THE IMPORTANCE OF REACTIVE OXYGEN SPECIES IN THE INDUCTION OF PLANT RESISTANCE TO HEAT STRESS

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Summary. Comparative studies of the effect of short-term heat hardening and exogenous Ca^{2+} ($CaCl_2$) on the heat resistance of wheat (*Triticum aestivum* L.) plantlets and the parameters of pro-antioxidative balance were carried out. It was shown that the influence of hardening and $CaCl_2$ caused an reversible increase of peroxides content in roots, as well as an increase of peroxidase and catalase activities. The antioxidant ionol (butilgidroksitoluol) eliminated the effect of the increase of heat resistance of *Triticum aestivum* L. plantlets caused by the action of hardening and $CaCl_2$ and also leveled the influence of hardening and $CaCl_2$ on the parameters of pro-antioxidative balance. A conclusion can be drawn that the increase of heat resistance caused by hardening and calcium ions occured with the intermediary of reactive oxygen species

***Key words**:* heat hardening, calcium, heat resistance, reactive oxygen species, peroxidase, catalase, ionol (butilgidroksitoluol), *Triticum aestivum* L.
Abbreviations: ROS - reactive oxygen species.

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INTRODUCTION

Similarity of phenomenological effects of plants heat hardening (including short-term) and the effect of exogenous calcium have been registered long time before (Barabalchuk, 1970). However, up to present it is not clear whether it means the existence of common mechanisms of calcium action and short-term hardening effect on plants heat resistance. There are the data proving that both calcium ions and short-term influence of high temperatures can increase thermostability of proteins (Alexsandrov, 1977).

At the same time, there is the grounding to suppose, that under the action of exogenous calcium and high temperatures there can be formed the identical signal messengers and can be activated the common ways of signal transduction (Gong et al., 1998). As a result of the signaling, connected with the action of calcium and hardening temperatures, there can be noticed the activation of gene expression providing formation of adaptive reactions.

It has been found out, that calcium ions under certain conditions are capable to stimulate formation of reactive oxygen species (ROS) in plant cells (Bolwer, Fluhr, 2000). On the other hand, it is known that heat hardening, at least the short-term one, can be accompanied with increase of the ROS content in cells (Dat et al., 1998). Such facts allow stating the hypothesis about the role of ROS in transduction of temperature signal (Suzuki, Mittler, 2006). It is supposed, that calcium and ROS are the components of the unified signal network (Kaur, Gupta, 2005). However, there are no direct sufficient proofs of the ROS role in the formation of heat resistance induced by the influence of short-term high temperature hardening or exogenous calcium.

The purpose of the present research was to carry out a comparison of the influence of short-term hardening and calcium ions on parameters of oxidation-reduction balance and heat resistance of wheat plantlets. With this in view treatment of plantlets roots with the antioxidant ionol (butilgidroksitoluol) was chosen as the methodical means allowing to regulate the pro-antioxidative status of plant cells (Shoring et al., 2000).

MATERIALS AND METODS

Seeds of wheat var. Donetskaja 48 were germinated in darkness at 20.0±0.1 °C during three days. After that the plant material was subjected to treatment presented in Table 1.

The conditions of hardening, concentration of $CaCl_2$ and ionol were chosen on the base of preliminary experimental results.

It is necessary to note that the procedure of use of antioxidant ionol in the experiments with wheat plantlets was probed without external mineral nutrition (Shoring et al., 2000). To avoid possible methodical artifacts connected with interreaction of ionol with components of nutrient mediums (e.g. with metals of variable valence) we did not apply any nutrient media.

After the end of the experimental procedures described in Table 1, the plantlets aged 6 days by the given moment, were placed in the light (2,0-2,5 klx) and in 3 days their surviving ability was estimated.

While estimating the pro-antioxidative effects of studied influences we analyzed the tissues of roots which, as in preliminary experiments it had been found out, were more sensitive to exogenous compounds, than propagules. We also estimated the total content of peroxides in roots (Sagisaka, 1976), as well as peroxidase (guaiacol peroxidase) (Ridge, Osborne, 1970) and catalase (Koroluk et al., 1988) activity.

To quantify the total content of peroxides the shot of plant material was homogenized in a cooled mortar in 5 % trichloroacetic acid, centrifuged for 10 min at 7000 x *g*. To 3 ml of supernatant 0.5 ml of 50 % of trichloroacetic acid, 2.5 M NH_4SCN and 10 mM $(NH_4)_2Fe(SO_4)_2$ each were added and the optical density at wave length of 480 nm was estimated. Concentration of peroxides was calculated by the calibration graph plotted on the basis of hydrogen peroxide.

