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BRIEF COMMUNICATION

Pre-treatment with H_2O_2 induces salt tolerance in barley seedlings

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Abstract

Barley seedlings were pre-treated with 1 and 5 μM H_2O_2 for 2 d and then supplied with water or 150 mM NaCl for 4 and 7 d. Exogenous H_2O_2 alone had no effect on the proline, malondialdehyde (MDA) and H_2O_2 contents, decreased catalase (CAT) activity and had no effect on peroxidase (POX) activity. Three new superoxide dismutase (SOD) isoenzymes appeared in the leaves as a result of 1 μM H_2O_2 treatment. NaCl enhanced CAT and POX activity. SOD activity and isoenzyme patterns were changed due to H_2O_2 pre-treatment, NaCl stress and leaf ageing. In pre-treated seedlings the rate of $^{14}CO_2$ fixation was higher and MDA, H_2O_2 and proline contents were lower in comparison to the seedlings subjected directly to NaCl stress. Cl^- content in the leaves 4 and 7 d after NaCl supply increased considerably, but less in pre-treated plants. It was suggested that H_2O_2 metabolism is involved as a signal in the processes of barley salt tolerance.

Additional key words: catalase, $^{14}CO_2$ fixation, malondialdehyde, NaCl, peroxidase, proline, superoxide dismutase.

Hydrogen peroxide is produced under various abiotic and biotic stresses and causes oxidative damage to plants. Some authors suggested that H_2O_2 plays a dual role in plants: at low concentration acts as a messenger involved in signaling and in triggering tolerance against various abiotic stresses, but at high concentrations H_2O_2 causes oxidative stress which leads to a loss of protein function, membrane integrity, and to programmed cell death (Asada 1996). It has been shown that H_2O_2 acts as a signal to induce defense gene expression (Desikan *et al.* 2000). Some of these genes encode antioxidant enzymes, and defense and stress-related proteins (Desikan *et al.* 2001). Noctor *et al.* (2002) call in attention to the potential significance of photorespiratory H_2O_2 in signaling and acclimation. Pre-treatment of rice seedlings with low concentrations H_2O_2 (< 10 μM) helped the survival under salt and heat stresses due to more green leaf tissue and higher quantum yield of PS 2 (Uchida *et al.* 2002). Nodal potato explants subcultured from H_2O_2-treated plantlets were resistant to a 15-h heat shock at 42 °C (Lopez-Delgado *et al.* 1998). Neto *et al.* (2005) reported that pretreatment of wheat seeds with H_2O_2 (1 - 120 μM) improved their salt tolerance by activating antioxidants and H_2O_2 scavenging. It was shown that exogenously applied H_2O_2 increased chilling stress tolerance, partly due to an enhanced antioxidative system (Prasad *et al.* 1994). The capacity of antioxidant defense system is often increased under stress conditions (Niknam *et al.* 2006, Mandhania *et al.* 2006, He and Zhu 2008), but in most situations the response is moderate (Foyer *et al.* 1994). The aim of this paper was to investigate effects of H_2O_2 pre-treatment for improving barley seedlings tolerance against NaCl stress and the role of the antioxidant defense system in this process.

Barley (*Hordeum vulgare* L. cv. Alfa) seedlings were grown in hydroponic culture in a growth chamber with a 12-h photoperiod, irradiance of 160 μmol m^{-2} s^{-1}, temperature of 25 - 22 °C and humidity of 60 %. Seedlings (4-d-old) were pre-treated with 1 or 5 μM H_2O_2 for 2 d. The plants were than supplied with water or 150 mM NaCl and were analysed after 4 and 7 d. Thus the four basic variants were analysed: not pre-treated and not

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Abbreviations: CAT - catalase; MDA - malondialdehyde; NBT - nitroblue tetrazolium; POX - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase; TBA - thiobarbituric acid; TCA - trichloracetic acid.
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salt-stressed, not pre-treated and salt-stressed, pre-treated and non salt-stressed, pre-treated and salt-stressed.

