Nitric Oxide Alleviates the Inhibition of Salinity Stress on Seed Germination and Seedling Growth of *Cynanchum bungei* Decne (Asclepiadaceae)

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Abstract. As a rare, endemic, important, and salt-sensitive medicinal plant species in China, *Cynanchum bungei* Decne seeds were treated to germinate with distilled water (control) or NaCl solutions in the presence or absence of nitric oxide (NO) donor sodium nitroprusside (SNP) to investigate the effects of exogenous NO on seed germination, seedling growth, and antioxidant enzyme activities under salt stress. Sixty mM NaCl significantly inhibited the germination and seedling growth of *C. bungei*. Exogenous SNP alleviated salt stress in a dose-dependent manner, as indicated by accelerating the seed germination, increasing germination index (GI), vigor index (VI), germination velocity (GV), shoot height (SH), taproot length (TL), shoot biomass (SB), root biomass (RB) as well as shortening mean germination time (MGT), and 0.1 mM SNP was the optimal concentration. SNP at 0.1 mM greatly increased the activities of superoxide dismutase (SOD) and catalase (CAT) under salt stress, which contributed to alleviate the oxidative stress induced by salt stress in *C. bungei* seeds. It is concluded that NO treatment is an effective practice to improve *C. bungei* seed germination under saline condition.

Soil salinity is a major limiting factor for agricultural land productivity. At least 20% of all irrigated lands in the world are salt-affected with some estimates being as high as 50% (Pitman and Läuchli, 2002). In China, there is a total area of 3.47×10^7 ha saline soil (Liu et al., 2008), and sodium chloride is the most common and thus the most troublesome salt composition. Although the salinity inhibition effects on plant growth vary at different stages, the first exposure and the most vulnerable phase of the plant to salinity stress usually occurs at germination and seedling stages. Thus, it is worthwhile to clear the influence mechanisms of salt stress on seed germination. Then suitable measures should be developed to improve seed germination, and thereby plant establishment, on saline soils. Salt stress can impose both ionic toxicity and osmotic stress on plants, which lead to nutritional imbalance and oxidative stress caused by reactive oxygen species (ROS) (Zhu, 2003). ROS are key components of signaling networks to regulate developmental processes (Kranter and Seal, 2013). However, the excess

of ROS can damage macromolecular and cellular structure (Kranter and Seal, 2013), and as a result, plant cells have developed different strategies to regulate their intracellular ROS concentrations by scavenging of ROS. Major ROS scavenging enzymes include SOD, peroxidase (POD), CAT, and ascorbate peroxidase (APX). Therefore, enhancing the activity of antioxidant enzymes in plant organs is necessary for improving a plant's tolerance to salt stress.

NO, as a crucial signaling molecule, is involved in multiple biological functions in plants. These include stimulation of seed germination (Sarath et al., 2006), modulation of plant growth and development (Gouvêa et al., 1997), regulation of plant maturation and senescence (Liao et al., 2013), suppression of floral transition (He et al., 2004), mediation of stomatal movement (Liu et al., 2003), and involvement of light-mediated greening (Zhang et al., 2006). NO has also been involved in responses to abiotic and biotic stresses such as drought (Fan and Liu, 2012), salt (Boldizsár et al., 2013), heat (Song et al., 2006a), and metal stresses (He et al., 2014), disease resistance (Asai et al., 2010), apoptosis (Pedroso et al., 2000), and ultraviolet-B radiation (Chen et al., 2003). NO effects on seed germination under salinity stress were confirmed in wheat (Duan et al., 2007), maize (Boldizsár et al., 2013), soybean (Vaishnav et al., 2013), cucumber (Fan et al., 2013a, 2013b), reed (Zhao et al., 2004), *Abaidopsis* (Zhao et al., 2007), etc.

Cynanchum bungei Decne (Baishouwu in China), a member of the Asclepiadaceae

family, is a perennial climbing shrub distributed in China. Its root has been used as a tonic medicine or health food for centuries in traditional Chinese medicine. *C. bungei* has useful activities including the antitumor and immune regulatory effect and is non-toxic (Gao et al., 2005). As a rare, endemic, and beneficial species, *C. bungei* is becoming more and more precious not only in China, but also in other countries such as Japan and Korea (Song et al., 2006b). With the increasing demand for this endangered wild resources (Zhang et al., 2010), seed propagation has already been used to solve supply and demand, protect germplasm resource, and enrich genetic diversity of this species (Zhang, 2012). The existing study showed *C. bungei* is sensitive to salt stress, especially during germination and as a seedling (Wang et al., 2012). Here, we suppose that exogenous NO application under salt stress can have a positive effect on *C. bungei* seed germination. In this article, the effects of exogenous NO treatment on seed germination, seedling growth, and antioxidant enzyme activity of *C. bungei* under salt stress were investigated to test the feasibility of this assumption. Meanwhile, the expected results would also suggest SNP, a NO donor, to be used as a seed coating to increase *C. bungei* seedling tolerance to salt stress.

