) seedlings and their tolerance to osmotic shock were investigated. During the initial 30 min after osmotic exposure, the increasing of hydrogen peroxide amount in roots and shoots (to a lesser extent) was observed, but the resistance of the seedlings and superoxide dismutase (SOD) activity decreased. Sometime later the decrease in hydrogen peroxide amount and the increase of seedlings tolerance to osmotic shock took place. SOD activity increased in 10 min after hardening osmotic exposure. Transient accumulation of hydrogen peroxide induced in this way was suppressed by the treatment of seedlings with sodium diethyldithiocarbamate (DDC), SOD inhibitor. DDC and hydrogen peroxide scavenger dimethylthiourea decreased positive hardening effect of osmotic exposure on the development of seedlings tolerance. It was concluded that SOD providing the generation of signal hydrogen peroxide pool took part in the induction of seedlings tolerance to osmotic shock development caused by preliminary hardening effect.

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Phenomenon of hardening of plants or increasing of their tolerance to injurious influence of harmful factors after preliminary temperate exposure to one or another factor has been studied for many decades (Aleksandrov, 1985; Talanova, 2009; Munir, Aftab, 2009). However, the molecular mechanisms of this phenomenon are not completely elucidated.

It is known that the early plant responses to action of stressors accompany with the transient increase in content of various signaling mediators – calcium ions, reactive oxygen species (ROS), nitric oxide, cAMP etc. (Kolupaev, Karpets, 2010). In recent years the evidences of the involvement of

ROS in the process of induction of plant resistance to damaging effects are appearing increasingly (Korsukova et al., 2013). After exposure of wheat seedlings to high hardening temperature there was observed a transient increase of hydrogen peroxide amount there (Kolupaev et al., 2013). In this case, hydrogen peroxide scavenger dimethylthiourea (DMTU), an inhibitor of NADPH-oxidase imidazole and SOD inhibitor sodium diethyldithiocarbamate (DDC), obstructed the development of heat resistance after hardening. Due to above mentioned fact, it was concluded that NADPH oxidase which generates superoxide anion radical and superoxide dismutase which converts it into hydrogen peroxide – a more stable ROS that performs signaling functions, took part in the formation of the signal that induced the development of heat resistance (Kolupaev et al., 2013). Also, we showed previously that temperate osmotic influence on wheat seedlings after a certain lag period caused an increase in their resistance to the injuring effects (osmotic shock), in other words, osmotic hardening effect is exhibited. Osmotic hardening as well as heat hardening caused transient accumulation of hydrogen peroxide in plant tissues and was suppressed by ROS-generated enzyme (NADPH-oxidase, extracellular peroxidase) inhibitors (Oboznyi, Kolupaev, 2012). These effectors substantially inhibited the development of resistance of seedlings to osmotic shock. However, the role of SOD as a possible link in formation of a pool of signal hydrogen peroxide under osmotic hardening exposure remained unexplored.

The aim of the study was to elucidate the involvement of SOD in the enhancing of hydrogen peroxide generation by root cells in response to the osmotic hardening and its importance for further development of resistance to injurious effects.

MATERIALS AND METHODS

The experimental object was etiolated seedlings of soft winter wheat (*Triticum aestivum* L.), cv. Elegiya, grown on purified tap water at 20°C. Three-day-old seedlings of respective types of treatment were transferred for 14–16 h to the solution of hydrogen peroxide scavenger dimethylthiourea (Sung at al., 2009) or Cu,Zn-SOD inhibitor (sodium diethyldithiocarbamate DDC, 5 mM) (Morita et al., 1999). Concentrations of these compounds were chosen in preliminary experiments (Kolupaev et al., 2013). Control seedlings were left on tap water.

Then seedlings were exposed to osmotic hardening (immersion of intact seedlings in 1M sucrose solution for 10 min with subsequent transferring to distilled water for 20 min) (Obozniy, Kolupaev, 2012). In the text below this exposure is called an osmotic hardening. With this method of hardening seedlings were also exposed to hypoxic stress. However, as special control experiments showed, the immersion of seedlings into water for 30 minutes did not cause the appearance of stress symptom (accumulation of hydrogen peroxide), this procedure hereinafter didn't inhibit the growth of wheat seedlings (data aren't shown). In connection with above mentioned facts we considered that studied effects were caused just by osmotic effect, but not by hypoxia. After hardening exposure seedlings pretreated with effectors were still kept on their solutions for 1 hour and then were transferred to purified tap water. Samples that were not treated DMTU or DDC were incubated in water all the time (control).

