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Salinity-induced Physiological Modification in the Callus from Halophyte *Nitraria tangutorum* Bobr.

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Abstract Little is known about the physiological adaptation mechanisms of the desert halophyte *Nitraria tangutorum* Bobr. to the environment. In this study, callus from *Nitraria tangutorum* Bobr. was used to investigate physiological responses to salinity and the regulatory function of nitric oxide (NO) on catalase (CAT) activity. Increased dry weight and soluble proteins were observed in the callus exposed to lower salinity (50 and 100 mM NaCl), whereas 200 mM NaCl led to significant decreases of these two growth parameters, and the levels of proline and soluble carbohydrates also were enhanced under NaCl treatment. In addition, short-term stress from 50 mM NaCl and the application of lower sodium nitroprusside (SNP, a NO donor) concentration resulted in decreased levels of malondialdehyde (MDA). In contrast, higher concentrations of NaCl and SNP induced significant oxidative damage in *Nitraria tangutorum* Bobr. callus. Analysis based on the fluorescent probe DAF-FM DA revealed that NaCl and SNP treatment led to enhanced levels of NO in the callus cells. Moreover, the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) reduced endogenous NO concentrations and abolished the enhancement in dry weight and the decrease in MDA level under 50-mM-NaCl treatment. CAT activity increased under salt stress, and the 50-mM-NaCl effect was alleviated by treatment with c-PTIO or the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine. We suggest that *Nitraria tangutorum* Bobr. callus exhibited tolerance to lower-salinity stress. We also showed that increased NO generation in response to salinity might be associated with regulation of growth, protection against oxidative damage, and excitation of CAT activity in *Nitraria tangutorum* Bobr. callus under salt stress.

Keywords Catalase · Homeostasis · Malondialdehyde · Nitric oxide · *Nitraria Tangutorum* Bobr · Salinity

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Introduction

High salinity is one of the most serious environmental stresses impeding plant growth and limiting crop production (Boyer 1982; Munns and others 2005). Salt-tolerant plants have evolved a variety of physiological responses that confer tolerance to salinity stress. Among these, osmotic adjustment involving osmoprotectants like proline and soluble sugar is an important physiological mechanism of salt adaptation in many plants (Miller and Smith 2008; Rosa and others 2009). Investigations of halophytes by Megdiche and others (2007) and Maggio and others (2000) have indicated that significant increased levels of proline and soluble carbohydrates are probably related to osmotic adjustment and the protection of membrane stability under salinity stresses. Further, Stewart and Lee (1974) have reported that the accumulation of proline is correlated with salt tolerance in the halophyte *Ameria maritime* Willd. Salinity is known to induce rapid and increased generation

of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), which leads to oxidative damage to plant cells (Shalata and Tal 1998). An effective ROS-scavenging system involving catalase (CAT; EC 1.11.1.6) is a critical component of salinity resistance (Moradi and Ismail 2007) because of its protective effect against oxidative damage under salinity stress (Li 2008). Catalase is one of the main H_2O_2-scavenging enzymes that dismutate H_2O_2 into water and O_2 (Asada 1992; Mittler 2002). Although the effects of NaCl on plant CAT have been investigated in a number of plant species, the results in previous studies have been different and even contradictory (Shalata and Tal 1998; Muscolo and others 2003; Sekmen and others 2007). Moreover, the precise mechanism by which the expression and metabolic regulation of this enzyme are affected by salinity treatment remains unclear.

Nitric oxide (NO) is a gaseous free radical that mediates many physiological processes at cellular and tissular levels (Desikan and others 2004). In recent years, increasing evidence has shown that NO plays multiple roles in plant growth and development (Beligni and Lamattina 2000; Lehnera and others 2009), such as stomatal closure, seed germination, and programmed cell death (Bright and others 2006; De Michele and others 2009; Guo and others 2009), and in plant responses to environmental stresses (Desikan and others 2004; Zhao and others 2004; Zhang and others 2007), including the activation of plant defense against abiotic stresses (Tanou and others 2009a). NO may act as an antioxidant involved in delaying ROS-induced programmed cell death in barley aleurone layers (Beligni and others 2002) and in reducing the levels of ROS and oxidized proteins and lipids in plants grown under nonstress conditions (Guo and Crawford 2005). In addition, NO is associated with the regulation of antioxidant enzyme activities. For instance, the application of sodium nitroprusside (SNP), a NO donor, significantly stimulates the activities of CAT and superoxide dismutase (SOD; EC 1.15.1.1) in *Kosteletzkya virginica* (Guo and others 2009) but inhibits the activities of tobacco CAT and ascorbate peroxidase (APX; EC 1.11. 1.11) (Clark and others 2000). In a recent study, Tanou and others (2009b) reported that SNP pretreatments increased antioxidant enzyme activities and induced related-isoform(s) expression under non-NaCl-stress conditions, and demonstrated that NO elicits long-lasting systemic primer-like antioxidant activity in citrus plants under physiological and NaCl-stress conditions. However, these studies described above show exogenous NO effects on plant physiology processes.

Nitraria tangutorum Bobr. is a typical desert halophyte, which is important ecologically and economically. Because of its superior tolerance to severe drought and high salinity, this species can grow in regions with high-saline soil and in arid and semiarid regions with high salinity in northwest China. A relationship in *Nitraria tangutorum* Bobr. between anatomical features and the adaptability to desert environments has been investigated by Yang and Furukawa (2006). However, little is known about the physiological and biochemical mechanisms responsible for its tolerance to environmental stresses. Salinity tolerance is a complicated whole-plant phenomenon, and it is necessary to understand the integration and expression of tolerant mechanisms from the cellular level to the whole-plant level (Dracup 1991). Cell and tissue culture of plants is one of the effective means to study basic biological processes (Golan-Goldhirsh and others 2004). A comprehensive study of the physiological adaptative mechanism at the plant-cell level would be of great help in understanding the processes controlling plant growth and survival in a hostile environment. Thus, we used the callus from *Nitraria tangutorum* Bobr. to investigate the adaptive responses to salinity and the relationship between the regulation of CAT activity and the generation of NO in the stress-signaling response after salt treatment.

