Journal of Stress Physiology & Biochemistry, Vol. 7 No. 2 2011, pp. 154-163 ISSN 1997-0838

ORIGINAL ARTICLE

INFLUENCE OF SALICYLIC AND SUCCINIC ACIDS ON ANTIOXIDANT ENZYMES ACTIVITY, HEAT RESISTANCE AND PRODUCTIVITY OF *PANICUM MILIACEUM* L.

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Received April 21, 2011

The influence of treatment of millet (*Panicum miliaceum* L.) seeds with the solutions of salicylic and succinic acids on the heat resistance of plantlets and activity of antioxidant enzymes – superoxide dismutase (SOD), catalase and peroxidase – in them have been investigated. In the micro-field experiment the influence of these acids on the millet yield was estimated. The action of salicylic (10 μM) and succinic (1 mM) acids caused the increase of plantlets resistance to the damaging heating that expressed in the rise of relative quantity of survived plantlets in 5 days after heating at the temperature of 47°C and in the reduced content of lipid peroxidation product malonic dialdehyde during the poststress period. The increase of activity of SOD, catalase and peroxidase took place in millet plantlets under the influence of salicylic and succinic acids. The increase of productivity of millet grain under the action of salicylic and succinic acids on 13,3-52,0 and 6,4-38,8% respectively depending on weather conditions in the field experiments was noted.

Key words: antioxidant enzymes / heat resistance / Panicum miliaceum L. / salicylic acid / succinic acid

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It is shown that presowing treatment of seeds or spraying of plants of different species with salicylic and succinic acids caused the increase of their crop capacity and resistance to abiotic and biotic stressors (Tarchevsky et al., 1999; Senaratna et al., 2000; Stoyanova, Doncheva, 2002), salicylic acid also influences on efficiency of rhizobial symbiosis (Glyan'ko et al., 2005). These compounds are currently used in the crop production practice. At the same time the physiological mechanisms of protective action of salicylic and especially succinic acids remain a little-studied that limits the possibilities of their practical use.

Capacity of exogenous salicylic acid to increase the resistance of plants to abiotic and biotic stress factors is bound, first of all, with the inhibition of catalase by it that leads to hydrogen peroxide accumulation (Janda et al., 1999). The consequence of H_2O_2 accumulation can be such effects as the activation of processes directed to the destruction of pathogens (an oxidative burst, synthesis of PRs-proteins, etc.) (Durner, Klessig, 1996; Wendehenne et al., 1998), the increase of activity of antioxidant enzymes (Liu et al., 2006; Wang, Li, 2006) and the accumulation of multifunctional low-molecular protectors (Kolupaev et al., 2007), which are important for the formation of plants resistance to abiotic stressors.

At the same time it is known that the catalase molecular forms of different plants organs can have unequal sensitivity to salicylic acid (Chen et al., 1997). In many cases the inhibition of enzyme is caused with high enough (not physiological) concentration of salicylic acid (Rao et al., 1997).

The ability of salicylic acid to activate the superoxide dismutase (SOD) has been revealed on certain species of plants (Rao et al., 1997) that, jointly with catalase inhibition, can be one of the probable reasons of increase of hydrogen peroxide content in plant tissues at the action of salicylic acid (Kolupaev et al., 2010). Salicylic acid can also exert the various (both activating and inhibiting) influence on the activity of peroxidase (Ruffer et al., 1995; Ananieva, Popova, 2002; Kolupaev et al., 2010), participating in the regulation of hydrogen peroxide pool and in the generation of superoxide anion-radical on the conditions of presence of surplus of reductants (Chen, Schopfer, 1999). The activating influence of salicylic acid on the alternative oxidase, that changes the intensity of reactive oxygen species formation in mitochondria, is also founded (Pavlova et al.., 2009).

The influence of exogenous succinic acid on the activity of antioxidant enzymes in connection with its capacity to induce resistance of plants to the abiotic stressors remains a little-studied till now. There are only certain papers in which the inhibition of catalase at some dicotyledonous by succinate has been shown (Tarchevsky et al., 1999; Panina et al., 2004). In this connection succinic acid is considered as the mimetic of salicylic acid (Tarchevsky et al., 1999). It is known also that hydrogen peroxide formation in mitochondria of plant cells depends on the content of succinic acid as substrate of oxidation (Casolo et al., 2006). It is possible to believe that the succinate-dependent H_2O_2 formation is capable to the influence on the activity of antioxidant enzymes.

