

ORIGINAL ARTICLE

Induction of Heat Resistance in Wheat Coleoptiles by 4-Hydroxybenzoic Acid: Connection with the Generation of Reactive Oxygen Species

Yastreb T.O., Kolupaev Yu.Ye.*, Vayner A.O.

V.V. Dokuchaev Kharkiv National Agrarian University, p/o «Communist-1», Kharkiv, 62483, Ukraine

* E-mail: plant_biology@mail.ru

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The effect of 4-hydroxybenzoic acid (4-HBA) on resistance of coleoptiles of 4-day-old etiolated seedlings of wheat (*Triticum aestivum* L., cv. Elegiya) to damaging heating (10 min at 43°C) and possible dependence of this effect on changes in the activities of enzymes producing and scavenging reactive oxygen species (ROS) were investigated. Treatment of coleoptiles with 10 µM 4-HBA resulted in enhancing of superoxide anion-radical generation and maintaining of hydrogen peroxide content there in. Increasing of the rate of ROS production was significantly suppressed by inhibitors of NADPH oxidase (α -naphthol) and peroxidase (salicylhydroxamic acid). Under the influence of 4-HBA the activities of superoxide dismutase and apoplasmic forms of peroxidase were increased. The activity of oxalate oxidase and catalase has not changed. Exogenous 4-HBA improved coleoptiles heat resistance and its effects were comparable with the influence of salicylic acid. Antioxidant agent BHT (butylhydroxytoluene), inhibitors of NADPH oxidase and peroxidase significantly reduced the increasing of wheat coleoptiles heat resistance, caused by 4-HBA action. It was concluded that 4-HBA influence on coleoptiles heat resistance is realized with the ROS mediation.

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Benzoic acid (BA) and its derivatives 2-hydroxybenzoic (salicylic acid – SA) and 4-hydroxybenzoic (4-HBA) acids are natural metabolites of plant phenylpropanoid pathway (Sircar, Mitra, 2009). SA is the most studied of these compounds. It belongs to the substances that combine the properties of a signal intermediate and stress phytohormone (Wang, Li, 2006).

Endogenous SA is involved in development of

plant resistance to biotic and abiotic stressors (Durner, Klessig, 1996; Kaplan *et al.*, 2004). Exogenous SA also increases the plant resistance to stress factors of different nature (Dat *et al.*, 1998; Kolupaev, Karpets, 2010; Lei *et al.*, 2010).

Physiological effects of BA and 4-HBA are investigated to a much lesser extent. It was shown that exogenous BA, as well as SA, increased the resistance of beans and tomatoes plants to heat

shock, water deficit and low temperature (Senaratna *et al.*, 2003). 4-HBA induced the resistance of wheat plants to low temperatures (Horwath *et al.*, 2007).

At the same time exogenous 4-HBA, as opposed to SA, did not enhance the cold tolerance of young maize plants (Horwath *et al.*, 2002). Also, 4-HBA didn't show the ability to induce synthesis of PR-proteins and to increase plant resistance to biotic stressors (Chen *et al.*, 1993). However, it was revealed the accumulation of 4-HBA in cucumber phloem fluids during infection of *Pseudomonas syringae*, associated with phenylalanine ammonia-lyase activation (Smith-Becker *et al.*, 1998).

The ability of exogenous SA to induce plant resistance is to a large extent due to the increased generation of reactive oxygen species (ROS), performing the signaling functions to induce protective responses to biotic and abiotic stressors (Dat *et al.*, 1998; Kolupaev *et al.*, 2011). It was shown by us, that rise of hydrogen peroxide content in cells of wheat coleoptiles under the SA influence was associated with increased activity of NADPH-oxidase and apoplastic peroxidases, and also superoxide dismutase which converts superoxide anion-radical to H₂O₂ (Kolupaev *et al.*, 2011). At the same time, the effect of 4-HBA on enzyme systems of plant cells, generating ROS, was not specifically studied.

The aim of our study was to investigate the effect of 4-HBA on the ROS generation by wheat coleoptiles and to establish its possible enzyme sources. Also, the possibility of coleoptiles heat resistance inducing by exogenous 4-HBA and the participation of ROS in this process were studied.