Osmotic injurious effect (osmotic shock) was created by immersion of seedlings into 1 M sucrose solution for 2 h, with following transferring to

distilled water for 1 h. In this case, the control seedlings were immersed for the appropriate time into purified tap water. As special experiments showed, the procedure did not cause any visible injuries for seedlings and slightly inhibited their growth in the next 4 days (linear sizes of seedlings differed from the sizes of the samples that were not exposed to such a procedure, no more than for 10-15%). After 4 days after exposure to osmotic shock relative number of survived seedlings was estimated.

Content of hydrogen peroxide was determined using the ferrothiocyanate method after its extraction with 5% TCA from the roots or shoots ground in the cold. The samples were centrifuged for 10 min at temperature of no higher than 4°C, and H_2O_2 concentration was determined in the supernatant using Mohr's salt and ammonium thiocyanate (Sagisaka, 1976).

In order to determine the activities of SOD (EC 1.15.1.1) and catalase (EC 1.11.1.6), the seedling roots were homogenized in the cold 0.15 M K,Na phosphate buffer (pH 7.6) with the addition of EDTA (0.1 mM), dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (0.5 mM) and detergent Triton X-100 (the final concentration of 0.1%). The supernatant obtained after centrifugation of homogenate at 8000 *g* for 10 min at 4°C was used for analysis. SOD activity was determined using the method based on ability of the enzyme to compete with the NBT for superoxide anions produced as a result of aerobic interaction of NADH and phenazine methosulfate (Kolupaev et al., 2005). Catalase activity was determined by the amount of broken down hydrogen peroxide (Kolupaev et al., 2005). The content of protein was determined according to Bradford using BSA as a standard (Bradford, 1976).

In order to determine the activity of guaiacol peroxidase (EC 1.11.1.7) plant material was homogenized in the 0.06 M K,Na phosphate buffer (pH 7.2) with the addition of EDTA (0.1 mM), dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (0.5 mM). Homogenate was centrifuged at 8000 *g* for 10 min at 4°C. Hydrogen peroxide was used as a substrate, as a reducer – guaiacole (Ridge, Osborne, 1970). It was maintained as 6.2 pH of the reactive mixture with the help of 0.06 M K,Na phosphate buffer.

Experiments were independently reproduced at least 3 times. Each individual experiment had three replicates with two recordings. The figures show the means and their standard deviations.

RESULTS

In control wheat seedlings tolerance to osmotic shock during the time of experiment did not change significantly (Fig. 1). During 1 hour after hardening effect there was significant decrease of resistance, after 6 h maximum resistance of hardened seedlings to osmotic shock was observed. Then there was gradual reduction of their resistance.

In the roots of control seedlings the amount of hydrogen peroxide during the observation period did not change significantly (Fig. 2 A). 10 min after osmotic hardening the level of H_2O_2 in the roots of wheat increased, after 15-30 min it reached a maximum and then gradually decreased, reaching control level after 6 h (Fig. 2 A).

In the shoots of control seedlings during the observation period the content of hydrogen peroxide did not change significantly (Fig. 2 B). After hardening osmotic exposure the content of H_2O_2 increased in shoots, as well as in roots, but this effect was less significant. Due to the greater sensitivity of the roots to osmotic exposure in

further investigations biochemical parameters were determined only in the roots.

SOD, catalase and guaiacol peroxidase activities in control seedling roots during 6 hours of observations did not change significantly (Fig. 3). Hardening osmotic effect significantly increased SOD activity in roots just after 10 minutes. Then, after 3-6 hours, the enzyme activity decreased slightly, but still exceeded more than 1.5 times the value of control (Fig. 3 A).

Catalase activity in 15-30 minutes after hardening osmotic exposure changed insignificantly, but subsequently its decrease occured. (Fig. 3 B).

The dynamics of guaiacol peroxidase activity was different. The enzyme activity increased just after 30 min after osmotic hardening subsequently it also was its gradual increase (Fig. 3 C).

Thus, the increase of hydrogen peroxide amount in the roots of hardened seedlings during the first 30 min after hardening osmotic exposure coincided in time with the increase of SOD activity (Fig. 2 and 3). Catalase activity, at this time decreased insignificantly, and guaiacol peroxidase activity on the contrary, slightly increased (Fig. 3). In this regard, a significant increase of the hydrogen peroxide content in roots for 30 minutes after osmotic hardening is unlikely to be connected with the changes in the activity of enzymes that reduce its pool. A more likely reason of this phenomenon may be the activation of SOD.