The peroxidase activity was quantified using 0.06 M K,Na-phosphatic Serensen buffer (pH 6.2) with addition of 0.5 M NaCl as an extragent. The homogenate was centrifuged during 15 min at 7000 x *g*, and after supernatant was used for the analysis. In a reactionary cuvette 0.75 ml of 0.07 % guaiacol, 2.25 ml of Serensen buffer, 0.75 ml of supernatant were mixed and, reckoning time, 0.75 ml of 0.15 % hydrogen peroxide was added. Extinction was estimated at λ=470 nm every 20 seconds during 2

min.

To estimate the catalase activity the plant material was homogenized in 0.1 M Tris-HCl-buffer (pH 7,6) and the homogenate was centrifuged during 15 min at 7000 x *g*. For the analysis 3 ml of supernatant was placed in test tubes and 3 ml of 0.3 % hydrogen peroxide was added. The mixture was incubated for 10 min. The reaction was stopped by adding 1 ml of 4 % ammonium molybdate. The extinction of the solution was quantified at 410 nm. The peroxide content in the sample was calculated with the calibration graph, using standard solutions of hydrogen peroxide.

Biochemical analyses was carried out right after the incubations of plantlets on solutions of ionol and (or) $CaCl_2$, as well as within certain time intervals after hardening and/or damaging heating (see below). At the same time the appropriate parameters also were estimated in the roots of plantlets which were not subjected to heating.

Each experiment was repeated 4 times independently. The average values and their standard deviations are shown in the tables.

RESULTS

Influence of hardening, Ca^{2+} ions, ionol and their combination on the heat resistance of wheat plantlets

Hardening and treatment of plantlets with $CaCl_2$ increased their survival after damaging heating approximately by identical average value. Antioxidant ionol caused some increase of heat resistance of plantlets, but its effects were much lower in comparison with actions of hardening and $CaCl_2$. Thus, antioxidant ionol has leveled the positive influence of both hardening and calcium ions on the heat resistance of wheat plantlets (Fig. 1).

Changes in peroxides content in roots of wheat plantlets under the influence of hardening, Ca^{2+} ions, ionol and their combination

The peroxides content in roots of control plantlets during the experimental observation did not change essentially (Table 2). During 1 h after the

damaging heating the peroxides content authentically did not change in roots of the control (not hardened) plantlets, but it increased during 24 h.

One-minute influence of hardening temperature (42 ºC) resulted in a short-term rise of peroxides content which was observed within 15 min after heating. However, within 1 h after the temperature influence the content of peroxides decreased to the control level, within 4-24 h after hardening this index did not essentially differ from value of the control either (Table 2). After the action of damaging temperature a decrease of peroxides content in roots of the hardened plantlets was registered. In roots of hardened plantlets, which were not exposed to the damaging heating, the peroxides content did not differ from the control within 48 h after hardening.

The treatment of plantlets with ionol solution reduced peroxides content in roots and this effect was kept during the whole period of experimental observation (Table 2). The peroxides content decreased in the samples treated with ionol after the damaging heating. The ionol also removed the temporary increase of peroxides content in roots caused by hardening. After damaging heating the peroxides content was essentially lower in the roots, subjected to the combined influence of hardening and ionol, than in corresponding control (Table 2). The peroxides content was also lower in

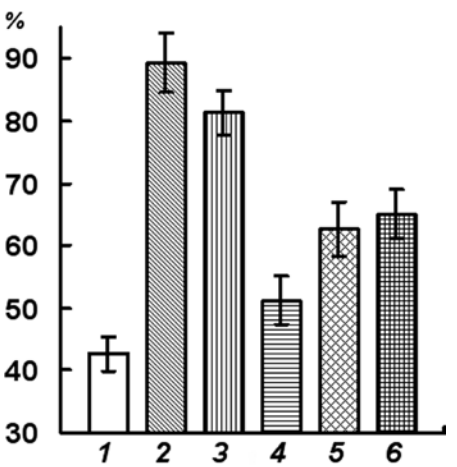

Fig. 1. Wheat plantlets survival (%) after damaging heating (45 ºC, 10 min).
1 - control, *2* - hardening (42 ºC, 1 min), *3* - $CaCl_2$ (50 mM), *4* - ionol (90 μM), *5* - hardening + ionol, *6* - $CaCl_2$ + ionol.

Table 1. The effects of hardening, $CaCl_2$ and ionol on parameters of pro-/antioxidative balance and heat resistance of wheat plantlets.