At the point of the beginning of our researches there were no studies in which the influence of salicylic and succinic acids would be compared on the activity of key enzymes, involved in the regulation of content of reactive oxygen species in cells, – SOD, catalase and peroxidase – in connection with the induction of plants resistance to abiotic stressors. This reason has determined the main purpose of our study – to investigate the influence of treatment of millet (*Panicum miliaceum* L.) seeds with the solutions of salicylic and succinic acids on the heat resistance of plantlets and the activity of antioxidant enzymes in them. In connection with the possibility of practical use of investigated acids in plant-growing (Shakirova, 2001) the study of seeds presowing treatment with salicylic and succinic acids influence on the productivity of millet in the conditions of north-east Forest-steppe of Ukraine was also included to the task of the research.

MATERIALS AND METHODS

Millet seeds of variety Konstantinovsky were treated with the solutions of salicylic or succinic acids in the concentration of range of 10^{-6}-10^{-2} M during one day (control – treatment with distilled water). Further the seeds of all variants were couched in the dark at the temperature of 23±1°C during 4 days in Petri dishes on the distilled water. Then the part of plantlets was subjected to the 10-minute damaging heating in the water thermostat at temperature of 47,0±0,2°C. Within one day after the heating the plantlets were exposed to diffused light (3000 lux), and still in 4 days their survival was estimated.

The activity of SOD (EC 1.15.1.1), catalase (EC 1.11.1.6) and peroxidase (EC 1.11.1.7) in shoots of plantlets was measured before the influence of hyperthermia and in 1 and 24 hours after heating.

At this time the visible appearances of injuries of plants were not observed yet. Simultaneously the enzymes activity was measured in the same age plantlets shoots which were heated.

For the measuring of SOD activity the samples of shoots were homogenised in the 0,15 M phosphatic buffer (pH 7,8) with the addition of detergent triton X-100 in the final concentration 0,1%. The homogenate was centrifuged during 15 minutes at 7000 g. The enzyme activity was estimated using the method described in the previous paper (Kolupaev et al., 2005). In its basis there is the capacity of SOD to compete with nitrotetrazolium blue for superoxide-anions which are formed as the result of aerobic interreaction of NADH and phenazine methasulfate. The optical density was detected at 530 nm.

Catalase activity was estimated by the amount of decomposed H_2O_2 (Kolupaev et al., 2005).

The activity of soluble peroxidase was detected by Ridge and Osborne method (Ridge, Osborne, 1970) with some changes (Kolupaev et al., 2005). The plant material was homogenized in 0,06 M K,Na-phosphatic Serensen buffer (pH 6,2). The homogenate was centrifuged at 7000 g during 15 minutes and then the enzyme activity was estimated in the supernatant. Guaiacol was used as the donor of hydrogen, and hydrogen peroxide – as the substrate. The optical density was measured at 440 nm.

The intensity of lipid peroxidation in the plant tissues was estimated by the content of compounds reacting with 2-tiobarbituric acid (basically malonic dialdehyde – MDA). Their content was analyzed as it was described earlier (Kolupaev et al., 2005).

Laboratory experiments were repeated 4-7 times. The mean values and their standard deviations are shown on the figures.

The influence of presowing treatment of millet seeds was estimated in the micro-field experiment which was placed at the Experimental field of the V.V. Dokuchaev Kharkiv National Agrarian University. Soil conditions represented with the typical chernozem on a loesslike loam. The experiment carried out on the generally accepted method of the field researches. The size of plots 1,5x1,5 m, the accounting area – 1 m^2, replication – fourfold.

RESULTS

Survival of millet plantlets after heating

The treatment of seeds with solutions of salicylic acid in concentration of 10 and 100 µM caused the significant increase of survival of millet plantlets after the damaging heating (fig. 1). Succinic acid showed the maximum protective effect in higher concentration – 100 μM - 1 mM.

Activity of antioxidant enzymes in millet plantlets

In the shoots of control plantlets (without treatment with acids) in 1 hour after heating the small increase of SOD activity was observed (fig. 2.A, II). However in 24 hours after the action of hyperthermia the enzyme activity in the heated plantlets was lower in comparison with the variant without stress (fig. 2.A, III, IV).

The significant increase of SOD activity was observed in the plantlets which have been grown from the seeds, treated with the solutions of salicylic acid in the concentration of 10 μM and 1 mM, (fig. 2.A, I). The succinic acid showed the activating influence on SOD only at the treatment of seeds with the higher concentration (1 mM).