Proline was determined by the method of Bates *et al*. (1973), and malondialdehyde and hydrogen peroxide according to Esterbauer and Cheeseman (1990). $^{14}CO_2$ fixation was determined radiometrically by the method described by Moll (1986). Native PAGE in 7.5 % gel was carried out by the method of Davis (1964). Cationic peroxidase was separated by the method of Reisfeld *et al*. (1962). Peroxidase (EC 1.11.1.7) iso-enzymes were detected according to procedure of Ornstein (1964). Superoxide dismutase (EC 1.15.1.1) isoenzymes were detected by the method of Greneche *et al*. (1991). Catalase (EC 1.11.1.6) isoenzymes were stained as described by Woodbury *et al*. (1971). Protein content was determined according to method of Lowry *et al*. (1951). Chlorophyll and carotenoids contents were determined according to Lichtenthaler (1987).

H_2O_2 generation, proline and MDA content increased and $^{14}CO_2$ fixation decreased after NaCl treatment, particularly in plants subjected for 7 d to salinity stress (Table 1). Exogenous H_2O_2 had no effect on $^{14}CO_2$ fixation, proline and MDA content and did not alter endogenous H_2O_2 content in the leaves (Table 1). H_2O_2 pre-treated seedlings exhibited lower proline content after salinization in comparison to plants treated only with NaCl. In the pre-treated seedlings $^{14}CO_2$ fixation decreased following NaCl application, but was about 2-fold higher than that in the non pre-treated NaCl-stressed seedlings.

Cl^- content in the leaves (Table 1) of the plants subjected to NaCl for 4 and 7 d increased approximately 10 and 20 times, respectively. As a result of pre-treatment with H_2O_2 the accumulation of Cl^- was lower both in roots and leaves. More pronounced was the effect of 1 μM H_2O_2 after 7 d of NaCl stress.

SOD activity and isoenzyme patterns changed as a result of H_2O_2 pre-treatment, salt stress and leaf ageing (Fig. 1*A*). Three new SOD isoenzymes appeared in 1 μM H_2O_2 pre-treated seedlings. According to our knowledge this is the first data about H_2O_2-induced SOD isoenzymes. NaCl inhibited SOD activity both in pre-treated and non pre-treated seedlings. Four SOD isoenzymes were detected in the root samples (Fig. 2*A*). Pre-treatment with H_2O_2 enhanced the activity of all isoenzymes especially at the end of the experimental period.

H_2O_2 (1 and 5 μM) had no effect on POX activity and isoenzyme patterns in the leaves while NaCl increased the activity of isoenzymes 7 and 8 (Fig. 1*B*). H_2O_2 pre-treatment had no effect on the POX activity and isoenzymes number in the roots (Fig. 2B) with exception of temporary increased isoenzymes 6 and 7. Four cationic POX isoenzymes were visualized in the leaf samples. The fastest moving isoenzyme 4 was the most active. The activity of isoenzyme 3 increased in the presence of NaCl (Fig. 3*A*). Four basic POX isoenzymes were visible in the roots as well (Fig. 3*B*). As a result of pre-treatment with 1 μM H_2O_2 a slight increase of enzyme activity was observed (Fig. 3*B*) but POX activity decreased after treatment with 5 μmol H_2O_2 (Fig. 3*B*). An enhancement of enzyme activity was established during the ageing (Fig. 3*B*). In the presence of NaCl the activity of all isoenzymes increased, especially isoenzyme 3, both in pre-treated and non pre-treated seedlings (Fig. 3*B*).

Three CAT highly active closely migrating isoenzymes were stained in the leaves (Fig. 1*C*). A slight decrease in the CAT activity was established in pre-treated seedlings at the end of the experimental period. H_2O_2 pre-treatment decreased CAT activity in the roots (data not shown).

H_2O_2 applied to growth solution in concentrations 1 and 5 μM 2 d before NaCl supply alleviated salt stress in barley seedlings. Lower H_2O_2 concentration had more pronounced effect than the higher one. Less proline and

Table 1. Contents of chlorophyll [mg g^{-1}(f.m)], carotenoids [mg g^{-1}(f.m)], Cl^- [mg g^{-1}(d.m.)], proline [μg g^{-1}(f.m.)], rate of CO_2 fixation [mg($^{14}CO_2$) g^{-1}(f.m.) h^{-1}], H_2O_2 [nmol g^{-1}(f.m.)] and MDA [μmol g^{-1}(f.m.)] contents in barley seedling pre-treated with 1 and 5 μM H_2O_2 before 150 mM NaCl for 4 and 7 d. The H_2O_2 pre-treated seedlings, not subjected to salt stress, were transferred to water for the next 4 and 7 d. Means ± SE, n = 3, *,**,*** - means significantly different from control at P = 0.05, 0.01, 0,001, respectively.