Variant	Treatment conditions
Control	Incubation on water (48 h) - damaging heating (45 °C, 10 min)* - incubation on water (24 h)
Hardening	Incubation on water (24 h) - hardening at 42 °C (1 min) - incubation on water (24 h) - damaging heating (45 °C, 10 min)* - incubation on water (24 h)
$CaCl_2$ - 50 mM	Incubation on water (24 h) - incubation on 50 mM solution of calcium chloride (24 h) - damaging heating (45 °C, 10 min)* - incubation on water (24 h)
Ionol - 90 μM	Incubation on 90 μM solution of ionol (48 h) - damaging heating (45 °C, 10 min)* - incubation on water (24 h)
Hardening + ionol - 90 μM	Incubation on 90 μM solution of ionol (24 h) - hardening at 42 °C (1 min) - incubation on 90 μM solution of ionol (24 h) - damaging heating (45 °C, 10 min)* - incubation on water (24 h)
$CaCl_2$ - 50 mM + ionol - 90 μM	Incubation on 90 μM solution of ionol (24 h) - incubation on 50 mM solution of calcium chloride with addition of 90 μM ionol (24 h) - damaging heating (45 °C, 10 min)* - incubation on water (24 h)

* - the appropriate control without damaging heating was in each variant of experiment.

roots of plantlets treated with ionol, but not exposed to hardening and/or damaging heating, within 24 h after the treatment and placing the plantlets on water (48 h from the observation beginning), than in corresponding control (Table 2).

$CaCl_2$ influence resulted in the increase of peroxide content which was valid 4 h after the treatment had begun. In 24 h the peroxide content decreased in the variant with $CaCl_2$. Within 4-24 h a decrease of peroxides content was observed in roots after the combined treatment of the samples with $CaCl_2$ and ionol in comparison with the variant with only $CaCl_2$ and control (Table 2).

Within 1-24 h after heating the peroxide content did not change essentially in the variants with calcium and with combined action of Ca^{2+} and ionol.

The peroxide content decreased in roots of the plantlets treated with $CaCl_2$ and were not subjected to the damaging heating, after placing them on water, and did not change essentially in the samples treated with combination of $CaCl_2$ and ionol (tab. 2).

Changes of peroxidase activity in roots of wheat plantlets under the influence of hardening, Ca^{2+} ions, ionol and their combination

The peroxidase activity did not change essentially in roots of control plantlets during the first 24 hours of experimental observation, but were increased within 48 h (Table 3). Within 1-4 h after hardening the increase of peroxidase activity was significant. Then, within 24 h the activity of the enzyme was a little decreased.

The heating at damaging temperatures resulted in the increase of enzyme activity in the roots of control and those subjected to plantlets hardening.

The ionol in itself did not influence significantly on the enzyme activity, but removed the effect of its increase under the influence of hardening temperature (Table 3). The treatment with ionol also reduced the effect of increase of peroxidase activity caused by the action of damaging temperature.

The peroxidase activity increased in the roots of plantlets treated with ionol and not subjected to the damaging heating after their placing on the water.

Table 2. Peroxide content (μmole/g of dry mass) in the roots of wheat plantlets.

Variants	Before hardening or treatment with $CaCl_2$	Time after hardening or beginning of treatment with $CaCl_2$ (h)				Time after damaging heating (h)		48 h after hardening or beginning of treatment with $CaCl_2$
		0,25	1	4	24	1	24	
Control	2.32±0.11	–	2.39±0.15	2.48±0.12	2.28±0.08	2.01±0.11	2.64±0.09	2.25±0.08
Hardening	–	2.91±0.14	2.33±0.17	2.49±0.10	2.11±0.10	1.47±0.11	1.33±0.12	2.11±0.13
$CaCl_2$	–	2.53±0.11	2.69±0.12	3.19±0.13	2.32±0.10	2.05±0.13	2.09±0.10	1.99±0.11
Ionol	1.75±0.09*	–	1.76±0.14	1.90±0.11	1.73±0.09	1.30±0.16	1.42±0.14	1.66±0.09
Hardening + ionol	–	1.92±0.17	1.68±0.12	1.78±0.15	1.68±0.09	1.11±0.12	1.21±0.10	1.59±0.12
$CaCl_2$ + ionol	–	1.93±0.12	1.90±0.10	1.83±0.09	1.83±0.12	1.79±0.14	2.01±0.12	1.99±0.10

* here and in Tables 3, 4 – values within 24 h after beginning of treatment with ionol.

The influence of calcium ions raised the peroxidase activity in roots, thus this effect being the most essential after 24 h the treatment with $CaCl_2$ had started (tab. 3). The subsequent damaging heating did not cause changes of peroxidase activity in the roots of plantlets treated with $CaCl_2$. The peroxidase activity changed insignificantly in roots of plantlets, which were treated with $CaCl_2$, but were not heated with the damaging temperature, within 24 h after placing them on the distilled water.