In the plantlets which have been grown from the seeds, treated with salicylic acid in both concentration, in 1 hour after the influence of hyperthermia the additional increase of SOD activity took place, and in 24 hours the enzyme activity decreased and differed from the appropriate values of control variant insignificantly. After 24 hours from the beginning of experimental observations in the plantlets which did not test stress influence in the variant with salicylic acid in concentration 10 μM the SOD activity was higher, than in control (fig. 2.A, IV).

In the samples treated with succinic acid some increase of SOD activity in 1 hour after the influence of hyperthermia was registered too (fig. 2.A, II). The values of enzyme activity in plantlets from the seeds treated with succinic acid in concentration of 1 mM were significantly above the control level at this point of experiment. In further (in 24 hours after the heating of plantlets) the enzyme activity decreased in the variants with the succinic acid, as well as in the other ones. Thus, however, this value was higher at the action of succinic acid in the concentration of 1 mM, than in the appropriate control (fig. 2.A, III). The SOD activity was also a little higher in the variant without heat stress in the plantlets grown from seeds, treated with succinic acid in the concentration of 1 mM, in comparison with the appropriate control in 24 hours from the moment of beginning of observations (fig. 2.A, IV).

In the plantlets of control variant (from the seeds without treatment with acids) the catalase activity did not change essentially in 1 hour after the heating, and by 24 hours it increased a little (fig. 2.B, II, III). After the heating the character of changes of catalase activity was the same in the plantlets which have been grown from seeds, treated with salicylic acid. Treatment with salicylic acid caused some increase of catalase activity in the plantlets (fig. 2.B, I). Though after heating in the variants with salicylic acid the enzyme activity differed a little from the control values (fig. 2.B, II, III). In the variant with treatment of seeds with succinic acid in concentration of 1 mM the increased enzyme activity in comparison with control before heating was observed. In 1 hour after the heating the additional increase of catalase activity took place in the plantlets from seeds treated with succinic acid. Then, in 24 hours the enzyme activity decreased (fig. 2.B, I-III). Thus, however, the absolute value of catalase activity was higher in the variant with succinic acid (1 mM) in 24 hours after the heating than in the appropriate control. In the samples which did not test the stress influence in 24 hours from the beginning of observations in the variants with salicylic and succinic acids in both concentration the catalase activity exceeded the appropriate value of control variant (fig. 2.B, IV).

In the shoots of plantlets of control variant some increase of peroxidase activity was registered during the 24 hours of experimental observations (fig. 2.C). The heat stress caused the increase of enzyme activity appreciable in 1 and 24 hours after heating.

The treatment of seeds with salicylic acid caused the increase of peroxidase activity in them (fig. 2.C, I). Higher level of activity of this enzyme was observed at the use of concentration of 10 µM after heating too in comparison with the appropriate control, in particular in 24 hours (fig. 2.C, II, III). The increased activity of peroxidase was also noted in not heated plantlets in the variant with salicylic acid in 24 hours of experimental observations (fig. 2.C, IV). The succinic acid caused the slight increase of peroxidase activity in the plantlets which were not exposed to the heating (fig. 2.C, I). However in 24 hours after the influence of hyperthermia in the samples treated with succinic acid in concentration of 1 mM the enzyme activity was significantly higher in comparison with the appropriate control (fig. 2.C, III). In 24 hours after the beginning of experiment the increased level of peroxidase activity was also registered in not exposed to the heating samples in comparison with the control (fig. 2.C, IV).

MDA content in millet plantlets

The content of lipid peroxidation product MDA in the millet plantlets of different variants before the damaging heating influence did not differ essentially (fig. 3). At the same time after the action of hyperthermia the increase of MDA content took place in the plantlets of control variant, while in all the experimental variants it did not change significantly. It allows confirming about the smaller degree of oxidative damages of plantlets in the variants with treatment of seeds with succinic and salicylic acids.

Productivity of millet grain

In the field conditions in 2009 the significant increase of millet grain yield under influence of salicylic acid was registered. In the variant with succinic acid only the tendency to the increase of productivity was noted (fig. 4). In 2010, differing extremely unfavorable terms (very dry and hot summer), effects of salicylic and succinic acids were considerably more substantial: the increase of grain yield was 52 and 39% respectively in comparison with control.