Materials and Methods

Seed source. Seeds of *C. bungei* were collected from the north-facing slope of Mount Tai (lat. 36°15'17" N, long. 117°06'15" E) in late fall and winter of 2012. Seeds were air-dried at room temperature and stored in darkness at 4 °C until Apr. 2013.

Experimental procedures. Germination tests were performed from May to Sept. 2013. Seeds were immersed in 75% ethanol for 5 min and then 5% sodium hypochlorite for 5 min for surface sterilization and then rinsed three times with deionized water and dried on filter paper.

Our preliminary experiment had shown that 60 mM NaCl decreased germination of *C. bungei* seeds ~40%. Based on this, 60 mM NaCl was used to induce salinity stress in this study. The treatments were as follows: distilled water [control (CK)], 60 mM NaCl, 60 mM NaCl + 0.01 mM SNP, 60 mM NaCl + 0.1 mM SNP, 60 mM NaCl + 0.5 mM SNP, and 60 mM NaCl + 1.0 mM SNP. Each treatment was replicated six times. Thirty-six seeds per treatment were placed in petri dishes (12 cm diameter × 1.5 cm depth) containing filter paper moistened with 10 mL of treatment solutions and germinated in incubators (HPG-280BX; Harbin Donglian Electronic Technology Development Ltd., Harbin, China) at 20 °C and a 12:12-h photoperiod (Zhang, 2012). The treatment solutions were renewed each day to maintain unaltered concentrations. The dishes were rearranged daily to avoid effects of potential temperature and light differences or gradients in the incubators.

Germination performance was estimated according to the rate of germinated seeds.

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Seeds were considered germinated when the radical had protruded through the seed coat. Final germination percentage (FGP) is the rate of germinated seeds on the 15th day. On the 20th day, SH and TL were measured. The shoot and roots of each seedling were dried at 60 °C for 48 h to quantify SB and RB. A series of parameters were as follows.

$$\text{Germination index, } GI = \sum \frac{Gt}{Tt}$$

where Gt is the number of germinated seeds in Day t , and Tt is the time corresponding to Gt in days.

$$\text{Vigor index, } VI = GI \times SH$$

$$\text{Mean germination time(d), } MGT = \frac{\sum TiNi}{\sum Ni}$$

where Ni is the number of the newly germinated seeds in times of Ti .

$$\text{Germination velocity, } GV = \sum \frac{Gi}{Ti}$$

where i is the number of counting periods when the germination was evaluated, Gi is the percentage of germinated seeds in the i period, and Ti is the number of days since the initiation of the assay.

$$\text{Root-to-shoot ratio, } RSR = \frac{TL}{SH}$$

The percentage of biomass allocated to

$$\text{root(\%), } BAR = \frac{RB}{RB + SB} \times 100$$

In this study, we found that 0.1 mM SNP was the most effective to alleviate NaCl stress on seed germination and seedling growth; this concentration of SNP was chosen for further study for ROS activity. Four treatments were set as CK, 0.1 mM SNP, 60 mM NaCl, 60 mM NaCl + 0.1 mM SNP. The germinating seeds were sampled for enzyme assays after 7 d of treatment.

Activity of SOD, POD, CAT, and APX were assayed according to Zhang and Li (2011). Germinating seeds were ground with ice-cold 50 mM phosphate buffer (pH 7.8) containing 1% polyvinylpyrrolidone and 0.1% mercaptoethanol. The homogenate was centrifuged at 13,000 g for 20 min at 4 °C and the supernatant was used for enzymatic activity assay. SOD activity was determined by its capacity to inhibit the photochemical reduction of nitro blue tetrazolium measured at 560 nm. POD activity was based on the oxidation of guaiacol using H_2O_2 at 470 nm. CAT activity was assayed spectrophotometrically by recording the decrease in absorbance at 240 nm. APX activity was determined in the presence of 0.5 mmol·L⁻¹ ascorbic acid and 0.5 mmol·L⁻¹ H_2O_2 by monitoring the decrease in absorbance at 290 nm. All spectrophotometric analyses were conducted on a Shimadzu (ultraviolet-2550, Kyoto, Japan) spectrophotometer.

