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Nitric oxide induced by polyamines involves antioxidant systems against chilling stress in tomato (*Lycopersicon esculentum* Mill.) seedling*#

Qian-nan DIAO§, Yong-jun SONG§, Dong-mei SHI, Hong-yan QI†‡

(*Collaborative Innovation Center of Protected Vegetable Surround Bohai Gulf Region, Key Laboratory of Protected Horticulture of Ministry of Education and Liaoning Province, College of Horticulture, Shenyang Agricultural University, Shenyang 110866, China*)

†E-mail: hyqiaaa@126.com

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Abstract: Polyamines (PAs) and nitric oxide (NO) are vital signals in modulating plant response to abiotic stress. However, to our knowledge, studies on the relationship between NO and PAs in response to cold stress in tomato are limited. Accordingly, in this study, we investigated the effects of putrescine (Put) and spermidine (Spd) on NO generation and the function of Spd-induced NO in the tolerance of tomato seedling under chilling stress. Spd increased NO release via the nitric oxide synthase (NOS)-like and nitrate reductase (NR) enzymatic pathways in the seedlings, whereas Put had no such effect. Moreover, H_2O_2 might act as an upstream signal to stimulate NO production. Both exogenous NO donor (sodium nitroprusside (SNP)) and Spd enhanced chilling tolerance in tomato, thereby protecting the photosynthetic system from damage. Compared to chilling treatment alone, Spd enhanced the gene expressions of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), and their enzyme activities in tomato leaves. However, a scavenger or inhibitor of NO abolished Spd-induced chilling tolerance and blocked the increased expression and activity due to Spd of these antioxidant enzymes in tomato leaves under chilling stress. The results showed that NO induced by Spd plays a crucial role in tomato's response to chilling stress.

Key words: Antioxidant enzymes, Chilling tolerance, Hydrogen peroxide, Nitric oxide, Spermidine, Tomato
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1 Introduction

Polyamines (PAs), mainly putrescine (Put), spermidine (Spd), and spermine (Spm), are a group of phytohormone-like aliphatic amine compounds. PAs exert influence in the plant life cycle, including cell division and elongation, morphogenesis, seed germination, flowering, and senescence (Igarashi and Kashiwagi, 2000; Bais and Ravishankar, 2002; Yoda *et al.*, 2006; Wimalasekera *et al.*, 2011; Gupta *et al.*, 2013). Furthermore, PAs also have impact on plants in response to diverse abiotic stresses, such as salinity (Zapata *et al.*, 2004; Shu *et al.*, 2012), drought (Yang *et al.*, 2007; Li *et al.*, 2015), oxidative stress (Rider *et al.*, 2007; Puyang *et al.*, 2015), high temperature (Cheng *et al.*, 2012; Mostofa *et al.*, 2014), and chilling stress (Nayyar, 2005; Yamamoto *et al.*, 2012). It was previously suggested that the elevated stress tolerance of plants due to PAs may be attributed to their polycationic nature at physiologic pH. PAs can interact with negatively charged macromolecules, which inhibits the phase change under stressed condition (Groppa and Benavides, 2008; Alcázar *et al.*, 2010). Additionally, PAs can directly or indirectly scavenge reactive oxygen species (ROS) and enhance the activities of antioxidant enzymes (Verma and Mishra, 2005; Parvin *et al.*, 2014).

‡ Corresponding author
§ The two authors contributed equally to this work
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ORCID: Qian-nan DIAO, http://orcid.org/0000-0001-5086-5166

Nitric oxide (NO) is a highly reactive gaseous molecule that regulates diverse plant growth and development processes, including seed germination, root growth, flowering, and senescence (Neill *et al.*, 2003; Besson-Bard *et al.*, 2008). Several studies have suggested that NO can participate in controlling the various plant responses toward diverse abiotic stresses. For example, Esim and Atici (2014) observed that exogenous NO (sodium nitroprusside (SNP)) can effectively alleviate chilling stress damage in maize seedlings. Tian and Lei (2006) reported that NO treatment improved the growth of wheat seedlings and relieved oxidative damage. In contrast, according to Tun *et al.* (2006), PAs induced accumulation of NO in *Arabidopsis thaliana* seedlings. Arasimowicz-Jelonek *et al.* (2009) presented evidence that PAs promoted NO synthesis in cucumber seedlings during drought stress. In light of the common functions of PAs and NO in abiotic stresses, it can be conjectured that NO is linked to PA-induced stress responses (Wimalasekera *et al.*, 2011).