Materials and Methods

Plant Material and Growth Condition

Seeds of *Nitraria tangutorum* Bobr. were obtained from the Minqin Desert Botanical Garden of Gansu province. The seeds were surface sterilized for 12 s with 75% (v/v) ethanol and then in 0.1% HgCl for 10 min. The seeds were rinsed six times and then soaked in sterile distilled water for 2 days. The embryos were extracted and incubated on 30 ml of 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 l^{-1} 6-benzyladenine and 1 mg l^{-1} naphthalene acetic acid for inducing callus. The callus was then separated from the initial explants and subcultured every 20 days. Different NaCl concentrations were added in the MS medium for salt stress. Sodium nitroprusside (SNP), N^{ω}-nitro-L-arginine (L-NNA), and c-PTIO (prepared with sterilized water) were added on the surface of the solid MS medium after filter sterilization. The callus was maintained at 24 ± 1.5°C in the dark for different periods according to the experiment, then evaluated and washed with distilled water; the excess water was blotted with filter paper.

Fresh Weight Increase and Dry Weight Measurement

The callus (300 mg) was stressed with different concentrations of NaCl. At the end of an 18-day incubation period, the fresh weight (FW) increase was calculated as the

difference between the final weight and the initial FW, and dry weight (DW) was also determined after the callus dried at 60°C for 48 h.

Soluble Protein Analysis by SDS-PAGE

The samples were collected from untreated and 12-day NaCl-treated callus for estimation of soluble proteins. The callus (1 g) was ground in chilled tris-(hydroxymethyl) amino methane (Tris)-HCl buffer (10 mM, pH 6.8), then centrifuged at 15,000 *g* for 20 min. The supernatants obtained were used for protein assay. The amount of soluble proteins was estimated according to the method of Bradford (1976) using bovine serum albumin as a standard.

The electrophoresis of proteins was performed as described by Laemmli (1970) with some modifications. Total proteins (70 μg) extracted from the callus were separated in 8% sodium dodecyl sulfate (SDS)-polyacrylamide separating gel and 4% stacking gels. Before fractionation, protein extraction was heated at 100°C for 3 min in 10 mM Tris–HCl buffer (pH 6.8) containing 2% SDS, 2.5% 2-mercapoethanol, 10% glycerol, and 0.005% bromophenol blue. After electrophoresis, the gel was stained for soluble proteins using Coomassie brilliant blue R-250.

Proline and Soluble Carbohydrate Assay

The amount of proline was estimated based on the ninhydrin method (Bates and others 1973) with some modifications. Fresh callus (0.5 g) was immediately homogenized in 5 ml 3% sulfosalicylic acid and then heated at 98°C for 10 min. The homogenate was centrifuged at 15,000 *g* at 4°C for 15 min, and then 0.25 ml supernatant was mixed with 0.75 ml 3% sulfosalicylic acid, 1 ml glacial acetic acid, and 2 ml 2.5% ninhydrin solution. The mixture was heated at 95°C for 60 min, and then the reaction was immediately finished in an ice bath. Toluene (4 ml) was added to the mixture, and the organic phase was extracted and monitored at 520 nm by spectrophotometry.

A modification of the method of phenol–sulfuric acid (Dubois and others 1956) was used to determine soluble sugar content. Fresh callus (0.5 g) was homogenized in 2 ml 80% absolute alcohol and maintained at 75°C for 10 min. After centrifugation at 5,000 *g* for 10 min, 40 μl of the supernatant was mixed with 100 μl of 80% phenol and 4 ml concentrated sulfuric acid, and the absorbance of the mixture at 490 nm was measured.

Lipid Peroxidation Analysis

Lipid peroxidation was measured according to the method of Zhou (2001) with some modifications. Callus (0.5 g) was immediately homogenized in 5 ml 0.25% thiobarbituric acid, heated at 98°C for 30 min, quickly cooled on ice, and then centrifuged at 12,000 *g* for 10 min. The absorbance of the supernatant was measured at 450, 532, and 600 nm.

Nitric Oxide Level Determination

The intracellular NO level was determined using a specific NO fluorescent probe, diaminofluorescein diacetate (DAF-FM DA, 5 mM in dimethyl sulfoxide; Beyotime Institute of Biotechnology, Haimen, China). The callus was incubated with DAF-FM DA at a final concentration of 10 μM at 37°C for 20 min in the dark. The extra DAF-FM DA was removed using three washings with NaH_2PO_4/Na_2HPO_4 buffer (PBS, pH 7.4) of 10 min each. Then the fixed callus cells were treated with 1% pectinase (in MS medium without agar) for 20 min. NO fluorescence was visualized using laser scanning confocal microscopy (LSM510 Meta, Zeiss), with excitation and emission settings at 495 and 515 nm. Images were taken and analyzed by Zeiss LSM 510 software. Nonspecific autofluorescence background was reduced by limiting pinhole and PMT gain. Also, the reduction of background noise was obtained by linear average of fluorescence intensity at each pixel using imaging software.

Catalase Activity Measurement

The overall procedure was carried out at 4°C. One gram of plant material was homogenized with 1 ml of chilled PBS buffer (50 mM, pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid and 1% polyvinylpyrrolidone. After centrifugation for 30 min at 15,000 *g*, the supernatant was collected for CAT activity determination.

CAT activity was measured according to the method of Aebi (1974). Briefly, 100 μl enzyme extraction was added to 3 ml reaction medium containing 50 mM PBS buffer (pH 7.0), and then the reaction was started by adding 15 mM H_2O_2. The change of absorption values was recorded at 240 nm for 3 min. CAT activity was estimated by calculating the initial rate of disappearance of H_2O_2.

Statistical Analysis

All values are represented by an average of at least three replicate measurements ± standard error (SE), and the significance of differences between the control and treatments was statistically evaluated by Student's *t*-test methods.

Results

Effect of NaCl and SNP on Growth and Soluble Total Proteins

The effects of NaCl on the growth of *Nitraria tangutorum* Bobr. callus were demonstrated by measuring fresh weight increase and dry weight. Only a slight reduction in fresh weight increase was observed in the callus after 18 days with 50- and 100-mM-NaCl treatment (Table 1). Also, these two NaCl concentrations resulted in about 37 and 18% enhancement in dry weight, respectively, compared with the control callus. Thus, *Nitraria tangutorum* Bobr. callus showed good growth in response to low salt concentrations. However, high salt concentrations inhibited the growth of the callus. Compared to the control, fresh weight increase was significantly reduced in the callus exposed to 200-mM-NaCl stress. The inhibition in the growth of the callus was also reflected in reduced dry weight under 200-mM-NaCl treatment (Table 1).