Data analysis. All data were subjected to analysis of variance followed by the Duncan's multiple range test using SPSS 11.5 software (SPSS Inc., Chicago, IL).

Results

Effect of SNP on seed germination of *C. bungei* under NaCl stress. *C. bungei* seeds used in the present study began to germinate at 4 d and had an average germination rate of 82.4% after 15 d under control conditions (Table 1). NaCl treatment significantly inhibited seed germination and only very few seeds began to germinate at 6 d and FGP was reduced to 50.9%. Exogenous SNP had a dose-dependent effect on germination performance of *C. bungei* seeds. SNP from 0.01 to 0.5 mM could accelerate the seed germination under saline conditions, and SNP at 0.1 mM showed maximum improvement. However, SNP at 1.0 mM aggravated the inhibitory effect of NaCl on FGP.

Compared with the CK, GI, VI, and GV of *C. bungei* seeds under salt stress were significantly lower; however, MGT was significantly longer (Table 2). SNP at 0.01 to 0.5 mM could increase GI, VI, and GV and shorten MGT, and 0.1 mM SNP was the most effective among all the treatments. However, 1.0 mM SNP had no significant effect on GI, VI, GV, or MGT under salt stress.

Effect of SNP on seedling growth of *C. bungei* under NaCl stress. SH, TL, SB, RB, RSR, and BAR were reduced by 67.4%, 76.3%, 51.3%, 64.4%, 27.5%, and 22.3% under NaCl stress, respectively, suggesting that NaCl inhibited seedling growth of *C. bungei* and root growth is more sensitive to NaCl than shoot growth (Table 3). Like these germination indices, SNP from 0.01 to 0.5 mM accelerated seedling growth under salt stress, and 0.1 mM SNP showed the best seedling performance in the presence of NaCl. However, 1.0 mM SNP aggravated the inhibition of seedling growth.

Effect of SNP on seed antioxidant enzymes activity of *C. bungei* under NaCl stress. SOD activity under salt stress was higher than that of the CK, and exogenously applied SNP increased SOD activity too; moreover, seed treatment with SNP under NaCl stress further improved SOD activity to be 1.3-fold of the NaCl-treated alone (Fig. 1A). SNP application alone enhanced POD activity over the CK. However, POD activity was unaffected by NaCl or NaCl plus SNP (Fig. 1B). SNP or salt stress alone increased CAT activity, and a further increasing in CAT activity was observed by SNP application under NaCl stress (Fig. 1C). No significant differences were found in APX activity among CK, SNP, NaCl, and NaCl + SNP (Fig. 1D).

Discussion

Salinity is a major abiotic stress factor that disrupts ionic homeostasis and imposes osmotic and toxicity stress to plants and consequently induces a reduction in seed

Table 1. Germination performance of *C. bungei* seeds at different concentrations of sodium nitroprusside (SNP) with 60 mM NaCl.

Treatment	Germination (%)														
	4 d	5 d	6 d	7 d	8 d	9 d	10 d	11 d	12 d	13 d	14 d	15 d			
Control	9.3 ± 1.4 a [*]	17.6 ± 1.4 a	30.6 ± 2.4 a	41.7 ± 2.4 a	50.0 ± 2.4 a	62.7 ± 1.4 a	72.2 ± 2.4 a	76.9 ± 1.4 a	79.6 ± 1.4 a	81.5 ± 3.8 a	82.4 ± 5.1 a	82.4 ± 5.1 a			
NaCl	0 ± 0 b	0 ± 0 c	0.9 ± 1.1 d	5.5 ± 0 e	18.5 ± 4.5 d	26.9 ± 1.4 d	34.2 ± 3.8 de	41.7 ± 2.4 d	44.4 ± 2.4 d	48.2 ± 1.4 d	50.8 ± 1.4 d	50.9 ± 1.4 d			
NaCl + 0.01 mM SNP	0 ± 0 b	1.9 ± 1.4 c	9.3 ± 1.4 c	17.6 ± 1.4 d	22.2 ± 0 d	27.8 ± 0 d	38.9 ± 2.4 d	50.0 ± 4.9 c	54.6 ± 3.8 c	61.1 ± 2.4 c	62.0 ± 2.8 c	62.0 ± 2.8 c			
NaCl + 0.1 mM SNP	8.3 ± 2.4 a	14.8 ± 1.4 a	29.6 ± 2.8 a	33.3 ± 2.4 b	38.0 ± 1.4 b	49.1 ± 3.8 b	56.5 ± 1.4 b	64.8 ± 1.4 b	70.4 ± 1.4 b	75.0 ± 2.4 b	75.0 ± 0 b	75.0 ± 2.4 b			
NaCl + 0.5 mM SNP	0 ± 0 b	7.4 ± 2.8 b	21.3 ± 1.4 b	25.0 ± 2.4 c	31.5 ± 1.4 c	38.0 ± 1.4 c	40.7 ± 3.8 cd	48.2 ± 3.8 c	50.0 ± 2.4 c	52.8 ± 2.4 d	57.3 ± 2.8 c	59.3 ± 1.4 c			
NaCl + 1.0 mM SNP	0 ± 0 b	0 ± 0 c	2.8 ± 1.4 d	6.5 ± 1.4 e	19.4 ± 2.4 d	25.9 ± 2.8 d	31.5 ± 1.4 e	38.0 ± 2.8 d	38.0 ± 2.8 e	43.5 ± 3.8 e	44.2 ± 1.4 e	44.4 ± 2.4 e			

