BIOLOGIA PLANTARUM 56 (2): 330-336, 2012

Exogenous H_2O_2 increased catalase and peroxidase activities and proline content in *Nitraria tangutorum* callus

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Abstract

Antioxidative responses and proline accumulation induced by exogenous H_2O_2 were investigated in the callus from halophyte *Nitraria tangutorum* Bobr. H_2O_2-treated callus exhibited higher H_2O_2 content than untreated callus. The activities of catalase (CAT) and peroxidase (POD) significantly increased in the callus treated with H_2O_2, while ascorbate peroxidase (APX) activity decreased. In addition, significantly enhanced proline content was observed in the callus treated by H_2O_2, which could be alleviated by H_2O_2 scavenger dimethylthiourea and calcium (Ca) chelator ethylene glycol bis-(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA). Moreover, γ-glutamyl kinase (GK) activity increased in H_2O_2-treated callus, but proline dehydrogenase (PDH) activity decreased significantly, and the reduction was partly abolished by EGTA or Ca channel blocker verapamil. Assays using a scanning electron microscope showed significantly enhanced Ca content in H_2O_2-treated callus.

Additional key words: antioxidative enzymes, calcium, γ-glutamyl kinase, proline dehydrogenase.

Introduction

The rapid and increased generation of reactive oxygen species (ROS), including hydrogen peroxide, is one of plant responses to stress conditions (Shalata and Tal 1998). This may initiate oxidative damage in plant cells resulting in disruption of metabolic function and loss of cellular integrity (Ozden *et al.* 2009). According to the review by Forman (2007), H_2O_2 can be important stress signal but also can be toxic to plants. Exogenous H_2O_2 resulted in increased Ca^{2+} influx in *Arabidopsis thaliana* root epidermis (Demidchik *et al.* 2007) and promoted stomatal closure in *Vicia faba* (Zhang *et al.* 2001) in a dose-dependent manner. Antioxidant enzymes including catalase (CAT; EC 1.11.1.6), peroxidase (POD; EC 1.11.1.7) and ascorbate peroxidase (APX; EC 1.11. 1.11) provide the first line of defense against ROS (Yahubyan *et al.* 2009, Mallik *et al.* 2011). Yu *et al.* (2003) reported that exogenous H_2O_2 decreased the activities of CAT and APX in the leaves of mung bean, while increased antioxidative enzyme activities were observed in some other plant species (Tsai *et al.* 2005, Ozden *et al.* 2009).

The accumulation of compatible organic solutes such as proline is another response of many plant species exposed to different abiotic stresses (Misra and Gupta 2005, Walker *et al.* 2010). Proline can function not only as osmoprotectant but also in detoxification of ROS and thus protecting membrane integrity (Ozden *et al.* 2009). It has been indicated that proline accumulation results from an increase in proline biosynthesis from glutamate and/or a decrease in proline degradation (Delauney and Verma 1993). The γ-glutamyl kinase (GK) and Δ^1-pyrroline-5-carboxylate synthetase (P5CS) are the most important enzymes of glutamate pathway (Szekely 2004). An increase in GK activity along with proline content elevation in plants under environmental stresses was observed (Misra and Gupta 2005, Gobinnathan *et al.* 2009). On the other hand, an inverse relationship of proline accumulation with proline dehydrogenase (PDH) activity was observed in numerous studies (Madan *et al.* 1995, Lee and Liu 1999). Increased proline content can also be achieved by synthesizing proline from ornithine involving

Received 2 January 2011, *accepted* 4 May 2011.

Abbreviations: APX - ascorbate peroxidase; ASA - ascorbate; CAT - catalase, DMTU - dimethylthiourea; EDTA - ethylenediaminetetraacetic acid; EGTA - ethylene glycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; GK - γ-glutamyl kinase; OAT - ornithine aminotransferase; PDH - proline dehydrogenase; POD - peroxidase; PVP - polyvinylpyrrolidone.

Acknowledgments: This work was financially supported by the National Natural Science Foundation of China (No. 30960064) and the Gansu Program Project for Science and Technology (1010RJZA027).

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ornithine aminotransferase (OAT; Delauney and Verma 1993). The correlations between proline accumulation and increased OAT activities have been demonstrated in diatom *Fragilariopsis cylindrus* by Krell *et al.* (2007). Although much supporting evidence on the effects of environmental stress on proline accumulation is available, the mechanisms of proline metabolism regulation in response to exogenous H_2O_2 are quite limited.