Previous research has provided evidence that the expression of proteins is regulated in plants depending on salt concentration (Sekmen and others 2007). Increased soluble protein content was observed in the callus of *Nitraria tangutorum* Bobr. exposed to low-concentration salinity treatment (50 and 100 mM) for 12 days. However, treatment with 200 mM NaCl led to a significant decrease in soluble protein content (Table 1). SDS–PAGE analysis also revealed increased contents of some soluble proteins in NaCl-treated callus. Moreover, newly synthesized bands were observed in the callus exposed to 50- and 100-mM-NaCl treatments (Fig. 1).

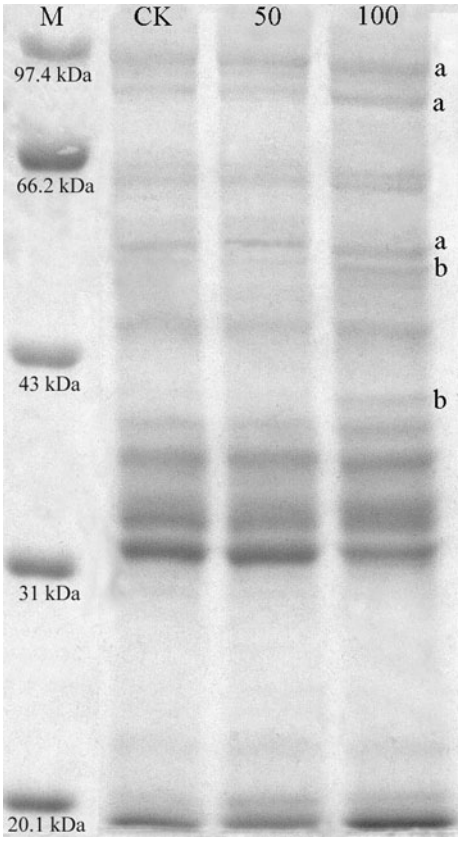

Fig. 1 SDS-PAGE analysis of soluble proteins. Total proteins (70 μg) are separated by SDS-PAGE. Lane M, marker; Lane CK, control; lane 50, 50-mM-NaCl treatment; lane 100, 100-mM-NaCl treatment. Small letters a and b indicate increased protein content and newly synthesized bands of proteins, respectively

NO plays multiple roles in plant growth and development (Lehnera and others 2009). The NO donor SNP is known to elevate NO levels in plants (Beligni and Lamattina 2000). The effect of different SNP concentrations on dry weights is shown in Fig. 2. Compared to the control, 10 μM SNP did not have any significant effects on dry weights of the callus, whereas 25 and 50 μM SNP led to about 25 and 20% increases in dry weight (Fig. 2A), indicating that NO might be involved in the regulation of callus growth. Furthermore, the presence of c-PTIO, a NO scavenger, partly abolished the elevation of dry weight under 50-mM-NaCl treatment (Fig. 2B).

As shown in Fig. 3, compared to the control, no significant changes in soluble protein content were found in the callus of *Nitraria tangutorum* Bobr. treated with SNP

Table 1 Effect of NaCl on the growth and soluble proteins of the callus

	NaCl concentration (mM)			
	0	50	100	200
Relative increase of fresh weight (%)	100 ± 1.56	95.64 ± 2.04	83.95 ± 1.12*	46.19 ± 1.83*
Dry weight (mg)	87.15 ± 2.09	119.53 ± 3.74*	102.17 ± 5.42	62.74 ± 2.55*
Soluble protein content (mg g^{-1} FW)	0.751 ± 0.014	0.925 ± 0.027*	1.166 ± 0.110*	0.656 ± 0.025

Nitraria tangutorum Bobr. callus was treated for 18 days with different NaCl concentrations, and then fresh weight (FW) increases and dry weights were measured. Values represented averages ± SE calculated from at least eight replicates. After treatment of NaCl for 12 days, the callus was collected for determination of soluble protein content. The experiments were done at least five times and the average values were ± SE represented

* Significantly different with $P < 0.05$ when compared with the control

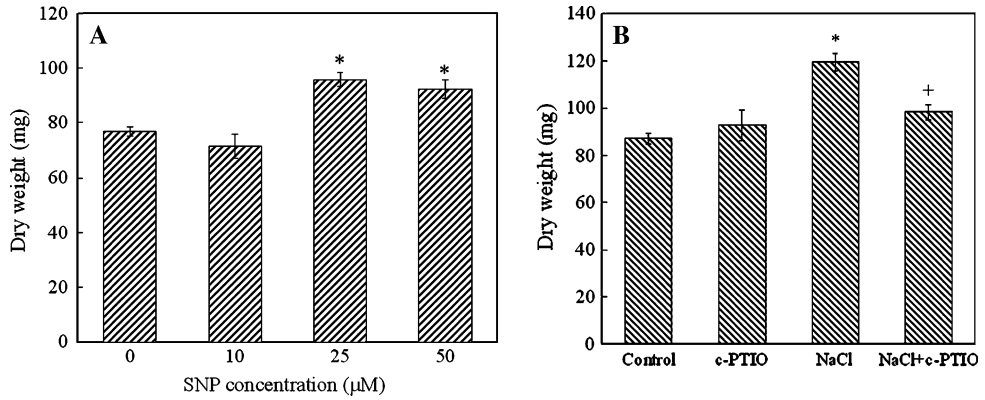

Fig. 2 **A** The amount of dry weight in *Nitraria tangutorum* Bobr. callus under NO donor SNP (0, 10, 25, and 50 μM) treatment for 18 days. **B** The changes in dry weight content induced by 50-mM-NaCl treatment for 18 days in the presence or absence of specific NO scavenger c-PTIO (250 μM). Values are mean ± SE of at least five independent measurements. * Significantly different with $P < 0.05$ when compared with the control. $^{+}$ Significant ($P < 0.05$) when compared with column NaCl

(10, 25, and 50 μM) or the NO scavenger c-PTIO (250 μM). Also, application of c-PTIO did not block the elevation in soluble protein content in response to 50-mM-NaCl stress for 3 days (Fig. 3B). These results suggest that NO might not be associated with regulation of soluble protein content in the callus of *Nitraria tangutorum* Bobr.