The ionol reduced the effect of peroxidase activity increase, caused by calcium that was shown practically at the all stages of experiment (Table 3). The peroxidase activity increased in the samples, treated with calcium chloride and ionol, after placing them on the water. It is necessary to note, that by this moment (48 h from the beginning of observation) the peroxidase activity increased in all variants, except for the variant with calcium chloride treatment. Possibly, that at this phase of experiment the increase of enzyme activity is not connected with exogenous influences, but with fluctuations of enzyme activity during development of plantlets (Kolupaev, Karpets, 2006).

Changes of catalase activity in roots of wheat plantlets under the influence of hardening, Ca^{2+} ions, ionol and their combination

The catalase activity did not change essentially in the control variant during the whole period of observations and was just slightly increased by 24-48 h of the experiment (Table 4). Hardening caused the increase of enzyme activity in the roots, the effect being observed already within 15 min after the influence of hardening temperature and kept during the next period of experimental observations.

The influence of damaging temperature resulted in decrease of catalase activity in the roots of control variant. At the same time the raised level of enzyme activity was kept in the roots of hardened plantlets after influence of damaging heating (Table 4).

Preliminary 24 h influence of ionol led to a decrease in catalase activity. The effect of ionol was leveled in 24 h after the placing plantlets on the water. The antioxidant partially removed the effect of catalase activity increase at combined action of hardening and ionol that was shown only

Table 3. Peroxidase activity (rel. units/(g of dry mass min)) in the roots of wheat plantlets.

Variants	Before hardening or treatment with $CaCl_2$	Time after hardening or beginning of treatment with $CaCl_2$ (h)				Time after damaging heating (h)		48 h after hardening or beginning of treatment with $CaCl_2$
		0,25	1	4	24	1	24	
Control	21.1±0.4	–	21.3±0.3	23.0±0.6	22.8±0.4	29.3±0.6	27.3±0.4	28.4±0.5
Hardening	–	22.8±0.4	24.8±0.4	26.9±0.5	23.9±0.3	29.9±0.5	27.4±0.7	29.2±0.5
$CaCl_2$	–	22.3±0.5	23.0±0.6	26.8±0.7	30.4±0.6	29.9±0.4	28.9±0.6	30.0±0.7
Ionol	20.2±0.6*	–	21.2±0.4	22.4±0.3	22.7±0.4	23.8±0.6	22.4±0.5	26.2±0.8
Hardening + ionol	–	20.3±0.7	20.6±0.5	21.9±0.6	19.6±0.7	25.5±0.6	23.2±0.4	27.1±0.3
$CaCl_2$ + ionol	–	21.4±0.5	20.9±0.4	23.8±0.4	23.8±0.5	23.7±0.7	24.6±0.8	29.1±0.7

through 24 h after hardening (Table 4). After the influence of damaging temperature the catalase activity was higher in the samples treated with ionol or subjected to combined influence of ionol and hardening than in corresponding control, but it was lower than in the variant with hardening.

Treatment of plantlets with $CaCl_2$ raised catalase activity in the roots (Table 4). This effect was kept during 24 h after the placing plantlets from the solution of $CaCl_2$ on the water. In the variant with calcium chloride the catalase activity was a little decreased after heating, though its absolute values exceeded the corresponding control values.

In the variant with combined treatment with calcium and ionol the antioxidant partially leveled the increase of enzyme activity, caused by Ca^{2+}ions. In 1 h after heating the catalase activity raised in the roots under the combined treatment with calcium chloride and ionol. Its absolute values were a little less than in the variant with only calcium treatment. Later, in 24 h after heating some decrease of activity there was (Table 4). Within 24 h after the treatment with $CaCl_2$ and ionol had been ended, the enzyme activity also was a little raised in roots of plantlets, which had not been subjected to the damaging heating (Table 4).

DISCUSSION

Both the short-term hardening and the influence of exogenous $CaCl_2$ caused an increase of heat resistance of wheat plantlets. Thus effects of both influences, causing increase of heat resistance, were substantially levelled by the antioxidant ionol (Figure 1). This fact in itself allows to assume the role of reactive oxygen species as signal messengers in the processes of induction of plants heat resistance by short-term influence of high temperatures (hardening) and treatment with exogenous calcium ions.