To determine the contribution of SOD in the accumulation of hydrogen peroxide caused by osmotic hardening, seedlings were treated with the inhibitor of this enzyme DDC. Treatment of seedlings with DDC completely eliminated the activating effect of osmotic hardening on SOD that was observed during the first hour (Fig. 4). In the roots of unhardened seedlings in the presence of DDC in the incubation solution decrease of SOD activity also was observed (Fig. 4). The effect of DDC on the activity of SOD was reversible. 5 hour after the transferring of seedlings on the medium without inhibitor (by 6th hour of observations), the activity of SOD in the variant with DDC didn't differ from control. At the same time, in the variant with the combined effect of DDC and osmotic hardening enzyme activity remained lower in comparison with the variant with hardening, but without inhibitor (Fig. 4).

In further experiments we investigated the influence of SOD inhibitor (DDC) and a scavenger of hydrogen peroxide (DMTU) on H_2O_2 content in the roots of seedlings in 15 minutes after hardening osmotic exposure, when there was a maximal generation of ROS. An increased level of hydrogen peroxide that was induced by hardening, was completely removed by DDC, which itself had no effect on the hydrogen peroxide content in the roots under experimental conditions. DMTU in used concentration had little effect on the content of H_2O_2 per se, however, significantly neutralized the effect of its increase, caused by osmotic hardening (Fig. 5).

If ROS are essential mediators to induce seedlings resistance to osmotic shock, then the modification of their generation should be reflected on the survival of seedlings after injurious effects. SOD Inhibitor DDC and hydrogen peroxide scavenger DMTU in themselves did not have a significant effect on the resistance of wheat seedlings to osmotic shock, but substantially neutralized the positive influence of osmotic hardening (Fig. 6). Thus, agents that reduce hydrogen peroxide amount in the roots of seedlings

after osmotic hardening decreased the development of resistance of seedlings to osmotic shock.

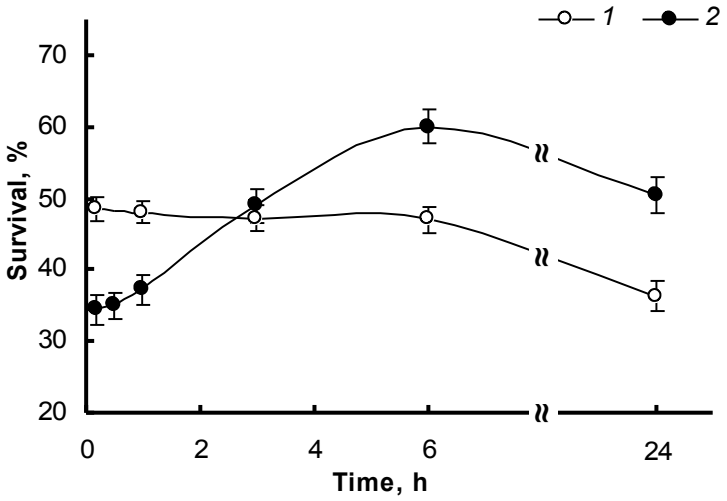

Figure 1. Survival (%) of wheat seedlings after osmotic shock (immersion of seedlings in 1 M sucrose solution for 2 h, with the further transfer to distilled water for 1 hour). Here and in Fig. 2, 3: *1* – control; *2* – hardening (immersion of intact seedlings in 1 M sucrose solution for 10 minutes and then transfer to distilled water for 20 minutes).