Effect of NaCl Stress on Osmotic Regulators Proline and Soluble Sugar

Proline is known to play an important role as an osmoprotectant (Delauney and Verma 1993) and in general is accumulated when plants are subjected to hyperosmotic stresses (Suriyan and Chalermpol 2009). The proline level in response to NaCl treatment was measured in *Nitraria tangutorum* Bobr. callus after subculture for 12 days. Figure 4 shows an approximately 1.42-, 3.57-, and 2.55-fold increase in proline content in the callus exposed to 50, 100 and 200 mM NaCl, respectively ($P < 0.05$). Soluble carbohydrates have also been mentioned as important osmoregulatory compounds in plants under salt stress (Hare and others 1998). In response to salinity, a salt-tolerant tomato genotype cv. *Pera*, compared with salt-sensitive cv. *Voglogradskij*, accumulates more soluble carbohydrates (Balibrea and others 2000). In *Nitraria tangutorum* Bobr. callus, solute sugar content significantly increased under salinity stress ($P < 0.05$). An approximately 25, 28, and 23% enhancement in the amount of soluble sugar was induced in the callus when exposed to 50, 100, and 200 mM NaCl for 12 days, respectively (Fig. 4). Therefore, in contrast to soluble sugar, proline possibly plays a more important role as an osmoprotectant in *Nitraria tangutorum* Bobr. callus subjected to salinity stress.

Effect of NaCl and SNP on Lipid Peroxidation

MDA, the product of lipid peroxidation, is the most prominent symptom of oxidative stress in plants (Yamamoto and others 2001). As shown in Fig. 5A, the amount of MDA increased about 27 and 70% in the callus treated with 100 and 200 mM NaCl, respectively, for 12 days in comparison to the control. In contrast, there was no significant change in MDA content after 12 days of

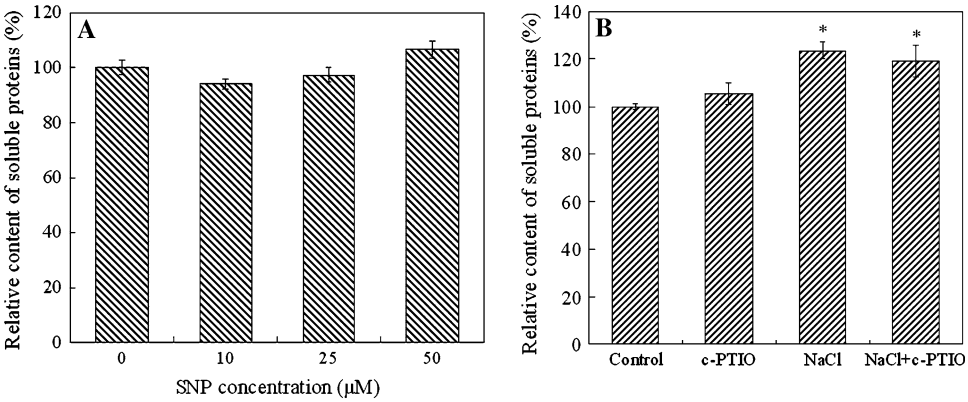

Fig. 3 **A** The amount of soluble proteins in *Nitraria tangutorum* Bobr. callus under NO donor SNP (0, 10, 25, and 50 μM) treatment for 3 days. **B** Effect of specific NO scavenger c-PTIO (250 μM) on the changes of soluble protein content induced by 50-mM-NaCl treatment for 3 days. Values are the mean ± SE of at least three independent measurements. *Significantly different with $P < 0.05$ when compared with column Control

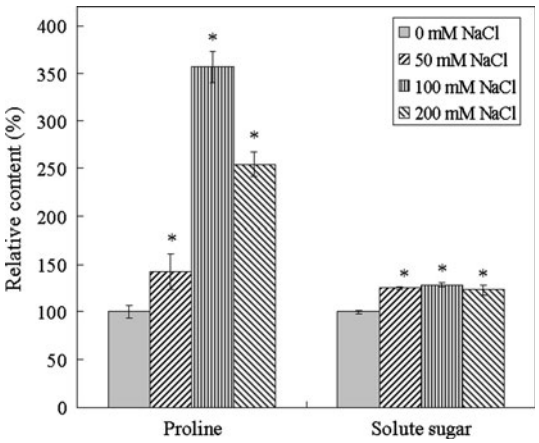

Fig. 4 The changes of proline and soluble sugar contents induced by NaCl treatment in the callus of *Nitraria tangutorum* Bobr. Data represent the means of at least five samples ± SE, with asterisks indicating significantly different means between unstressed and stressed callus ($P < 0.05$)

treatment with 50 mM NaCl. Further, a time-response experiment of lipid peroxidation was investigated in the callus stressed with 50 mM NaCl. At 3 and 6 days with NaCl treatment, the amount of MDA decreased about 29 and 15%, respectively, compared with that of the control, but it increased to the level of the control at 12 days of treatment (Fig. 5B).

To determine the effect of NO on lipid peroxidation levels in *Nitraria tangutorum* Bobr. callus, the NO donor SNP was used. Figure 6A shows that lower amounts of MDA were mediated in the callus treated with 10 or 25 μM SNP for 3 days compared with that of the control. However, the degree of lipid peroxidation observably increased when the callus was treated with 100 μM SNP ($P < 0.05$). Thus, it can be suggested that NO plays an important role in protecting the callus cells from oxidative damage, but high NO concentrations mediated significant oxidative damage in *Nitraria tangutorum* Bobr. callus. In addition, the treatment of the callus cells with the NO scavenger c-PTIO significantly increased the MDA content compared with that of untreated callus cells (Fig. 6B). This result supports the conclusion that NO was required for protecting *Nitraria tangutorum* Bobr. callus from the oxidative damage to membrane lipids. It is also suggested that a basal level of NO production might occur in the callus cells. Furthermore, the NaCl-induced decrease in lipid peroxidation was alleviated by treatment with c-PTIO (Fig. 6B), indicating that NO production played an important role in suppressing lipid peroxidation in the callus of *Nitraria tangutorum* Bobr. under salinity stress.