A shrub *Nitraria tangutorum* Bobr. (*Zygophyllaceae*) is distributed in desert areas in the north-west of China. A relationship between anatomical features and the adaptability to stress conditions has been investigated by Yang and Furukawa (2006). However, a comprehensive study of adaptation mechanism at the cellular level would be of great help in understanding the processes controlling plant growth and surviving in a hostile environment. Recently, we found that the callus from *N. tangutorum* is an ideal material for studies of adaptations to salinity stress (Yang *et al.* 2010). In this paper *N. tangutorum* callus was used to investigate antioxidative responses and proline accumulation after H_2O_2 treatment.

Materials and methods

Seeds of *Nitraria tangutorum* Bobr. were surface sterilized for 12 s in 75 % (v/v) ethanol, for 10 min in 0.1 % $HgCl_2$, and were rinsed 6 times with sterile distilled water. The embryos were extracted and incubated on growth regulator-free Murashige and Skoog (MS) solid medium. The cotyledons from aseptic seedlings were cut to about 0.3 cm and cultured on MS solid medium supplemented with 0.3 mg dm^{-3} 6-benzyladenine and 1 mg dm^{-3} α-naphthaleneaetic acid for inducing callus. The callus was then separated from the initial explants and was subcultured every 18 - 20 d. H_2O_2, dimethylthiourea (DMTU), ethylene glycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) or verapamil were added on the surface of the solid MS medium after filter sterilization. The callus was maintained at temperature of 24 ± 1.5 °C and 10-h photoperiod with irradiance of 150 μmol m^{-2} s^{-1}, then washed by distilled water and the excess water was blotted with filter paper.

Callus (1 g) was ground with 1 cm^3 of chilled NaH_2PO_4/Na_2HPO_4 buffer (PBS, 50 mM, pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1 % (m/v) polyvinylpyrrolidone (PVP). After centrifugation for 30 min at 15 000 *g*, the supernatant was collected for the measurement of CAT and POD activities. A modification of the method of Aebi (1974) was used to assay CAT activity. Briefly, 0.1 cm^3 of the enzyme extract was added to 3 cm^3 of 50 mM PBS buffer (pH 7.0). After 5 min pre-incubation at 25 °C, 15 mM H_2O_2 was added and the absorbance changes were recorded at 240 nm for 3 min. An absorbance change of 0.01 units per min was defined as 1 unit of CAT activity. POD activity was measured following a modification of the method of Rao *et al.* (1996). The enzyme extract (0.005 cm^3) was mixed with 3 cm^3 of reaction mixture containing 50 mM PBS (pH 6.5) and 20 mM guaiacol. After pre-incubation at 25 °C for 5 min, 0.04 cm^3 of 0.05 % (v/v) H_2O_2 was added to initiate the reaction. The changes of the absorbance at 470 nm within 3 min were recorded. One unit of POD activity was defined as an absorbance change of 0.1 units per min.

Fresh callus (1 g) was ground with 1 cm^3 of chilled 50 mM PBS buffer (pH 7.0) containing 1 mM EDTA and 1 mM ascorbate (ASA). After centrifugation for 30 min at 15 000 *g*, the supernatant was collected. The assay of APX activity was performed as described by Nakano and Asada (1981) with some modifications. The assay was carried out in a reaction mixture consisting of 50 mM PBS (pH 7.0), 0.5 mM ASA, 0.1 mM H_2O_2 and 0.1 cm^3 of the enzyme extract. The changes in the absorbance at 290 nm were recorded at 25 °C for 1 min after the addition of H_2O_2. One unit of APX activity was defined as an absorbance change of 0.1 units per min.

The measurement of H_2O_2 content was performed as described by Sergiev *et al.* (1997). Fresh callus was ground in an ice bath with 2 cm^3 of 0.1 % (m/v) trichloroacetic acid. The homogenate was centrifuged at 12 000 *g* for 20 min and 0.7 cm^3 of the supernatant was mixed with 0.7 cm^3 10 mM PBS buffer (pH 7.0) and 0.7 cm^3 1 M KI. H_2O_2 content was estimated by measuring the absorbance at 390 nm based on a standard curve.