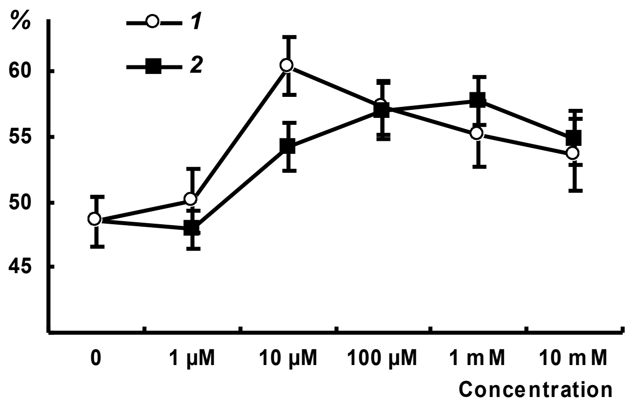

Fig. 1. Survival (%) of millet (*Panicum miliaceum* L.) plantlets after heating (47ºC, 10 min). *1* – salicylic acid; *2* – succinic acid. The zero point on the axis of abscisses corresponds to the survival of plantlets of control variant.

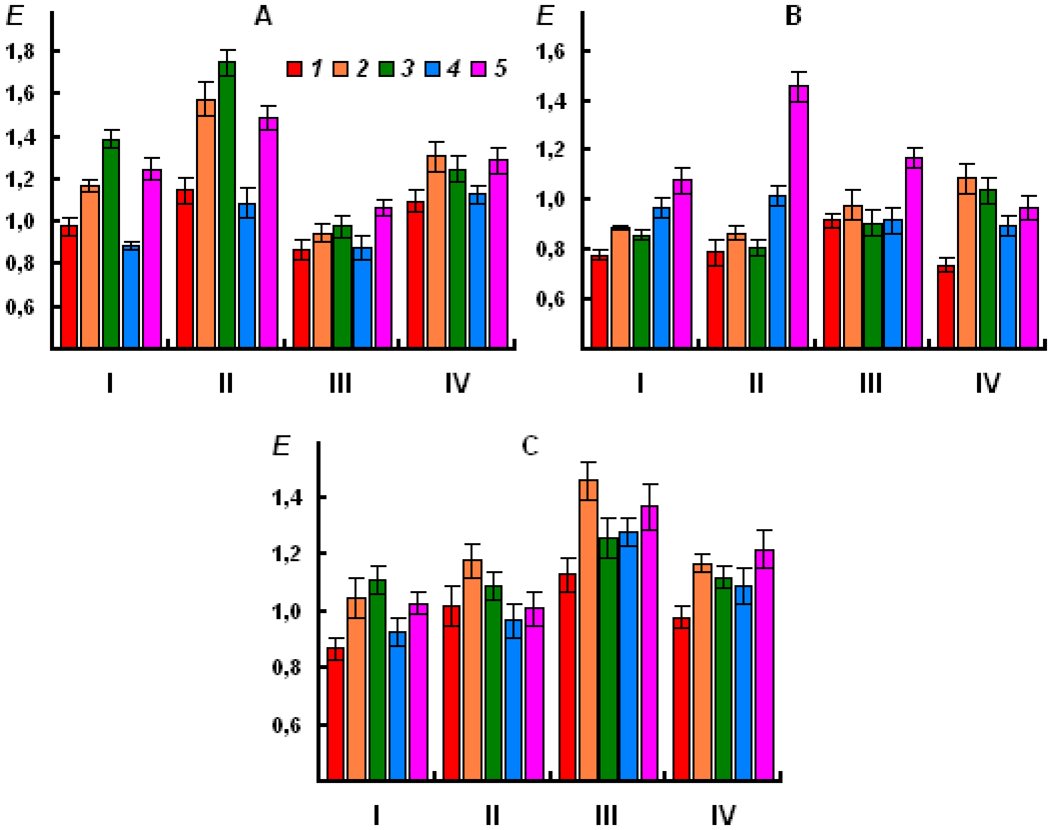

Fig. 2. Activity (*E*, relative units/(g · min)) of SOD (A), catalase (B) and peroxidase (C) in millet plantlets. *1* – control; *2, 3* –salicylic acid 10 µM and 1 mM, respectively; *4*, *5* –succinic acid 10 µM and 1 mM, respectively. I – before heating; II, III – in 1 and 24 hours after heating, respectively; IV – in 24 hours after incubation of not heated plantlets.