MATERIALS AND METHODS

The object of the investigation was segments of

the coleoptiles detached from 4-day-old etiolated seedlings of winter wheat (*Triticum aestivum* L.) cv. Elegiya, which are the object model, sensitive to exogenous phytohormones and hormone-like compounds, in particular, to SA (Kolupaev *et al.*, 2011).

Preparation of plant material has been described previously (Kolupaev *et al.*, 2011). After separation from seedlings, coleoptiles were incubated in Petri dishes in sterile 2% sucrose solution with the addition of the penicillin sodium salt (10⁵ units/l) for 14-16 h, then the segments of experimental variants were incubated for 2 h in the 10 μM solutions of 4-HBA, SA or BA, prepared at 2% sucrose solution. Concentrations of effectors were selected on the basis of preliminary experiments. The coleoptiles of the control variant were incubated in 2% sucrose. In separate series of experiments coleoptiles were treated with an antioxidant BHT (butylhydroxytoluene, 5 μM) (Shorning *et al.*, 2000), an inhibitor of NADPH-oxidase α-naphthol (1 μM) (Hung *et al.*, 2006) and peroxidase inhibitor salicylhydroxamic acid (SHA, 500 μM) (Mori *et al.*, 2001). Concentrations of these compounds were also selected in preliminary experiments. In the variants with combined treatment these effectors were added to the basic incubation medium for coleoptiles (2% sucrose + 10⁵ units/l of penicillin) 1 h before the insertion of 4-HBA.

After incubation of coleoptiles in solutions of investigated effectors, the segments were exposed to potentially lethal heating in water ultrathermostat in sterile distilled water at 43±0.1°C for 10 min, and then placed into Petri dishes containing 2% sucrose solution with addition of penicillin (10⁵ units/l). After 3 days after heating the damage of coleoptiles was estimated by the

appearance of specific brown coloring and the loss of turgor.

After processing coleoptiles with investigated acids the rate of superoxide anion-radical generation by coleoptiles, the hydrogen peroxide contents, the activities of cell walls peroxidase and loosely-coupled extracellular peroxidase, superoxide dismutase (SOD), catalase, and oxalate oxidase activities were determined.

A release of superoxide anion-radicals from the segments of coleoptiles to external solution was determined by the reduction of nitro blue tetrazolium (NBT) (Shorning *et al.*, 2000). 15 coleoptiles were placed into every test tubes with 5 ml of 0.1 M phosphate buffer (pH 7.6) containing 0.05% NBT, 10 μ M EDTA, and 0.1% Triton X-100. The samples were agitated for 1 h on a shaker (120 rpm), and then optical density of the solution of incubation was determined at 530 nm. In order to test the specificity of $O_2^{\cdot-}$ generation, some samples were supplemented with SOD (50 units/ml). SOD suppressed the generation of superoxide anion-radical by no less than 90%. We assumed that the amount of reduced NBT depended on the content of $O_2^{\cdot-}$. Superoxide producing activity was assessed as changes in optical density of the reaction mixture for 1 h of incubation per segment.

A hydrogen peroxide content was determined by the formation of complex with Xylenol orange (Bindschedler *et al.*, 2001). Prior to analysis the reagent was prepared by mixing 1 ml of 25 mM Mohr's salt solution in 2.5 M sulfuric acid with 100 ml of 125 μ M Xylenol orange solution in 100 mM sorbitol. In order to determine H_2O_2 content the weighted coleoptiles were homogenized in the cold in 0.01 mM Na-phosphate buffer (pH 6.2), the homogenate was centrifuged for 15 min at 8000 g,

the supernatant was added to 10-fold volume of this reagent and incubated at room temperature for 30 min. After centrifugation at 8000 g for 10 min optical density of the solution was determined at 560 nm.

In order to determine peroxidase (EC 1.11.1.7) activity coleoptiles were homogenized in 0.01 M Na-phosphate buffer (pH 6.2). The homogenate was centrifuged at 8000 g for 15 min. The precipitate was repeatedly washed with initial buffer, the ion-bound peroxidase fraction was extracted from the sediment by 1 M NaCl (Ranieri *et al.*, 1996). After that, the extract of bound forms of enzymes were dialyzed against the initial buffer for 14-16 h. Enzyme activity was determined by the method of Ridge and Osborne (1970). Hydrogen peroxide was used as a substrate and guaiacol – as a donor of hydrogen. The optical density of colored product was measured at 440 nm.