Proline analysis was performed according to Bates *et al.* (1973) with some modifications. 0.5 g of fresh callus was immediately homogenized in 5 cm^3 of 3 % (m/v) sulfosalicyclic acid solution, and then heated at 98 °C for 10 min. After centrifugation at 15 000 *g* for 15 min, 0.25 cm^3 supernatant was added to 3.75 cm^3 the reaction medium containing 0.56 % (m/v) sulfosalicyclic acid, 0.25 % (v/v) glacial acetic acid and 1.25 % (m/v) ninhydrin solution. The mixture was kept at 95 °C for 60 min, and then the reaction was stopped quickly by an ice bath. Toluene (4 cm^3) was added to the mixture, the organic phase was extracted and monitored at 520 nm by spectrophotometer.

Proline dehydrogenase (PDH) activity was measured as described by Rena and Splittstoesser (1975) with a slight modification. Fresh callus (0.5 g) was homogenized in the ice-cold extraction buffer (100 mM sodium phosphate, 1 mM cysteine, 0.1 mM EDTA, pH 8.0). After centrifugation at 15 000 *g* for 10 min at 4 °C, the supernatant was used as crude enzyme preparation for measurement of PDH activity. The reaction buffer containing 100 mM Na_2CO_3-$NaHCO_3$ (pH 10.3), 10 mM

NAD, 20 mM L-proline and the crude extract was incubated at 32 °C for 5 min, and then PDH dependent NAD reduction was monitored at 340 nm for 4 min. One unit of PDH activity was defined as an absorbance change of 0.001 units per min.

Ornithine δ-aminotransferase activity was assayed with ninhydrin according to Kim *et al*. (1994). About 1 g of callus was immediately homogenized in 100 mM potassium phosphate buffer (pH 7.9) containing 1 mM EDTA, 15 % glycerol and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 15 000 *g* for 15 min at 4 °C and the supernatant was collected for OAT activity measurement. Then, 1 cm^3 of the reaction mixture consisted of 50 mM Tris-HCl (pH 8.0), 50 mM L-ornithine, 5 mM β-ketoglutarate, 0.05 mM pyridoxal phosphate and the appropriate amount of crude enzyme extract was incubated at 37 °C for 20 min. After the addition of 0.3 cm^3 of 3 M perchloric acid and 0.2 cm^3 of 2 % ninhydrin, the reaction was stopped by boiling for 5 min. The precipitate was collected by centrifugation (13 000 *g*, 30 min, 4 °C) and completely dissolved with ethanol, and then the absorbance was recorded at 510 nm. The absorbance of 0.01 at 510 nm was defined as one unit of OAT activity.

About 2 g of callus was ground in 2 cm^3 TD buffer containing 50 mM Tris-HCl buffer (pH 7.0), 1 mM dithiothreitol and 10 % (m/v) glycerol. After centrifugation at 16 000 *g* for 20 min, the supernatant was collected and precipitated by adding solid ammonium sulphate (40 % saturation). Then, the soluble fraction obtained by centrifugation (16 000 *g* for 20 min) was saturated with dry ammonium sulphate to a concentration of 80 % (m/v). After centrifugation at 15 000 *g* for 15 min at 4 °C, the pellet was collected and completely dissolved with 1 cm^3 TD buffer. The crude enzyme solution was obtained after a 24 h dialysis against TD buffer at 4 °C. Glutamyl kinase activity was assayed by the method of Smith (1984) with some modifications. A total volume of 1 cm^3 assay mixture containing 50 mM glutamate, 10 mM ATP, 20 mM $MgCl_2$, 100 mM oxammonium hydrochloride, 50 mM Tris-HCl buffer (pH 7.0) and an appropriate amount of enzyme was incubated at 37 °C for 30 min and then the reaction was stopped by adding 1 cm^3 stop solution (5.5 % $FeCl_3$, 2.0 % $HClO_4$, 2 M HCl). The precipitate was removed by centrifugation, and the absorbance of the supernatant at 535 nm was recorded against a blank identical to the one mentioned above but lacking ATP. The changes of absorbance of 0.01 units per hour at 535 nm were defined as one unit of GK activity.