H_2O_2	NaCl	Chl *a*+ *b*	Carotenoids	Leaf Cl^-	Root Cl^-	Proline	CO_2	H_2O_2	MDA
Control		1.760±0.004	0.295±0.003	0.003	0.005	61±1.3	13.5±0.1	2250±93	0.020±0.001
-	4 d	1.450±0.026**	0.270±0.004**	0.048±0.006	0.042±0.008	480±43***	2.1±0.18***	3100±15***	0.027±0.003
1	4 d	1.550±0.019	0.286±0.007	0.036±0.007	0.031±0.003	310±28*	4.3±0.36**	2600±87**	0.022±0.001
1	-	1.720±0.017	0.292±0.003	0.004	0.003	73±4*	14.1±0.12*	2200±113	0.022±0.002
5	4 d	1.605±0.011**	0.284±0.002*	0.036±0.004	0.034±0.005	580±22	3.8±0.3**	2700±154**	0.024±0.001
5	-	1.775±0.032	0.216±0.011**	0.004	0.005	48±2.4**	12.2±0.22**	2450±35	0.021±0.001
Control		1.474±0.007	0.271±0.009	0.003	0.007	42±1.1	7.4±0.52	2770±132	0.019±0.002
-	7 d	1.357±0.024**	0.247±0.021*	0.068±0.004	0.056±0.009	4440±20***	1.3±0.02***	3800±93**	0.038±0.003**
1	7 d	1.320±0.048	0.275±0.021	0.051±0.008	0.044±0.009	3060±180**	2.6±0.14***	3100±58**	0.025±0.002*
1	-	1.396±0.046	0.262±0.003	0.003	0.008	50±5***	8.6±0.22	2780±87	0.018±0.001
5	7 d	1.347±0.027	0.277±0.012	0.055±0.006	0.048±0.006	3440±210*	3.1±0.08***	3150±154*	0.034±0.002
5	-	1.779±0.044*	0.319±0.015	0.004	0.007	36±2***	8.2±0.22	2850±134	0.019±0.002

MDA accumulation might be related to lower Cl^- content in the leaves. It has been shown that the physiological disturbances in citrus by salinity are associated with Cl^- rather than Na^+ accumulation (Moya *et al*. 2003). Treatment with H_2O_2 was shown to induce stomatal closure (Middleton and Teramura 1993). We did not establish any decrease of $^{14}CO_2$ fixation (Table 1) as a result of H_2O_2 treatment. This is indirect indication that in our experiments there was no H_2O_2-induced stomatal closure. The reduced NaCl uptake, measured by Cl^- content in the leaves might be one of the reasons for the established barley seedlings tolerance to salt stress after pre-treatment with H_2O_2. According to Bowler and Fluhr (2000) H_2O_2 is produced in response to variety of stimuli and it is likely that H_2O_2 mediates cross-talk between signalling pathways. In the present study H_2O_2 (1 and 5 μM) had no effect of proline, MDA and even H_2O_2 contents in the leaves, which means that exogenous H_2O_2 itself did not act as a stress factor. Exogenous H_2O_2 might play a signalling role in triggering acclimation of barley seedlings to salt stress implying a complex mechanism for cellular regulation of oxidative status (Neto *et al*. 2005). Our data suggest that H_2O_2 pre-treatment could directly or indirectly activate antioxidant enzymes leading to higher resistance to salt stress. SOD activity increased in the leaves and in the roots in acclimated seedlings, suggesting that H_2O_2 pre-treated plants had a better ability of superoxide scavenging. Additionally H_2O_2 may increase salt tolerance in barley seedlings by decreasing Na^+ and Cl^- uptake.