The activity of extracellular peroxidase was determined in the incubation solution after 1-h-long agitation on a shaker (120 rpm) of 15 coleoptiles segments in test tubes with 5 ml of phosphate buffer (pH 6.2) with the addition of 0.1% Triton X-100, with H_2O_2 as a substrate and guaiacol as a reducing agent.

In order to determine SOD (EC 1.15.1.1) and catalase (EC 1.11.1.6) activities, the segments of coleoptiles were homogenized in the cold in 0.15 M phosphate buffer (pH 7.6) with the addition of detergent Triton X-100 (final concentration 0.1%). The supernatant obtained after homogenate centrifugation at 8000 g was used for the analysis. SOD activity was determined using the method based on ability of the enzyme to compete with 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 decomposed H_2O_2 (Kolupaev *et al.*, 2005).

Oxalate oxidase was extracted from plant material by 0.01 M Na-phosphate buffer (pH 6.2). In order to determine the enzyme activity (Viletic, Sukalovich, 2000; Troshina *et al.*, 2007), 0.05 M succinate buffer (pH 3.8), 0.15% chromogenic substrate ortho-phenylenediamine and 2.5 mM oxalic acid were added to the enzymatic extract into reaction cell. The samples were incubated in dark for 5 or 10 minutes, whereupon the optical density was measured at 490 nm.

Experiments were conducted in 3–4 biological replicates and repeated independently at least 3 times. Figures show the means and their standard deviations.

RESULTS AND DISCUSSION

Pretreatment of coleoptiles with 4-HBA considerably improved the superoxide anion radical and hydrogen peroxide generation therein, and its effects were comparable with the action of SA. BA did not provide a similar activity (Fig. 1). Exogenous SA, 4-HBA, but not BA, increased the resistance of wheat coleoptiles to damaging heating. Thus, the physiological activity of exogenous organic acids largely depends on their ability to cause increased ROS generation by plant cells.

The intensification of superoxide anion-radical generation by wheat coleoptiles under the effect of 4-HBA may be associated with increased activity of NADPH-oxidase (Glyanko *et al.*, 2009) and/or certain forms of peroxidase (Minibaeva *et al.*, 2001). In order to verify this assumption, we used the inhibitor analysis. The generation of $O_2^{\cdot-}$, caused by the 4-HBA action were largely

suppressed both an inhibitor of NADPH-oxidase α -naphthol and peroxidase inhibitor SHA (Fig. 2). At the same time inhibitors of these enzymes by themselves slightly decreased the formation of superoxide anion-radical by wheat coleoptiles. Antioxidant agent BHT also decreased intensification of superoxide anion radical generation induced by 4-HBA (Fig. 2).

4-HBA caused activation of loosely-coupled extracellular and ion-bound forms of peroxidase (Table 1). These enzyme forms may be involved in ROS generation (Minibaeva *et al.*, 2001; Graskova *et al.*, 2004).

The SA ability to increase SOD activity in wheat coleoptiles has been previously shown by us (Kolupaev *et al.*, 2011). 4-HBA also enhanced the SOD activity (Table 1). Activation of SOD may be the reason of maintaining the hydrogen peroxide content in coleoptiles under the 4-HBA influence.

Hydrogen peroxide can be accumulated also in relation with reduction of catalase activity. At some objects the ability of aromatic acids to inhibit this enzyme has been demonstrated (Senaratna *et al.*, 2003). However, in our experiments under the 4-HBA influence catalase activity did not change (Table 1). Previously, we showed that there were no effect of SA on the catalase activity in coleoptiles of wheat cv. Elegiya (Kolupaev *et al.*, 2011).

In ROS generation also an oxalate oxidase can participate (Berna, Bernier, 1999). This enzyme activity was increased under SA influence at plants of wheat (Troshina *et al.*, 2007). However, the activity of oxalate oxidase did not change under the influence of 4-HBA in our experiments (Table 1).

Thus, main reasons for the hydrogen peroxide accumulation in wheat coleoptiles under the 4-HBA effect are apparently the increasing of NADPH-

oxidase and peroxidase forms activities, involved in superoxide anion-radical generation, and also SOD, which converts $O_2^{\cdot-}$ into hydrogen peroxide (Fig. 2, Table 1).