Mineral elements were measured using a scanning electron microscope *(Philips Electronics*, Eindhoven, The Netherlands) fitted with a *Kenex* (Valencia, CA, USA) energy dispersive X-ray detector as described by Vázquez *et al*. (1999) with some modifications. The callus was placed directly on the aluminum stage, quickly frozen under vacuum. The examination time of each sample was less than 10 min to avoid cell distortion. At least four to five cells per sample were examined. The results were calculated by expressing the atomic number for a particular element in a given cell as a percentage of the total atomic number for all the elements measured (K, Na, Ca, Mg, P, S and Cl) in the cell.

The amount of soluble proteins was estimated according to the method of Bradford (1976) using bovine serum albumin as a standard.

Each experiment was repeated at least three times. Statistical comparisons were carried out using *SPSS v. 13.0* software.

Results

The CAT activity significantly increased in callus treated with different H_2O_2 concentrations for 1 and 3 d as compared to that of the control, but decreased to the level of the control after 6-d treatment. H_2O_2 induced significant enhancement in POD activity during the whole stress period (Table 1). After exposure the callus to 2 μM H_2O_2 for 1, 3 and 6 d, we found about 7, 48 and 18 % increase in POD activity as compared to the control, respectively. Similary, POD activity increased by 152, 132 and 117 % of the control value when exposed to 10 μM H_2O_2 for 1, 3 and 6 d, respectively. In contrast, no significant change in the activity of APX was found in the callus treated with 2 μM H_2O_2, and 1 d of 10 μM H_2O_2 treatment resulted in a slight but not significant increase in APX activity, whereas significant decrease (about 20 and 39 %, respectively) in this enzyme activity was detected at 3 and 6 d of 10 μM H_2O_2 treatment (Table 1).

The amount of endogenous H_2O_2 in the callus

Table 1. The changes of catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) activities [U mg^{-1} protein] in *Nitraria tangutorum* callus after treatment with 0, 2 and 10 μM H_2O_2 for 1, 3 or 6 d. Means ± SE of at least three independent measurements. Different letters within the same row indicate statistically significant difference at the 0.05 level.

	0 μM H_2O_2	2 μM H_2O_2	10 μM H_2O_2
CAT 1 d	66.33 ± 5.29a	73.11 ± 4.37b	79.61 ± 6.47c
3 d	64.33 ± 4.78a	71.76 ± 4.52b	73.33 ± 2.31b
6 d	73.17 ± 4.53a	74.11 ± 2.60a	75.72 ± 3.67a
POD 1 d	85.35 ± 9.52a	91.51 ± 2.41a	129.75 ± 16.32b
3 d	163.33 ± 22.42a	241.92 ± 17.94b	215.47 ± 19.23b
6 d	136.00 ± 4.14a	160.90 ± 14.86b	158.79 ± 6.31b
APX 1 d	143.88 ± 11.16a	156.31 ± 22.10a	153.70 ± 18.80a
3 d	192.75 ± 17.46a	173.25 ± 14.88a	155.60 ± 20.31b
6 d	147.39 ± 18.97a	134.50 ± 18.94a	90.17 ± 3.71b

Table 2. The changes of endogenous H_2O_2 content [ng g^{-1}(f.m.)] in *N. tangutorum* callus after treatment with 0, 2 and 10 μM H_2O_2 for different time periods. Means ± SE from at least four independent measurements. Different letters within the same column indicate statistically significant difference at the 0.05 level.

H_2O_2 [μM]	6 h	12 h	1 d	3 d	6 d
0	48.49 ± 3.19a	66.73 ± 2.36a	84.29 ± 2.49a	111.19± 2.36a	56.61 ± 0.53a
2	63.37 ± 2.79b	82.69 ± 6.04b	94.93 ± 2.4b	133.88 ± 9.26b	72.58 ± 1.06c
10	78.85 ± 2.01c	98.39 ± 7.82b	96.79 ± 5.09b	152.51 ± 7.79b	67.26 ± 1.06b

Table 3. The changes in relative proline content in *N. tangutorum* callus after treatment with 2 and 10 μM H_2O_2 for 1 or 3 d. 100 % corresponded to 612.86 ± 27.22 and 649.39 ± 18.52 μg g^{-1}(f.m.) in untreated callus growing for 1 and 3 d, respectively. Means ± SE of at least four independent measurements. Different letters within the same column are significantly different at the 0.05 level.