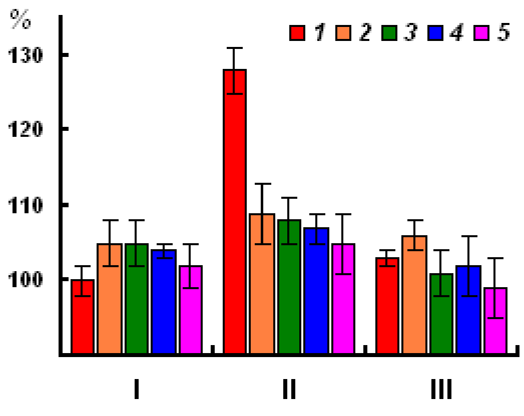

Fig. 3. MDA content (% relative to control before heating) in millet plantlets.
1 – control; *2, 3* –salicylic acid 10 µM and 1 mM, respectively; *4*, *5* –succinic acid 10 µM and 1 mM, respectively. I – before heating; II – in 24 hours after heating; III – in 24 hours after incubation of not heated plantlets.

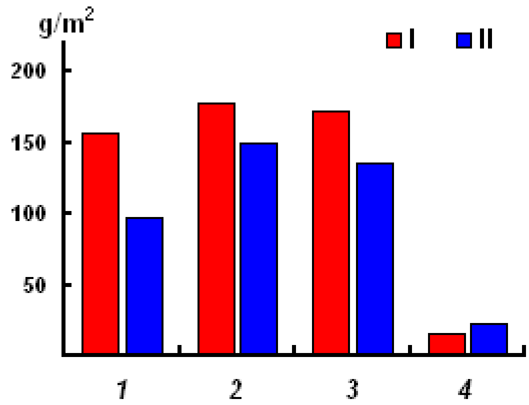

Fig. 4. Influence of presowing treatment with salicylic and succinic acids on the yield of millet (g/m^2) in 2009 (I) and 2010 (II). *1* – control; *2* – salicylic acid 10 µM; *3* – succinic acid 1 mM; *4* – least essential difference.

DISCUSSION

Under the action of salicylic and succinic acids the increase of activity of SOD, playing the role of «primary border» in cell protecting against reactive oxygen species, was registered in our experiments (Alscher et al., 2002). This function of SOD connects with elimination of superoxide radical and such way this enzyme indirectly reduces the possibility of formation of hydroxylic radicals, singlet oxygen, peroxynitrite and other reactive oxygen species, which by reason of its high reactionary ability cannot be removed by protein enzymes.

The pretreatment of seeds with the solutions of salicylic and succinic acids stimulated the catalase activity (fig. 2) that can be bound to the intensifying of its synthesis. The induction of expression of catalase gene under the influence of salicylic acid is shown earlier on corn plants (Guan, Scandalios, 1995).

The treatment of millet seeds with salicylic acid caused the increase of peroxidase activity at all stages of experiment. The increase of peroxidase activity under the influence of salicylic acid is shown earlier too on the example of other cereals – barley (Ananieva, Popova, 2002) and wheat (Kolupaev et al., 2010).

The succinic acid did not render the essential influence on the enzyme activity before heating and in 1 hour after it. However in 24 hours after the heating the value of peroxidase activity in the variant with the succinic acid exceeded the value of control level. It is necessary to note that there are the data about the ability of succinic acid in high enough millimolar concentration to increase the peroxidase activity of isolated roots of wheat in the scientific publication (Chasov et al., 2002).

Thus, in given paper for the first time the identical activating influence of salicylic and succinic acids on the key antioxidant enzyme SOD has been proved. Its activation can lead to the hydrogen peroxide accumulation. It is necessary to note that reactionary ability of hydrogen peroxide and its toxic action on cellular structures is much less in comparison with superoxide and other radical forms of ROS (Foyer, Noctor, 2009). Thus, the activating influence of salicylic and succinic acids on SOD can have the protective importance in itself. Along with it the increase of hydrogen peroxide content can play the role of signal ensuring the formation of protective reactions, in particular, the activation of antioxidant system (Foyer, Noctor, 2009). As it was already noted, under the influence of both acids there was not only the increase of SOD activity but also and activity of catalase and peroxidase in plantlets, exposed to the damaging action of hyperthermia.

The smaller content of lipid peroxidation products in plantlets after the damaging heating also authenticate in favor of activation of antioxidant system under the influence of seeds treatment with salicylic and succinic acids (fig. 3). Apparently, more effective functioning of antioxidant system held back the development of damages caused by heating and promoted the higher survival of plantlets after the stress (fig. 1).

Finally, the more essential increase of millet grain productivity in obviously adverse 2010 also testifies to positive influence of salicylic and succinic acids on resistance of millet plants to abiotic stressors.

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