The biochemical analyses carried out as a whole confirm this assumption. Both influences (hardening and $CaCl_2$) caused effect of "oxidative stress", which was expressed in the increase of total peroxides content in roots (Table 2). After the influence of hardening temperature this effect was short-term, but it was prolonged at the incubation of roots in the $CaCl_2$ solution.

Table 4. Catalase activity (mmole of H_2O_2/(g of dry mass min)) in the roots of wheat plantlets.

Variants	Before hardening or treatment with $CaCl_2$	Time after hardening or beginning of treatment with $CaCl_2$ (h)				Time after damaging heating (h)		48 h after hardening or beginning of treatment with $CaCl_2$
		0,25	1	4	24	1	24	
Control	7.84±0.12	–	7.75±0.16	8.04±0.12	8.45±0.11	7.22±0.21	6.27±0.19	8.66±0.15
Hardening	–	8.90±0.14	8.72±0.15	9.39±0.10	10.09±0.12	9.40±0.15	9.13±0.16	9.68±0.25
$CaCl_2$	–	8.09±0.14	8.38±0.17	8.15±0.13	11.51±0.14	9.59±0.12	8.95±0.16	10.43±0.19
Ionol	6.79±0.16*	–	7.25±0.15	7.24±0.11	7.75±0.12	8.18±0.16	7.45±0.14	8.85±0.13
Hardening + ionol	–	8.61±0.11	8.47±0.18	9.03±0.15	9.58±0.13	8.90±0.30	7.80±0.10	10.50±0.25
$CaCl_2$ + ionol	–	6.86±0.12	7.58±0.16	7.46±0.16	7.05±0.10	8.96±0.18	8.15±0.18	8.95±0.19

By the example of mustard plantlets it is shown, that the heat hardening caused the temporary increase of hydrogen peroxide content in tissues, and then the heat resistance of plantlets has been raised (Dat et al., 1998). On the wheat plantlets we have registered the similar effect, which was suppressed by the ionol. Such suppression of the "oxidative stress" effect by the antioxidant, as stated above, blocked the development of heat resistance.

The facts of intensifying of ROS generation under the influence of exogenous calcium have been registered before too. So, on the example of cut roots (Minibaeva et al., 1997) and isolated wheat coleoptiles (Kolupaev and Karpets, 2007) the intensifying generation of superoxide radical is shown at their treatment with 5-10 mM $CaCl_2$ solution. Such induction of oxidative stress by calcium can be connected with the increase of NADPH-oxidase activity (Sagi and Fluhr, 2006). It is known, that the 91 kD subunit of NADPH-oxidase has two Ca^{2+}-bonding sites (Keller et al., 1998). It is not excluded, that exogenous calcium induces the primary wave of ROS formation, what causes calcium channels opening. The calcium entry in cytosol leads to the additional activation of ROS-generating systems (Kaur and Gupta, 2005). It is supposed, that the both interconnected processes of change of the cells calcium status and the ROS content in cells can be involved in the development of adaptive reactions to various stressors (Bolwer and Fluhr, 2000). The significant leveling of the positive calcium influence by antioxidant on the heat resistance of wheat plantlets, registered by us, can be considered as the argument in favour of such assumption.

Both hardening and exogenous calcium caused the activity increase of peroxidase and catalase, the enzymes participating in maintenance of hydrogen peroxide balance, to some extent in roots of wheat plantlets (Tables 3, 4). It is necessary to note, that the phenomenon of activity increase of antioxidative enzymes (in particular, catalases and superoxide dismutases) in wheat plants has been registered earlier by the example of long action of acclimatization temperature 34 °C (Zhou, 1995). The effects of activity increase of various antioxidative enzymes, including catalases, peroxidases, superoxide dismutases, under the influence of exogenous calcium was shown by the example of different plants (Bakardjieva et al., 2000; 2001; Chen et al., 2004; Kolupaev et al., 2005).

In the given paper we have succeeded to demonstrate, that the increase of peroxidase and catalase activity in the roots of wheat plantlets, induced both by hardening and exogenous calcium, was substantially removed by the antioxidant. It is possible to believe, that the primary intensifying of ROS generation under the influence of hardening and calcium serves as the signal for activation of antioxidative enzymes. As it is known, the activation of some antioxidative enzymes is also possible under the influence of exogenous ROS on plants, in particular, hydrogen peroxide (Kolupaev, Karpets, 2007; Upadhayaya et al., 2007). Possibly, both hardening influence and pretreatment with exogenous calcium "prepared" the plant antioxidative system for the adequate functioning under the conditions of damaging high temperatures action that was one of the ways of heat resistance induction. Thus, the increase of heat resistance of wheat plantlets under the influence of short-term hardening and calcium chloride, apparently, took place with intermediary of ROS.

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