It is known that NADPH-oxidase is localized in plasma membrane and forms superoxide anion-radical on its outer surface (Sagi, Fluhr, 2006). In etiolated plants and in non-green parts of them SOD is contained in cytoplasm in significant amounts, but was revealed in apoplast as well

(Ogawa *et al.*, 1997; Miller *et al.*, 2010). It is possible that apoplastic SOD, that converts superoxide anion-radical in H_2O_2 , promotes penetration of the ROS into cytoplasm and discharging of their signaling functions therein. At the same time the possibility of superoxide anion-radical penetration in protonated form to cytoplasm (Sagi, Fluhr, 2006), followed by its conversion to hydrogen peroxide by intracellular forms of SOD, is not ruled out.

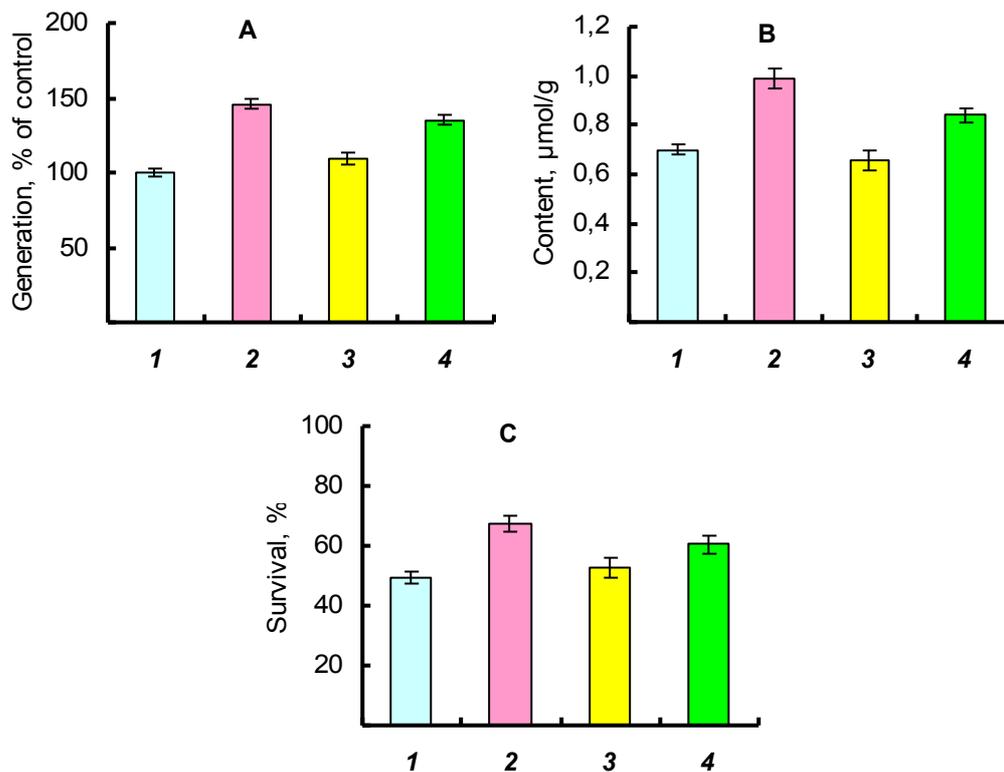


Figure 1. Generation of superoxide anion-radical (A), a content of hydrogen peroxide (B) in wheat coleoptiles and their survival (%) after heating (C). 1 – control, 2 – SA (10 µM), 3 – BA (10 µM), 4 – 4-HBA (10 µM).

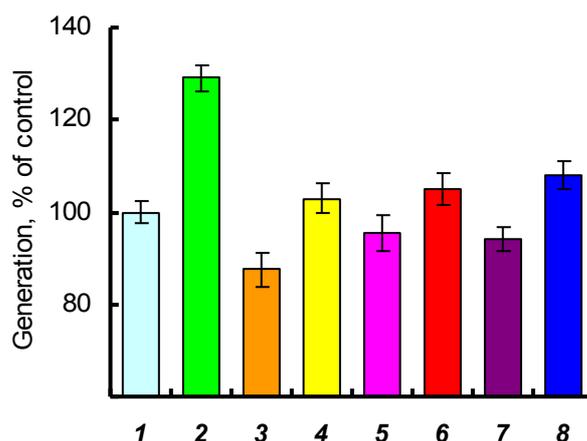


Figure 2. Effect of 4-HBA, BHT and inhibitors of prooxidant enzymes on the generation of superoxide anion-radical. 1 – control, 2 – 4-HBA (10 μM), 3 – BHT (5 μM), 4 – 4-HBA (10 μM) + BHT (5 μM), 5 – α-naphthol (1 μM), 6 – 4-HBA (10 μM) + α-naphthol (1 μM), 7 – salicylhydroxamic acid (500 μM), 8 – 4-HBA (10 μM) + salicylhydroxamic acid (500 μM).