H_2O_2 [μM]	1 d	3 d
0	100.00 ± 2.57a	100.00 ± 1.87a
2	123.05 ± 5.96b	141.28 ± 2.02b
10	120.58 ± 2.93b	117.04 ± 4.32b

increased significantly in comparison with the control after treatment with 2 or 10 μM H_2O_2, the maximum was reached after 3 d treatment and then H_2O_2 content slightly decreased (Table 2).

Compared to the control, there was about 23 and 21 % elevation in the of proline content after 2 and 10 μM H_2O_2 treatments for 1 d, but after treatments with these two concentrations of H_2O_2 for 3 d, about 41 and 17 % increase in proline content was observed, respectively (Table 3). The addition of 5 mM DMTU, a H_2O_2 scavenger, blocked H_2O_2-induced elevation in proline content in the callus (Fig. 1), suggesting that the elevation in proline accumulation was due to H_2O_2. Furthermore,

Table 4. The changes of proline dehydrogenase (PDH), ornithine aminotransferase (OAT) and γ-glutamyl kinase (GK) activities [U mg^{-1} protein] in *N. tangutorum* callus after treatment with 0 (control), 2 and 10 μM H_2O_2 for 1 or 3 d. Means ± SE from at least 3 independent measurements. Different letters within the same column indicate statistically significant difference at the 0.05 level.

	PHD		OAT		GK	
H_2O_2 [μM]	1 d	3 d	1 d	3 d	1 d	3 d
0	36.82 ± 0.29a	62.93 ± 1.25a	0.212 ± 0.013a	0.235 ± 0.009a	22.93 ± 1.25a	27.52 ± 0.72a
2	30.37 ± 0.37b	46.23 ± 0.52b	0.203 ± 0.018a	0.241 ± 0.012a	27.17 ± 0.81b	36.11 ± 2.33b
10	25.87 ± 1.51c	45.96 ± 2.00b	0.227 ± 0.014a	0.215 ± 0.009a	24.25 ± 0.52a	33.35 ± 1.87b

Table 5. The effects of 4 mM EGTA and 10 μM verapamil on the changes of proline dehydrogenase (PDH) and γ-glutamyl kinase (GK) activities [U mg^{-1} protein] in *N. tangutorum* callus after treatment with 0, 2 and 10 μM H_2O_2 for 3 d. Means ± SE from at least three independent measurements. Different letters within the same row indicate statistically significant difference at the 0.05 level.

	Control	2 μM H_2O_2	2 μM H_2O_2 + EGTA	2 μM H_2O_2 + verapamil	10 μM H_2O_2	10 μM H_2O_2 + EGTA	10 μM H_2O_2 + verapamil
PDH	62.93 ± 1.25a	46.23 ± 0.52b	57.69 ± 1.85a	56.21 ± 1.61a	45.96 ± 2.00b	59.82 ± 3.32a	56.63 ± 1.99a
GK	27.52 ± 0.72a	36.11 ± 2.33b	38.13 ± 3.69b	35.49 ± 2.19b	33.35 ± 1.87b	32.99 ± 1.19b	36.76 ± 3.88b

Ca chelator EGTA (4 mM) also reversed the increase of proline content caused by 2 μM H_2O_2. The similar results were found in the callus exposed to 10 μM H_2O_2 in the absence and in the presence of 4 mM EGTA.

Further study was to examine whether the changes of PDH activity was associated with H_2O_2-induced proline accumulation. Exogenous H_2O_2 caused a significant reduction of PDH activity after 2 and 10 μM H_2O_2 treatment for 1 d in comparsion with the control. After 3-d treatment, approximately 27 % decrease in PDH activity was observed in the callus under 2 or 10 μM H_2O_2 treatment. In addition, we found that the treatment with H_2O_2 induced only non-significant change in OAT activity (Table 4). In contrast, H_2O_2 treatment led to a

significant elevation in GK activity. GK activity continued to increase in 2 μM H_2O_2-treated callus. A slight but not significant enhancement in GK activity was detected in the callus after treatment with 10 μM H_2O_2 for 1 d, whereas in the callus treated for 3 d, this enzyme activity significantly elevated and reached about 121 % of the control values (Table 4).