Effect of NaCl and SNP on NO Levels

The callus cells were incubated with the fluorescent probe DAF-FM DA, and the average green fluorescence was quantified using Zeiss LSM 510 software. As illustrated in Figs. 7 and 8, DAF-FM DA fluorescence was detected in untreated cells, and the application of the NO donor SNP significantly increased green fluorescence in the callus compared with the control, but the NO scavenger c-PTIO partly diminished DAF-FM DA fluorescence. These results suggest that the fluorescence was caused by NO production in the callus cells. It has been reported that the exposure of plants to various abiotic stresses induces the generation of NO (Neill and others 2002; Desikan and others 2004). We further measured NO levels in NaCl-treated callus cells using DAF-FM DA. Remarkably, the production of endogenous NO in the callus significantly increased under 50-mM-NaCl treatment alone for 3 days (observed as green fluorescence, Fig. 8Ab, B), whereas a slight signal of NO fluorescence was observed in the callus cells treated with NaCl in the presence of L-NNA, an inhibitor of NO production via nitric oxide synthase (NOS; EC 1.14.13.39) activity, or in the presence of c-PTIO (Fig. 8Ac, Ad, B). These data indicate that NaCl induced increased NO production in the callus of *Nitraria tangutorum* Bobr., which might be partly dependent on NOS activity.

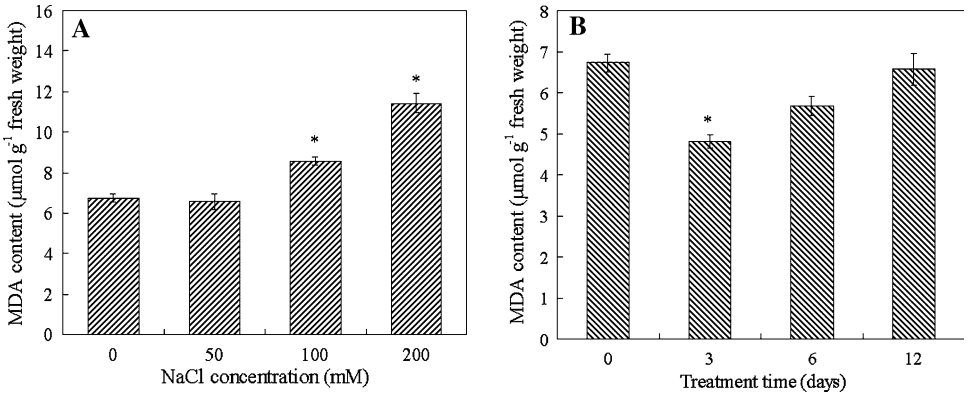

Fig. 5 The changes of the amount of MDA in *Nitraria tangutorum* Bobr. callus after treatment with different NaCl concentrations (0, 50, 100, and 200 mM) for 12 days (**A**) or with 50 mM NaCl for 3, 6, and 12 days (**B**). Data represent the mean ± SE of at least five independent measurements. *Significant difference between unstressed and stressed callus ($P < 0.05$)

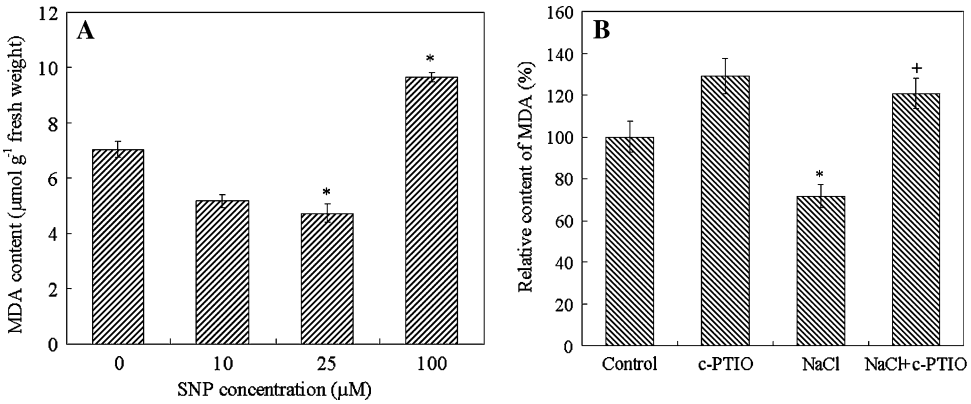

Fig. 6 **A** MDA level in *Nitraria tangutorum* Bobr. callus under NO donor SNP (0, 10, 25, and 100 μM) treatment for 3 days. **B** Specific NO scavenger c-PTIO (250 μM) reverses the decrease of lipid peroxidation induced by 50-mM-NaCl treatment for 3 days. * Significant ($P < 0.05$) when compared with the control. +Significantly different with $P < 0.05$ when compared with column NaCl. Values are the means ± SE of at least five independent measurements

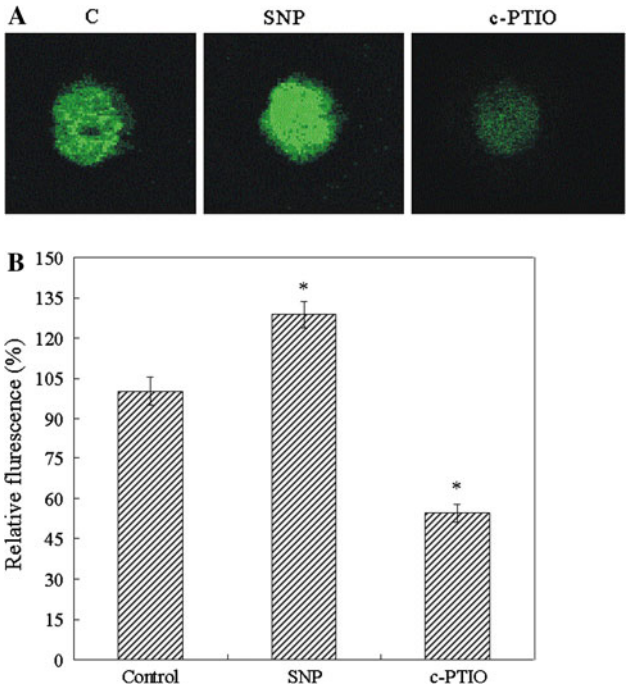

Fig. 7 **A** NO content in *Nitraria tangutorum* Bobr. callus cells is visualized as green fluorescence based on the fluorescent probe DAF-FM DA using laser scanning confocal microscopy. C, the control; SNP, 25 μM; c-PTIO, 250 μM. **B** Mean relative DAF-FM DA fluorescence densities for the callus cells corresponding to **A** C, SNP, and c-PTIO. Values represent the averages of at least five cells for each treatment and bars indicate SE. * Significant differences between the control and treated callus ($P < 0.05$)

NaCl Induced NO Generation Involved in the Regulation of CAT Activity

CAT is one of the major antioxidant enzymes that eliminate H_2O_2 by converting it into oxygen and water (Asada 1992; Wang and others 2004). A decrease in CAT activity was observed in Kikuyu grass after exposure to salt stress (Muscolo and others 2003). However, in some plants salt stress has been shown to enhance CAT activity (El-baky and others 2003). As shown in Fig. 9A, an approximately 15 or 35% increase in CAT activity was measured in the callus after 50- or 100-mM-NaCl treatment for 12 days, respectively, whereas the enzyme activity decreased about 26% of the control value at 12 days of treatment with 200 mM NaCl. We next examined the duration of the salinity effect on CAT activity. At 3, 6, and 12 days of treatment with 50 mM NaCl, CAT activity increased by about 45, 24, and 15%, respectively, in comparison with the control (Fig. 9B).