Many potential sources of NO production exist in plants; among them, the nitric oxide synthase (NOS) and nitrate reductase (NR) enzymatic pathways have been the focus of most studies (Guo *et al*., 2003; Wimalasekera *et al.*, 2011). NR has been found to be the source of NO in *Arabidopsis*, tobacco, sunflower, alfalfa, spinach, and maize (Desikan *et al.*, 2002; Rockel *et al*., 2002; Dordas *et al*., 2003; Planchet *et al*., 2005). In animals, NO is synthesized via NOS. Although mammalian-type NOS is intricate (Guo *et al*., 2003; Zemojtel *et al*., 2006), NOS-like activity has been found extensively in plants, and inhibitors of mammalian NOS can suppress NO production in plants (Neill *et al*., 2008; Tewari *et al.*, 2013). NO and hydrogen peroxide (H_2O_2), as universal signal transduction molecules, have been shown to be involved in controlling many physiological functions in plants (Finkel and Holbrook, 2000; Bright *et al*., 2006; Dickinson and Chang, 2011). A growing number of studies show that there is a relationship between NO and H_2O_2. During plant responses to various stresses or stimuli, NO and H_2O_2 production often occur in parallel or in short succession (Bright *et al*., 2006; Pasqualini *et al.*, 2009). Interestingly, evidence has been found suggesting that H_2O_2 can be also generated via PA catabolic pathways, through diamine oxidase (DAO) and polyamine oxidase (PAO) activities (Martin-Tanguy, 2001; Kusano *et al.*, 2007; Hussain *et al.*, 2011; Gupta *et al.*, 2013). In *Zea mays*, PAO modulates H_2O_2 production during wound healing (Angelini *et al.*, 2008). In the development of soybean lateral roots, DAO and PAO play important roles in H_2O_2 formation (Su *et al.*, 2006).

Chilling is a main abiotic stress factor that directly influences plant growth and productivity. It has been suggested that Put, Spd, and NO have effects on plant responses to chilling stress (Neill *et al.*, 2003; Cuevas *et al.*, 2008; Li *et al.*, 2014). We have recently shown that Put and Spd accumulated to some extent in tomato seedlings in response to low temperature (Song *et al.*, 2015). However, limited studies exist on whether PAs are involved in NO production under chilling stress, or whether PAs can enhance chilling tolerance by inducing NO production. Therefore, we performed a series of experiments using tomato seedlings to clarify these problems. The aims of this study were as follows: (1) to study which type of PAs can induce NO accumulation under chilling stress; (2) to clarify the possible mechanism underlying PA-induced NO synthesis under chilling stress; and (3) to determine whether NO production induced by PAs can enhance chilling tolerance.

2 Materials and methods

2.1 Plant materials, growth, and treatment conditions

Seeds of tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) were germinated and grown in 12 cm×12 cm plastic pots containing peat moss in a greenhouse (temperature 25 °C (day)/15 °C (night), natural light, relative humidity 60%) in September 2014 at Shenyang Agricultural University, China. The seedlings were watered daily. The tomato plants at the five-leaf stage were treated as follows.

The seedlings were subjected to three treatments: (1) H_2O+chilling (as control); (2) 1 mmol/L Put+chilling; and (3) 1 mmol/L Spd+chilling. In order to carry out chilling treatment, the seedlings were transferred to a phytotron. The environmental conditions were as follows: light irradiation of 600 μmol/(m^2·s) and temperature of 4 °C. Put and Spd treatments were carried out by spraying over the whole leaves of tomato seedlings (five-leaf-old), which were then

exposed to 25 °C (day)/15 °C (night) for 24 h before chilling treatment. Samples for physiological and biochemical analyses (including NO and H_2O_2 contents, NR activity, and NOS-like activity) were harvested at 0, 12, and 24 h after the treatment.

To further investigate whether Spd induced NO, before chilling treatment (4 °C) some seedlings were pretreated with 1 mmol/L methylglyoxal-bis(guanylhydrazone) (MGBG, an inhibitor of Spd synthesis), 200 μmol/L 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO, a scavenger of NO), or distilled water, and treated with Spd or distilled water 12 h later. Some seedlings with distilled water treatment at 4 °C served as the control. The leaves for NO analysis were harvested at 24 h after the treatments.