Table 6. The changes of calcium percentage [% of total elements measured] in *N. tangutorum* callus treated with 0, 2 and 10 μM H_2O_2. Means ± SE of at least three experiments with replicated measurements. Different letters within the same column indicate statistically significant difference at the 0.05 level.

H_2O_2 [μM]	6 h	12 h	1 d	3 d
0	16.23 ± 0.60a	16.28 ± 0.47a	16.72 ± 0.38a	18.03 ± 0.69a
2	18.86 ± 0.61b	19.65 ± 0.35b	19.13 ± 0.40b	21.95 ± 0.95b
10	21.57 ± 0.57c	19.91 ± 0.28b	22.84 ± 0.39c	22.12 ± 0.52b

To determine the relationship between Ca content and PDH activity, callus was treated with different H_2O_2 concentrations in the presence of Ca chelator EGTA (4 mM). Compared to the control, the treatment of 2 μM H_2O_2 for 3 d induced remarkable decrease in PDH activity, but the reducted PDH activity was reversed by the presence of 4 mM EGTA. The similar results were found in the callus exposed to 10 μM H_2O_2 in the absence and in the presence of 4 mM EGTA (Table 5). In addition, Ca channel blocker verapamil significantly blocked the inhibition of PDH activity under H_2O_2 treatment. These data suggested that Ca content elevation might be responsible for H_2O_2-induced decrease of PDH activity. However, the application of EGTA and verapamil did not affect the elevation of GK activity in callus exposed to different H_2O_2 concentrations for 3 d (Table 5).

Ca content was about 16 and 18 % in the untreated callus cultured for 1 and 3 d, respectively. Upon exposure to 2 or 10 μM H_2O_2, Ca content significantly increased (Table 6).

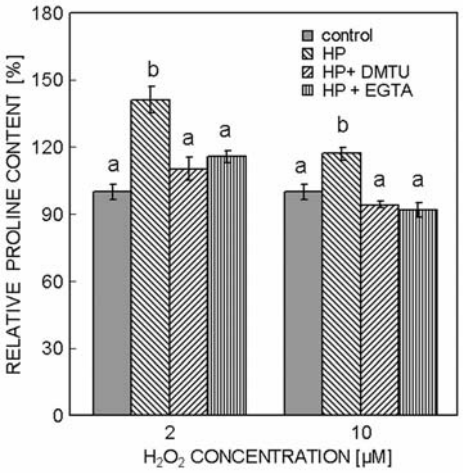

Fig. 1. The effects of 5 mM DMTU or 4 mM EGTA on H_2O_2-induced accumulation of proline in the callus of *N. tangutorum* callus. (HP - hydrogen peroxide). Control content of proline (100 %) corresponded to 649.39 ± 18.52 μg g^{-1}(f.m.) in untreated callus growing for 3 d. Means ± SE of at least four independent measurements. Within each set of experimental, bars with different letters are significantly different at the 0.05 level.

Discussion

It has been demonstrated that the exogenous H_2O_2 have to have signaling role (Forman 2007) as it can change the redox status of the surrounding cells (Lin and Kao 1998). For example, oxidative stress induced by H_2O_2 dramatically increased CAT activity but decreased POD and APX activities in the leaves of *Vitis vinifera* (Ozden *et al*. 2009). Similarly, exposure of Chinese water chestnut to H_2O_2 treatment inhibited POD activity (Peng *et al.* 2008). However, exogenous H_2O_2 enhanced the activity of APX and the expression of *OsAPX* gene in rice roots (Tsai *et al.* 2005). In the present study with *N. tangutorum* callus, different H_2O_2 concentrations induced significant increases in the activities of CAT and POD, while a remarkable decrease in APX activity was found after exposure to 10 μM H_2O_2. The effects of exogenous H_2O_2 can be due to increased endogenous H_2O_2 content. Significant increase in the endogenous H_2O_2 content was observed in the leaves of *Vitis vinifera* (Ozden *et al.* 2009) and in rice roots (Tsai *et al*. 2005) after exogenous H_2O_2 treatment. However, application of H_2O_2 did not alter the endogenous H_2O_2 content in *Vigna radiata* immediately after the treatment (Yu *et al.* 2003). Moreover, H_2O_2 treatment resulted in a decrease in endogenous H_2O_2 content in rice leaves (Lin and Kao 1998). CAT, POD and APX constitute main H_2O_2 scavenging system in cells, and the changes of these enzyme activities can regulate intracellular H_2O_2 levels (Mittler 2002). Even though significantly elevated activities of CAT and POD were detected in *N. tangutorum* callus under H_2O_2 treatment, we found a remarkable enhancement in endogenous H_2O_2 content in the callus during the early stage of H_2O_2 stress. We assumed that the enhancement of endogenous H_2O_2 content might be due to H_2O_2 diffusion through the plasma membrane into the callus cells. Supporting this hypothesis, Antunes and Cadenas (2000) and Sousa-