It has been demonstrated that exogenous NO, provided by using a variety of NO donors, inhibits the activity of CAT in tobacco (Clark and others 2000). However, ABA-triggered NO generation induced the stimulation of CAT activity in *Stylosanthes guianesis* (Zhou and others 2005). To determine whether exogenous NO affects CAT activity, the NO donor SNP was used to treat *Nitraria tangutorum* Bobr. callus. We found an approximately 28, 33, and 51% increase in CAT activity after exposure of the callus to 10, 25, or 50 μM SNP for 3 days, respectively (Fig. 10). This indicates that exogenous NO mediated the excitation of CAT activity in the callus in a dose-dependent manner. The treatment of *Nitraria tangutorum* Bobr. callus with NaCl not only increased CAT activity, it also led to NO production. Therefore, we further studied the relationship between endogenous NO production and the changes of CAT activity in the callus under NaCl treatment. To this purpose, the callus was treated with 50 mM NaCl for 3 days in the presence or absence of the NOS inhibitor L-NNA or NO scavenger c-PITO. The inhibition of NOS activity by L-NNA partly abolished the NaCl-induced excitation of CAT activity (Fig. 11). In agreement with this result, c-PTIO significantly blocked the excitation of CAT activity under NaCl treatment (Fig. 11). These results

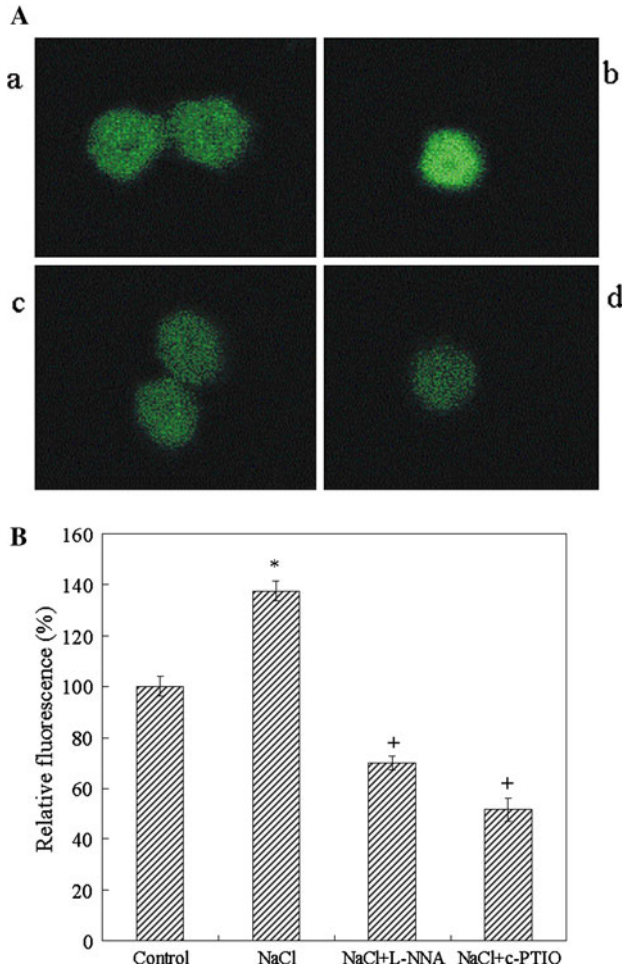

Fig. 8 **A** Endogenous NO level is visualized using a specific fluorescent probe DAF-FM DA in *Nitraria tangutorum* Bobr. callus cells after treatment with 50 mM NaCl for 3 days in the absence or presence L-NNA or c-PTIO. Aa, control (without treatment); Ab, NaCl (50 mM); Ac, NaCl (50 mM) + L-NNA (0.5 mM); Ad, NaCl (50 mM) + c-PTIO (250 μM). **B** Mean relative DAF-FM DA fluorescence densities for the callus cells corresponding to Aa, b, c, d. Values represent the means of at least five cells for each treatment and bars indicate SE. Significant difference ($P < 0.05$) between the control and NaCl treatment is marked by asterisk. $^{+}$ Significant ($P < 0.05$) when compared with column NaCl

suggest that NOS-dependent NO production might be related to NaCl-induced excitation of CAT activity in the callus of *Nitraria tangutorum* Bobr.

Discussion

It is necessary to understand the integration and expression of tolerant mechanisms from the cellular level to the whole-plant level, and in vitro culture provides an excellent means to study the physiological and genetic processes of the plant at cell and tissue levels (Dracup 1991). Fresh weight increase and dry weight are often measured to reveal the growth of plants and cells in response to environmental stresses. In the present study we observed that lower salt concentrations induced a slight reduction in the fresh weight increase and significant elevation in dry weight in *Nitraria tangutorum* Bobr. callus as compared with the control, which suggest a cellular tolerance to lower salinity in this halophytic species. However, the inhibition of the callus's growth was reflected in a decreased fresh weight increase and dry weight under 200-mM-NaCl treatment (Table 1). A similar result was obtained in rice callus (Ahmad and others 2009). To improve adaptation to salt, related gene expression was induced to produce salt-induced proteins in plants (El-baky and others 2003; Yildiz 2007). The data in our study indicate that lower-dose NaCl treatment of the callus resulted in a significant increase in the amount of soluble proteins (Table 1). Furthermore, SDS–PAGE analysis indicates that some newly synthesized bands were induced in the callus treated with 50 or 100 mM NaCl (Fig. 1). These results suggest that NaCl might induce the regulative expression of related genes in *Nitraria tangutorum* Bobr. callus for adapting to stress. However, high NaCl concentrations (200 mM) resulted in a significant decrease in soluble protein content. NO plays multiple roles in plant growth and development (Beligni and Lamattina 2000;