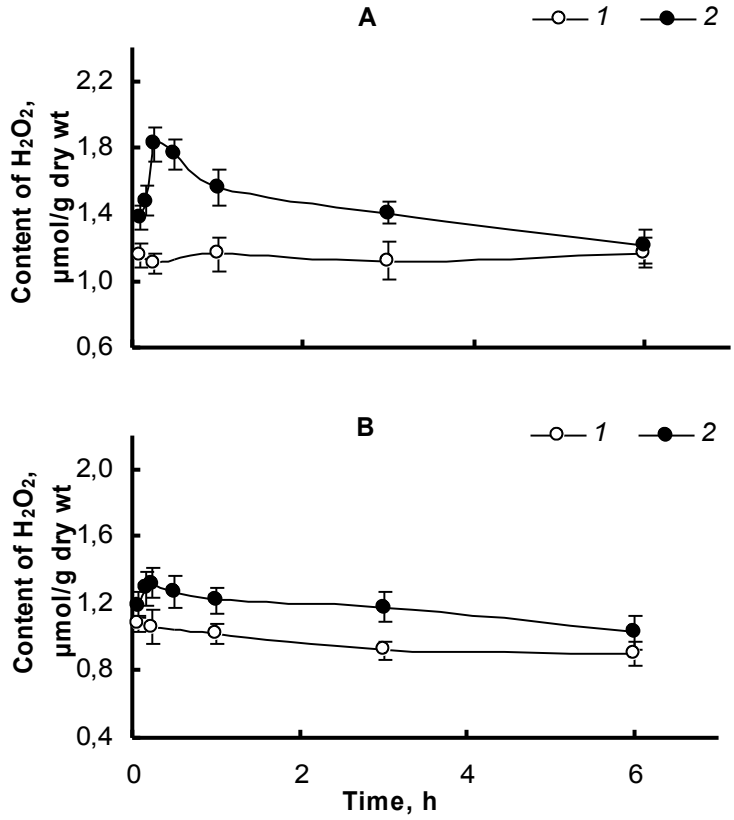

Figure 2. Dynamics of hydrogen peroxide content in the roots (A) and shoots (B) of wheat seedlings.

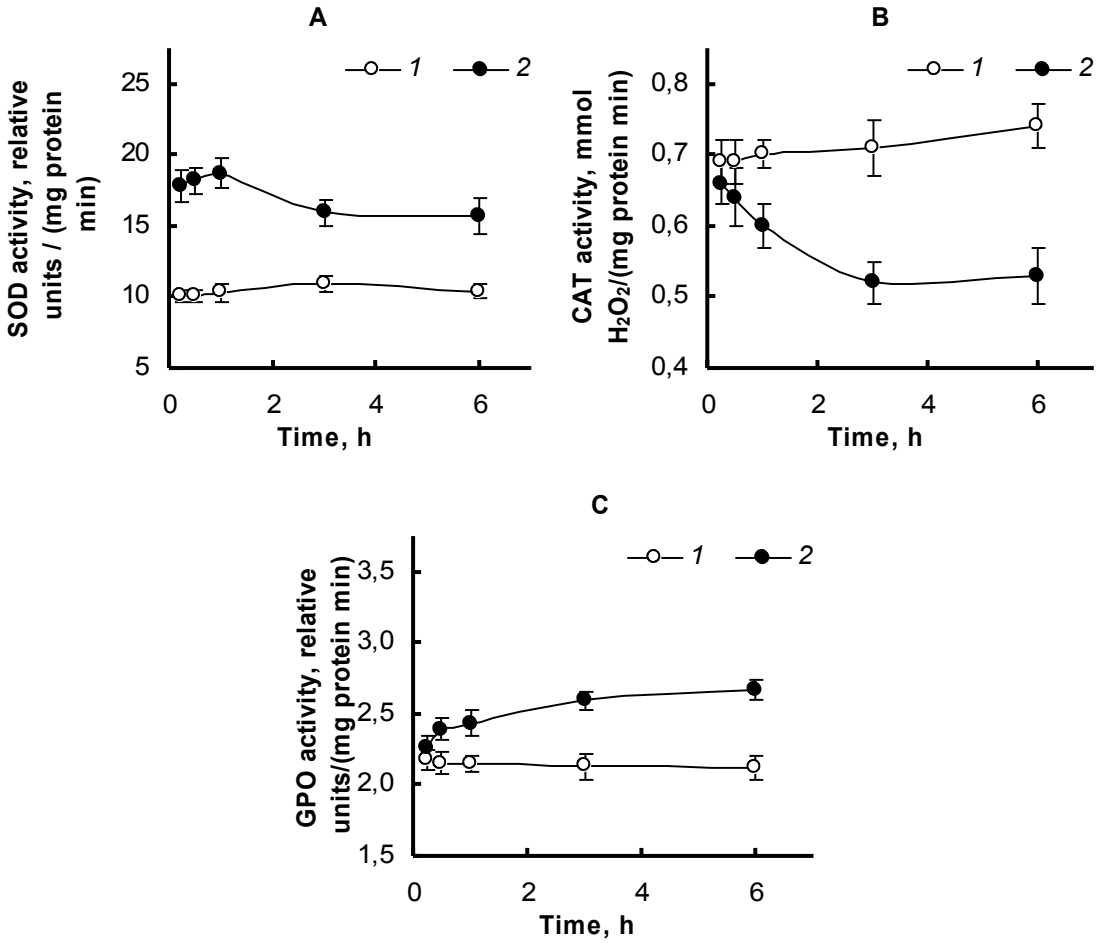

Figure 3. SOD (A), catalase (CAT) (B) and guaiacol peroxidase (GPO) (C) activities in the roots of wheat seedlings.