^{*}Within a column, values followed by different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$).

germination rate and seedling establishment. In this study, our results also showed that salinity inhibited seed germination and seedling growth of *C. bungei*. Nitric oxide is a bioactive molecule that has successively become known as an important signal not only in plant disease resistance, but also in the process of seed germination, growth, development, and responses against abiotic stress (Neill et al., 2003). Exogenous NO has a strong stimulating effect on seed germination under stress or non-stress conditions (Neill et al., 2003; Sarath et al., 2006). It has been reported that exogenous NO promoted the germination of NaCl-treated *Suaeda salsa* seeds (Li et al., 2005), cucumber seeds (Fan et al., 2013a), etc. In this article, exogenous NO can alleviate the

inhibitory effect of salt stress on *C. bungei* seeds in a dose-dependent manner and 0.1 mM SNP showed the most effective alleviation. However, 0.05 mM SNP was the most effective concentration to accelerate cucumber seed germination under NaCl stress (Fan et al., 2013a). Priming of seeds with 0.06 mM SNP for 24 h markedly alleviated the inhibition on wheat seed germination by salt stress (Duan et al., 2007). The discrepancy between studies may be caused by the different sensitivity on NO in different plant species or different treatment methods of NO donor. NO resulted in a more rapid increase of β -amylase activity in seeds, which could promote seed germination under salt stress (Duan et al., 2007), osmotic stress (Zhang et al., 2003), and normal conditions

(Zhang et al., 2005), indicating that NO is involved in the intrinsic mechanism of seed germination under different conditions.

NO is known to enhance de-etiolation and promote greening in young seedlings (Beligni and Lamattina, 2000). In this experiment, the leaves of *C. bungei* seedlings treated with SNP were also observed greener than that of the CK, which is perhaps because that NO improves internal iron availability in plants (Graziano et al., 2002). SNP application also increased seedling growth as compared with salt treatment in this study. The possible reasons are: 1) NO may enhance salt tolerance in plants by increasing K^+ accumulation and decreased Na^+ accumulation (Duan et al., 2007); and 2) NO participates in enhancement of photosynthesis by inducing the photosynthetic pigments under salt stress (Fan et al., 2007). Moreover, SNP application led to better root growth, which could be advantageous for *C. bungei* seeds to grow in unfavorable soils with high pH, little water, and low nutrients, etc.

Exogenous NO was reported to improve seed germination under salt stress by enhancing antioxidant capability in wheat (Zheng et al., 2009), barley (Li et al., 2008), maize (Sun et al., 2007), cucumber (Fan et al., 2013), etc. In accordance with these findings, we observed that activities of both SOD and CAT in the seeds significantly increased because of exogenous NO application in the present study (Fig. 1A and C) and which might have contributed to the alleviated oxidative stress in the germinating *C. bungei* seeds and thereby improved germinating rate under salt stress. It is well known that the antioxidant enzymes such as SOD, CAT, and POD play a significant role in scavenging ROS in salt-stressed plants (Ashraf, 2009). Therefore, in this study, SNP improved salt stress tolerance either as the inducer of the antioxidant system or the direct scavenger of ROS, which has accumulated in response to stress stimuli. POD and APX activities under salt stress had no significant change after SNP application in the present study (Fig. 1B and D), indicating that the antioxidative role of exogenous NO against salt is not the result of the modulation of POD and APX.