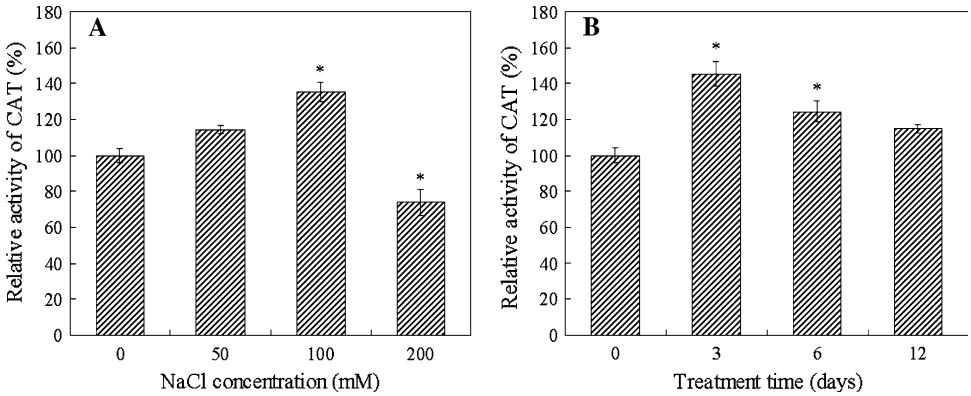

Fig. 9 The changes in CAT activity in *Nitraria tangutorum* Bobr. callus treated with different NaCl concentrations (0, 50, 100, and 200 mM) for 12 days (**A**) or with 50 mM NaCl for 3, 6, and 12 days (**B**). Values are the average ± SE of at least three experiments with replicated measurements. * Significant differences between the control and NaCl-stressed callus ($P < 0.05$)

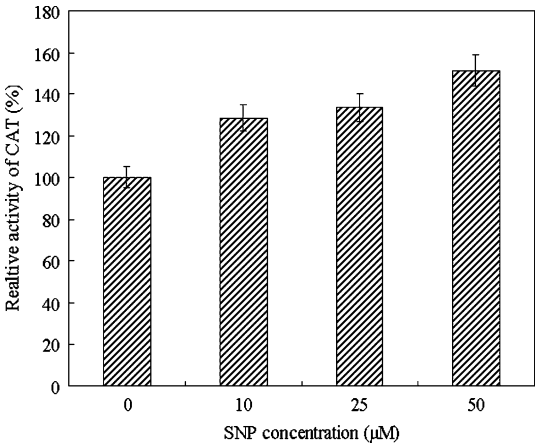

Fig. 10 The changes in CAT activity induced by the NO donor SNP in *Nitraria tangutorum* Bobr. callus. Values represent the average ± SE calculated from at least four independent experiments

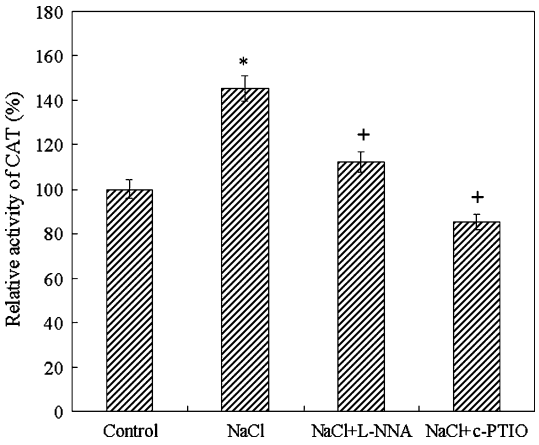

Fig. 11 The effects of L-NNA or c-PTIO on NaCl-induced excitation of CAT activity in the callus of *Nitraria tangutorum* Bobr. Values are the means of at least four replicates and bars indicate SE. Significant differences ($P < 0.05$) between the control and NaCl treatment is indicated by asterisks. $^{+}$ Significantly different with $P < 0.05$ when compared with column NaCl

Lehnera and others 2009), including the regulation of soluble protein content (Tanou and others 2009b). In *Lens culunaris* Medik., SNP application induces the enhancement in total soluble proteins depending on the concentrations, and the most obvious increase is observed in the application of 700 μM SNP (Çiğdem SE and others 2008). However, during H_2O_2-induced oxidative stress, a low concentration of NO prevents the accumulation of soluble proteins in *Nicotiana plumbaginifolia* cells (Dubovskaya and others 2007). In *Nitraria tangutorum* Bobr. callus, SNP treatment induced a significant increase in dry weight (Fig. 2A) but did not have a significant effect on the amount of soluble proteins (Fig. 3A). Moreover, the addition of c-PTIO did not abolish NaCl-induced elevation of soluble protein content in the callus (Fig. 3B) but partly reversed the increase of dry weight under 50-mM-NaCl treatment (Fig. 2B). These results suggest that NO might be associated with regulation of growth in *Nitraria tangutorum* Bobr. callus under salinity stress.

Maintaining osmotic homeostasis by accumulating metabolically compatible compounds such as carbohydrates and proline may be among the important adaptive mechanisms of salinity tolerance in plants (Rosa and others 2009). It has been suggested that soluble sugar content instead of proline content is a better marker for selecting salt-tolerant wheat genotypes (Kerepesi and Galiba 2000) and drought tolerance improvement in durum wheat (Al Hakimi and others 1995). In the halophyte *Sesuvium portulacastrum*, treatment with 100 mM NaCl induced significantly increased levels of proline in the leaves, but it had no effect on soluble sugar content (Slama and others 2007). Also, in some plants and cell cultures, it has been demonstrated that the changes in proline levels are correlated with their ability to tolerate or adapt to saline conditions (Pandey and Ganapathy 1985; Chowdhury and others 1993; Radyukina and others 2007). Similarly, in the present study, salt-treated callus of *Nitraria tangutorum* Bobr. accumulated high amounts of proline in comparison with the control, and the maximum amount of proline was observed in 100-mM-NaCl-treated callus, whereas NaCl treatment led to only a slight but significant increase in soluble sugar level (Fig. 4). Thus, there is a greater accumulation of proline than soluble carbohydrates in the response of the callus to salinity stress, suggesting that *Nitraria tangutorum* Bobr. callus may be adapted to salinity via a high capacity for the accumulation of proline.