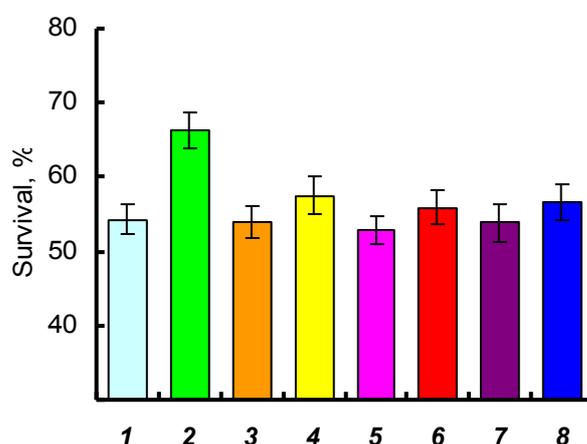


Figure 3. Survival of wheat coleoptiles (%) after damaging heating. 1 – control, 2 – 4-HBA (10 μM), 3 – BHT (5 μM), 4 – 4-HBA (10 μM) + BHT (5 μM), 5 – α-naphthol (1 μM), 6 – 4-HBA (10 μM) + α-naphthol (1 μM), 7 – salicylhydroxamic acid (500 μM), 8 – 4-HBA (10 μM) + salicylhydroxamic acid (500 μM).

Table 1. Activity (relative units/(g dry weight / min)) of enzymes generating and converting ROS in wheat coleoptiles

Treatment	Loosely-coupled extracellular peroxidase*	Ion-bound peroxidase of cell walls	SOD	Catalase	Oxalate oxidase
Control	0.118±0.008	7.10±0.24	12.7±0.5	6.32±0.09	276.0±8.0
4-HBA, 10 μM	0.173±0.010	7.94±0.18	17.3±0.7	6.31±0.08	263.4±8.2

* activity of this enzyme is given in rel. units/(coleoptiles' segment · min).

Previously, in wheat coleoptiles the connection between the SA ability to activate ROS generation

by plant cells and to induce the development of coleoptiles heat resistance has been shown by us

(Kolupaev *et al.*, 2011). Most probably ROS are involved in the 4-HBA effect implementation as well. Thus, antioxidant BHT, inhibitors of NADPH-oxidase (α -naphthol) and peroxidase (SHA) significantly reduced the positive effect of 4-HBA on wheat coleoptiles heat resistance (Fig. 3). At the same time these substances did not significantly affect the coleoptiles survival after damaging heating by themselves.

Thus, this work showed the increasing of the heat resistance of wheat coleoptiles under the 4-HBA influence. Important intermediaries into its physiological effects implementation, apparently, are the ROS.

The obtained results of inhibitor analysis suggest that in wheat coleoptiles the enzymatic sources of ROS formation stimulated by 4-HBA are NADPH-oxidase and apoplastic peroxidase, because inhibitors of these enzymes (α -naphthol and SHA) partially leveled the increased ROS generation and maintained heat resistance of coleoptiles (Figs. 2 and 3). Elevated levels of hydrogen peroxide in coleoptiles treated with 4-HBA, observed in our experiments, to some extent may be due to increase in SOD activity (Table 1).

This way, we can assume that the protective effects of 4-HBA are associated with a modification of enzymes involved in ROS formation. Their activation can lead to formation and transmission into genetic apparatus the signal that induces nonspecific protective reactions, such as increased expression of antioxidant enzymes genes (Kolupaev, Karpets, 2010; Lei *et al.*, 2010). In this regard, the physiological effects of 4-HBA may be similar to the effects of SA (Horvath *et al.*, 2007).

It is possible that 4-HBA, as well as SA, can be synthesized in plants in response to stressors

(Smith-Becker *et al.*, 1998) and perform safety functions. Of course, this assumption requires special studies.

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