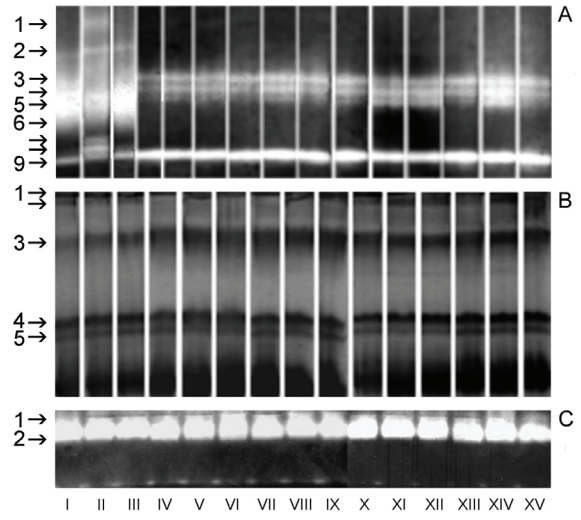

Fig. 1. Isoenzyme profiles of SOD (*A*), POX (*B*) and CAT (*C*) in the leaves of barley seedlings pre-treated with 1 and 5 μmol H_2O_2 before salinization with 150 mM NaCl for 4 and 7 d. 2 d after H_2O_2 treatment: I - control, II - 1 μM H_2O_2, III - 5 μM H_2O_2. 4 d after NaCl supply: IV - control, V - NaCl, VI - 1 μM H_2O_2 + NaCl, VII - 1 μM H_2O_2, VIII - 5 μM H_2O_2 + NaCl, IX - 5 μM H_2O_2. 7 d after NaCl supply: X - control, XI - NaCl, XII - 1 μM H_2O_2 + NaCl, XIII - 1 μM H_2O_2, XIV - 5 μM H_2O_2 + NaCl, XV - 5 μM H_2O_2. 50 μg protein per lane was loaded.

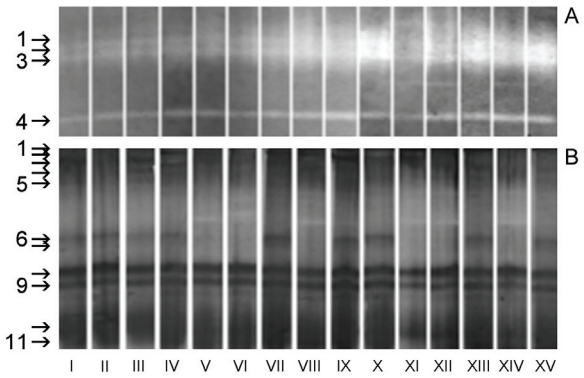

Fig .2. Isoenzyme profiles of SOD (*A*) and POX (*B*) in the roots of barley seedlings pre-treated with 1 and 5 μmol H_2O_2 before salinization with 150 mM NaCl for 4 and 7 d. 25 μg protein per lane was loaded. The experimental details are the same as in Fig. 1.

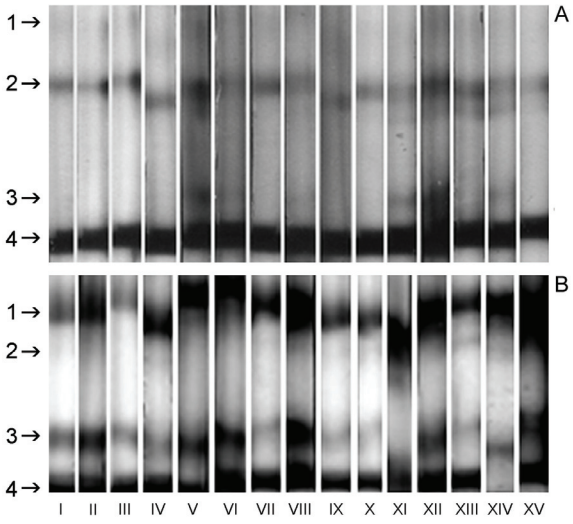

Fig. 3. Isoenzyme profiles of cationic POX in the leaves (*A*) and in the roots (*B*) of barley seedlings pre-treated with 1 and 5 μmol H_2O_2 before salinization with 150 mM NaCl for 4 and 7 d. 100 μg protein per lane was loaded for the leaves and 50 μg for the roots. The experimental details are the same as in Fig. 1.

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