The amount of MDA reflects lipid peroxidation levels in plants exposed to different environmental stresses (Yamamoto and others 2001; Koca and others 2007). Salt stress is manifested as an oxidative stress (Gueta-Dahan and others 1997), which causes increased MDA content in plants (El-baky and others 2003; Sekmen and others 2007). In response to NaCl treatment for 12 days, no significant difference was observed in MDA content between untreated and 50-mM-NaCl-treated callus, but higher salt concentrations (100 or 200 mM) increased lipid peroxidation (Fig. 5A). Also, *Nitraria tangutorum* Bobr. callus had lower levels of MDA under 50-mM-NaCl stress for 3 or 6 days (Fig. 5B). In agreement with the present results, decreased MDA levels were observed in the salt-tolerant species *Behary Red* cv. (El-baky and others 2003) and *Plantago maritime* (Sekmen and others 2007) under salt stress, suggesting that these plants and cells are better protected against oxidative damage. It has been shown that exogenous NO decreases MDA content under environmental stresses (Wang and Yang 2005). For example, exogenous application of 60 μM SNP decreases MDA content in *Kosteletzkya virginica* under salt stress,

indicating that NO may confer salt tolerance in plants by preventing oxidative membrane damage (Guo and others 2009). As shown in our experiment, different doses of the NO donor SNP exhibited dual roles in lipid peroxidation in *Nitraria tangutorum* Bobr. callus. Ten or 25 μM SNP decreased the degree of lipid peroxidation, but treatment with 100 μM SNP significantly increased the oxidative damage in the callus (Fig. 6A). Therefore, low NO concentrations could protect the callus from oxidative damage, whereas high NO concentrations caused oxidative stress on *Nitraria tangutorum* Bobr. callus. In addition, treatment with the NO scavenger c-PTIO alone, which reduces endogenous NO levels (Fig. 7), resulted in enhancement of MDA content in the callus compared with that in the untreated callus (Fig. 6B), further supporting the conclusion that low NO concentrations protect the callus from oxidative damage and suggesting that NO production might occur under basal conditions. Taking into account that increased NO production is mediated in plants exposed to various environmental stresses (Arasimowicz and Floryszak-Wieczorek 2007; Liu and others 2007), we further measured NO levels in the callus cells using the special fluorescent probe DAF-FM DA. NO green fluorescence was observed in untreated cells (Figs. 7A and 8Aa). Meanwhile, significant fluorescent levels were detected in SNP- and NaCl-treated cells, corresponding to basal NO production (Figs. 7A and 8Ab), and the presence of the NOS inhibitor L-NNA or NO scavenger c-PTIO reduced the signal of NO fluorescence in the callus under NaCl treatment (Fig. 8Ac, Ad, B). These data prove that salinity stress resulted in increased NO production in the callus, which might be dependent on NOS activity. In agreement with NO protection of *Cassia tora* L. against aluminum-induced oxidative stress (Wang and Yang 2005), the decrease of NO accumulation using the NO scavenger c-PTIO could reserve lower MDA levels induced by 50-mM-NaCl treatment for 3 days (Fig. 6B). This demonstrates that NO generation played an important role in protecting *Nitraria tangutorum* Bobr. callus from NaCl damage.

One of the plant's responses to salinity stress is the enhancement in antioxidant enzyme activities, which provides protection from oxidative damage and develops plant tolerance to environmental stress (Sekmen and others 2007). It has been suggested that upregulation of CAT is one component of the tolerant adaptations of halophytes to high salinity (Pang and others 2008). Bor and others (2003) observed higher activity of CAT in wild salt-tolerant *B. maritima* compared with that of cultivated beet *B. vulgaris* cv. *Ansa* under salinity. A similar observation has been reported for wild salt-tolerant tomato species and salt sensitive cultivated tomato (Koca and others 2007). In the present study, the activity of CAT was elevated in 50- or 100-mM-NaCl-stressed callus but decreased in 200-mM-NaCl-treated callus (Fig. 9A). In addition, the increased rate of CAT activity had a tendency to decline with prolonged stress. The highest increase (about 45%) and the lowest increase (only 15%) relative to control values were observed in the callus stressed with salinity for 3 or 12 days, respectively (Fig. 9B). These results indicate that CAT played an important role in scavenging H_2O_2 and in protecting the callus from oxidative damage during the early treatment with NaCl. A previous study indicated that exogenous NO inhibits CAT activity in tobacco (Clark and others 2000). Conversely, in *Kosteletzkya virginica*, the application of SNP significantly stimulates the activity of CAT (Guo and others 2009). The results from the present study indicate that the treatment of *Nitraria tangutorum* Bobr. callus with the NO donor SNP induced significant excitation of CAT activity (Fig. 10). Thus, the regulatory effects of exogenous NO on plant antioxidant enzyme activities may be dependent on different plant species. Previous work has demonstrated that endogenous NO generation is involved in the regulation of CAT and APX activities in plants in response to ABA (Zhou and others 2005). In the callus of *Nitraria tangutorum* Bobr., we found that the NO scavenger c-PTIO or the NOS inhibitor L-NNA partly abolished the NaCl-induced increase of CAT activity in the callus (Fig. 11), indicating that NOS-dependent NO production might be responsible for NaCl-induced excitation of CAT activity.

Taken together, higher salinity induced inhibition of growth and significant oxidative damage of *Nitraria tangutorum* Bobr. callus. However, in response to lower salinity, increased dry weights and soluble proteins were observed in the callus, and also high levels of proline were induced by NaCl with increasing salinity stress. In addition, the short-term stress of 50-mM salinity resulted in decreased levels of MDA in the callus. These characteristics are commonly associated with the response of salt-resistant plant cells or tissues to salinity. Therefore, the callus from *Nitraria tangutorum* Bobr. may be ideal for studies on the adaptations of plants to salinity stress. Our results also indicated that increased NO generation induced by salinity might be associated with the regulation of growth, protection against oxidative damage, and excitation of CAT activity in *Nitraria tangutorum* Bobr. callus under salt stress. Finally, our present data are in excellent agreement with recent findings that enlighten the existence of an interplay between the H_2O_2 and NO-signaling pathways in NaCl-stressed plants (Tanou and others 2009a).

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