Conclusion

Exogenous NO is able to improve seed germination and seedling growth of *C. bungei* in a dose-dependent manner, and 0.1 mM SNP produces the most effective improvement. Exogenous NO greatly promoted the

Table 2. Germination index (GI), vigor index (VI), germination velocity (GV), and mean germination time (MGT) of *C. bungei* seeds at different concentrations of sodium nitroprusside (SNP) under 60 mM NaCl.

Treatment	GI	VI	GV	MGT (d)
Control	4.32 $\pm$ 0.12 a ^z	14.88 $\pm$ 0.43 a	0.63 $\pm$ 0.01 a	7.64 $\pm$ 0.39 d
NaCl	1.98 $\pm$ 0.06 d	2.22 $\pm$ 0.07 e	0.25 $\pm$ 0.01 e	9.67 $\pm$ 0.31 a
NaCl + 0.01 mM SNP	2.54 $\pm$ 0.12 c	3.97 $\pm$ 0.19 d	0.32 $\pm$ 0.02 d	9.43 $\pm$ 0.06 a
NaCl + 0.1 mM SNP	3.78 $\pm$ 0.12 b	11.86 $\pm$ 0.38 b	0.53 $\pm$ 0.02 b	8.13 $\pm$ 0.29 cd
NaCl + 0.5 mM SNP	2.75 $\pm$ 0.13 c	8.46 $\pm$ 0.41 c	0.38 $\pm$ 0.02 c	8.69 $\pm$ 0.20 bc
NaCl + 1.0 mM SNP	1.79 $\pm$ 0.11 d	1.65 $\pm$ 0.10 e	0.23 $\pm$ 0.01 e	9.38 $\pm$ 0.02 ab

^zWithin a column, values followed by different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Fig. 1. Effect of SNP on SOD (A), POD (B), CAT (C), and APX (D) of *C. bungei* seed under salt stress. Bars represent $\pm$ SE. Letters indicate significant difference ($P \leq 0.05$) according to Duncan's multiple range tests. CK = control; SNP = 0.1 mM SNP; NaCl = 60 mM NaCl; NaCl + SNP = both SNP and NaCl treatments. SNP = sodium nitroprusside; SOD = superoxide dismutase; POD = peroxidase; CAT = catalase; APX = ascorbate peroxidase.

Table 3. Shoot height (SH), taproot length (TL), shoot biomass (SB), root biomass (RB), root-to-shoot ratio (RSR), and the percentage of biomass allocated to root (BAR) of *C. bungei* at different concentrations of sodium nitroprusside (SNP) under 60 mM NaCl.

Treatment	SH (cm)	TL (cm)	SB (kg·m ⁻²)	RB (kg·m ⁻²)	RSR	BAR (%)
Control	3.44 $\pm$ 0.17 a ^z	4.51 $\pm$ 0.12 a	2.57 $\pm$ 0.04 a	0.90 $\pm$ 0.10 a	1.31 $\pm$ 0.05 a	25.86 $\pm$ 2.17 a
NaCl	1.12 $\pm$ 0.11 bc	1.07 $\pm$ 0.12 e	1.25 $\pm$ 0.08 d	0.32 $\pm$ 0.05 d	0.95 $\pm$ 0.10 b	20.10 $\pm$ 1.80 b
NaCl + 0.01 mM SNP	1.56 $\pm$ 0.28 b	1.78 $\pm$ 0.17 d	1.40 $\pm$ 0.17 d	0.50 $\pm$ 0.01 c	1.16 $\pm$ 0.14 ab	26.19 $\pm$ 2.53 a
NaCl + 0.1 mM SNP	3.14 $\pm$ 0.12 a	3.99 $\pm$ 0.23 b	2.05 $\pm$ 0.09 b	0.76 $\pm$ 0.06 ab	1.27 $\pm$ 0.05 a	27.01 $\pm$ 0.76 a
NaCl + 0.5 mM SNP	3.08 $\pm$ 0.23 a	3.56 $\pm$ 0.15 c	1.69 $\pm$ 0.03 c	0.61 $\pm$ 0.07 bc	1.16 $\pm$ 0.05 ab	26.44 $\pm$ 1.70 a
NaCl + 1.0 mM SNP	0.72 $\pm$ 0.06 c	0.69 $\pm$ 0.10 e	0.77 $\pm$ 0.05 e	0.14 $\pm$ 0.01 e	0.95 $\pm$ 0.13 b	15.27 $\pm$ 2.27 c

^zWithin a column, values followed by different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$).

activities of SOD and CAT and alleviated the oxidative stress induced by salt stress in *C. bungei* seeds. Therefore, exogenous NO treatment on *C. bungei* seeds may be an option to accelerate seed germination under saline conditions.

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