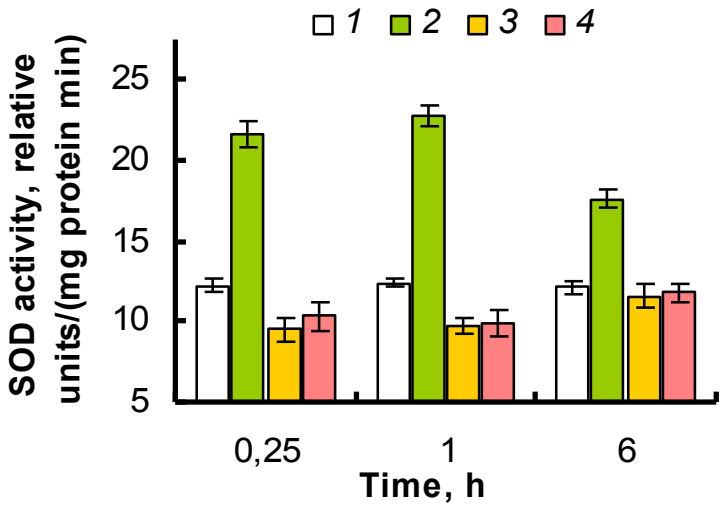

Figure 4. Influence of osmotic hardening and DDC on SOD activity in the roots of wheat. *1* – control; *2* – hardening; *3* – DDC (150 μM); *4* – hardening + DDC(150 μM).

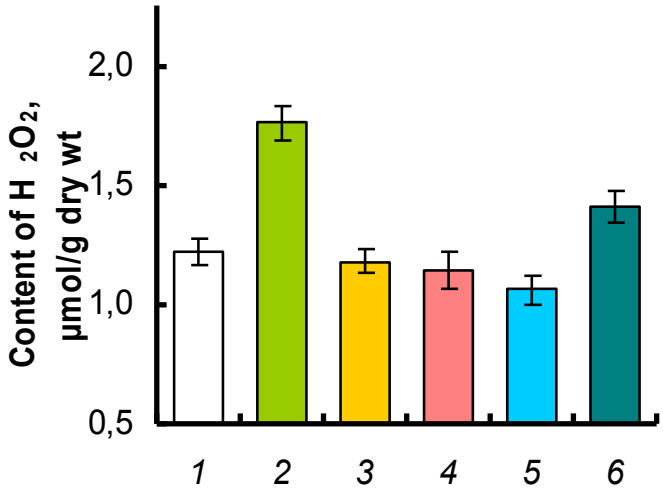

Figure 5. Hydrogen peroxide content in the roots of wheat seedlings 15 min after osmotic hardening. Here and on Fig. 6: *1* – control; *2* – hardening; *3* – DDC (150 μM); *4* – hardening + DDC (5 mM); *5* – DMTU (150 μM); *6* – hardening +DMTU (150 μM).

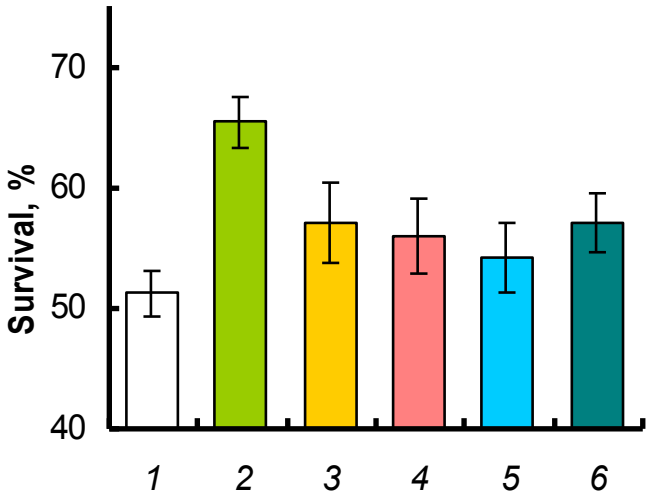

Figure 6. Influence of DDC and DMTU on survival (%) of wheat seedlings after osmotic shock.