Lopes *et al.* (2004) reported that upon H_2O_2 application, the endogenous H_2O_2 content was dependent on the extent of the intracellular consumption of H_2O_2 and on the permeability properties of cell barriers to H_2O_2.

Accumulation of free proline in response to different environmental stresses seems to be wide-spread among plants (Banu *et al.* 2009, Thippeswamy *et al.* 2010). Proline can protect plants from stress through different mechanisms, including osmotic adjustment, detoxification of ROS, protection of membrane integrity, and stabilization of proteins/enzymes (Ozden *et al.* 2009, Pei *et al.* 2010). In some plants and cell cultures, it has been demonstrated that the changes of proline contents are correlated with their ability to tolerate or adapt to stress conditions (Delauney and Verma 1993, Hare *et al.* 1999, Xue *et al.* 2010). The present data showed that proline content was higher in *N. tangutorum* callus growing on medium containing H_2O_2 in comparison with the control. Because the presence of 5 mM DMTU suppressed H_2O_2-induced proline accumulation, we can suggest that proline accumulation in our experiments was a consequence of increasing endogenous H_2O_2 content. Forman (2007) suggested that exogenous H_2O_2 may mimic signaling induced by endogenously produced H_2O_2.

The change in intracellular Ca^{2+} content is considered as a signal involved in the regulation of physiological function in higher plants exposed to stress conditions (Lee and Liu 1999). Moreover, we found that Ca^{2+} chelator EGTA could abolish the enhancement of the amount of proline induced by H_2O_2 treatment for 3 d, indicating that Ca might be positively associated with exogenous H_2O_2-induced proline accumulation.

Proline degradation is catalyzed by the mitochondrial enzyme PDH (Hare *et al.* 1999) and positive correlations have been demonstrated between increased proline content and the inhibition of PDH activity under stress conditions (Madan *et al.* 1995, Lee and Liu 1999). In *N. tangutorum* callus, the H_2O_2 treatment caused the significant decrease in PDH activity and the elevation in GK activity, while no significant changes in OAT activity. No significant change in the PDH activity was observed in the callus treated with Ca^{2+} chelator EGTA or Ca^{2+} channel blocker verapamil (data not shown). However, inhibition of PDH activity induced by H_2O_2 was eliminated by the addition of EGTA or verapamil, suggesting that enhanced Ca^{2+} content might be important for this process. Conversely, Lee and Liu (1999) demonstrated that the Ca^{2+} efflux is involved in the induction of proline accumulation under abiotic stress *via* the inhibition of PDH activity in plants. It has been indicated that Ca^{2+} may also be involved in the regulation of GK activity in plants exposed to environmental stresses. For example, the application of $CaCl_2$ decreased GK activity in NaCl-treated *Pennisetum* seedlings (Gobinathan *et al.* 2009), but a positive effect of Ca^{2+} content on NaCl-induced expression of the *P5CS* gene was found in *Arabidopsis thaliana* (Knight *et al.* 1997). The present data showed that EGTA and verapamil did not affect increased GK activity induced by H_2O_2 treatment. Taken into account that Ca^{2+} content is mediated in plants exposed to various environmental stresses (Coelho *et al.* 2002, Lecourieux *et al.* 2002), we further measured Ca^{2+} content in the callus using a scanning electron microscope. In *N. tangutorum* callus, H_2O_2 treatment induced significant increase in the amount of Ca^{2+}, which might be responsible for proline accumulation *via* the decrease in PDH activity.

In conclusion, the application of exogenous H_2O_2 callus led to significant enhancements in the activities of CAT and POD as well as decrease in APX activity in *N. tangutorum* callus. The increase of Ca^{2+} content, the inhibition of PDH activity and the elevation of GK activity was essential for the proline accumulation under H_2O_2 treatment.

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