DISCUSSION

The exposure of wheat seedlings to osmotic hardening exposure improved their resistance to further osmotic shock. However, the improving of tolerance caused by hardening was preceded by decrease in the development of resistance which was observed during the first hour after osmotic hardening (Fig. 1). Similar appropriateness of changes in resistance were observed under the induction of heat resistance of plants with hardening heating (Kolupaev et al., 2013).

Increased level of hydrogen peroxide in seedlings shoots and especially roots for the first 30 minutes after hardening osmotic exposure (Fig. 2)

can be considered as the result of reversible injuries occurring after such effect, because there was a decrease of the resistance of seedlings to osmotic shock at that time. On the other hand, increasing of the ROS generation immediately after the start of hardening can serve as a signal required for the induction of protective stress systems and further enhancing the stability of the seedlings to injurious osmotic stress. The sum of our results confirms this point of view.

Increase of H_2O_2 content in plant tissues can be caused by increased activity of enzymes that generate ROS, and decreased activity of antioxidant enzymes. Our experiments carried out earlier show that increased content of hydrogen peroxide in the organs of wheat seedlings immediately after the osmotic hardening are likely to be connected with increased activity of ROS-generating enzymes, such as NADPH oxidase and extracellular peroxidase. Inhibitors of these enzymes imidazole and salicylhydroxamic acid significantly reduced the elevation in the H_2O_2 content caused by heat and osmotic hardening effects (Oboznyi, Kolupaev, 2012). At the same time, the transformation of superoxide anion radicals generated by the mentioned enzymes in a more stable ROS, hydrogen peroxide, is apparently not spontaneous, but is due to the activation of SOD. The inhibition of hydrogen peroxide accumulation caused by osmotic (Fig. 5) and heat (Kolupaev et al., 2013) hardening effects by SOD inhibitor confirms that supposition.

A large content of SOD in etiolated plants and in non-green parts of plants is present in the cytoplasm but it was also detected in the apoplast (Ogawa et al., 1997; Miller et al., 2010). One can assume that just apoplastic SOD that transforms NADPH oxidase-generated superoxide anion radical into H_2O_2 ensures penetration of ROS molecules into the cytoplasm and performance of signal functions by them. At the same time, it is possible that protonated superoxide anion radical can get into cytoplasm (Sagi, Fluhr, 2006) and be subsequently transformed into hydrogen peroxide by intracellular forms of SOD.

The intensification of hydrogen peroxide formation involving SOD is apparently necessary factor to the further improvement of wheat seedling heat tolerance. For instance, treatment of seedling before hardening with antioxidant agent DMTU or SOD-inhibitor DDC considerably reduced their heat tolerance (Fig. 6). One can assume that ROS participate in the activation of some stress-protective responses.

Data on the dynamics of SOD and soluble peroxidase activity show that one of the reasons of the positive influence of osmotic hardening on resistance of wheat seedlings to osmotic shock may be an increase in the activity of these enzymes (Fig. 3). Their high activity observed after 6 h after osmotic hardening coincided in time with the maximal development of the seedling resistance to this factor (Fig. 1). Though, the increase in peroxidase activity was accompanied by decrease in the activity of another enzyme involved in hydrogen peroxide metabolizing – catalase (Fig. 3). Some publications describe the examples of original catalase-peroxidase “interchangeability”. Moreover, it is known that under high concentration of hydrogen peroxide peroxidase can perform catalase activity (Mika et al., 2004).

Naturally, the hardening osmotic effect can induce other protective reactions which are necessary for adaptation to osmotic shock, in particular the accumulation of low molecular weight polyfunctional protectors – proline and sugars (Oboznyi et al., 2013). It is known that

proline has chaperone properties and can stabilize many proteins, including antioxidant enzymes, under the action of dehydration and other stress factors (Szabados, Savoure, 2009). In this case, there are data (though mostly indirect) that the synthesis of low molecular weight protectors, including proline, can be controlled by the ROS (Hofmann et al., 2003).

On the whole, on the basis of carried out researches it can be assumed that a short-term increase in the signal pool of ROS which occurs after osmotic hardening connected with the elevation of activity of enzymes that generate superoxide anion radical, in particular NADPH oxidase (Oboznyi, Kolupaev, 2012), and SOD which converts it into a stable form – hydrogen peroxide. Thus, SOD, apparently, serves not only as the antioxidant enzyme, but also as apart of cell signaling.

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