Also, to investigate the effect of Spd on the major enzymatic pathway of NO, some seedlings were treated with distilled water at 4 °C in a phytotron and used as the control. Other seedlings were treated with distilled water, 200 μmol/L N^G-nitro-L-arginine methyl ester (L-NAME, an inhibitor of NOS), or 200 μmol/L tungstate (an inhibitor of NR); the pretreatment was done from 6:00 p.m. to 6:00 a.m. for 3 d. After 12 h, the seedlings were sprayed with 1 mmol/L Spd, and then subjected to chilling stress for 24 h.

To investigate whether PAs induced NO via the production of H_2O_2, before chilling treatment these seedlings were treated with catalase (CAT, 100 U/ml; H_2O_2 scavenger) or distilled water and then sprayed with Put and Spd 12 h later, respectively. Some seedlings subjected to distilled water treatment at 4 °C served as the control. The leaves for NO analysis were harvested at 12 and 24 h after the treatments.

To investigate the effect of NO on F_v/F_m (maximum quantum efficiency of photosystem II (PSII), the ratio of variable fluorescence and maximum fluorescence) and electrolyte leakage, the tomato seedlings were sprayed with 200 μmol/L SNP (an NO donor) for 12 h daily for 3 d (the treatments were as described above), and then exposed to chilling stress for 24 h after a 12-h pretreatment. Other seedlings were pretreated with distilled water, 200 μmol/L L-NAME, 200 μmol/L tungstate, or 200 μmol/L PTIO. The pretreatment was done for 12 h daily for 3 d. After 12 h, the seedlings were treated with 1 mmol/L Spd, and then exposed to chilling stress for 24 h. The seedlings that were subjected to 25 or 4 °C for 24 h served as control and chilling control, respectively.

To investigate the effect of NO in the antioxidant system induced by Spd, the tomato seedlings were sprayed with distilled water, 200 μmol/L L-NAME, 200 μmol/L tungstate, or 200 μmol/L PTIO. After 12 h, the seedlings were treated with 1 mmol/L Spd and exposed to chilling stress for 24 h. The seedlings subjected to 25 or 4 °C for 24 h served as control and chilling control, respectively.

The third and fourth fully expanded leaves were sampled from 12 uniform tomato seedlings for each treatment. All leaf samples were repeatedly washed with distilled water, then frozen in liquid N_2, and stored at −80 °C for subsequent analysis.

2.2 Determination of electrolyte leakage

Electrolyte leakage was measured based on the method of Sairam and Srivastava (2002). Leaf samples (0.2 g) were rinsed three times with deionized water and placed in 20 ml distilled water at 25 °C for 3 h, and the initial electrical conductivity of the solution (E_1) was measured. Leaves were incubated at 100 °C for 30 min and cooled to room temperature, and then the final electrical conductivity (E_2) was measured. The relative electrolyte leakage was determined as E_1/E_2 and expressed as percent.

2.3 NO release determination

NO content was measured by the method of Murphy and Noack (1994) with slight modifications. Leaf samples (0.5 g) were placed in 100 U of CAT and 100 U of SOD for 5 min to remove endogenous ROS before adding 10 ml of 5 mmol/L oxyhemoglobin (HbO_2). After incubation, NO was determined by assaying the conversion of HbO_2 to methemoglobin (metHb), and the NO content was estimated by using the formula of $\varepsilon(A_{401\ (metHb)}-A_{421\ (HbO_2)})$, where ε is extinction coefficient of 77 ml/(mol·cm), and $A_{401\ (metHb)}$ and $A_{421\ (HbO_2)}$ are the absorbance of metHb at 401 nm and HbO_2 at 421 nm, respectively.

2.4 NO detection by confocal microscopy

NO detection was carried out according to Corpas *et al.* (2006) with small modifications, by binding to the cell-permeable, NO-sensitive fluorescent dye 3-amino, 4-aminomethyl-2',7'-difluorescein diacetate (DAF-FM DA, Beyotime). Epidermal fragments of tomato were incubated in 1 ml of 5 μmol/L DAF-FM DA (10 mmol/L Tris-HCl buffer, pH 7.2) at 25 °C for

20 min. After washing with fresh loading buffer three times, the fluorescence images of NO were observed with a Zeiss Axiovert 200 M inverted microscope equipped with a confocal laser scanner (Zeiss LSM 510). Excitation and emission were at 495 and 515 nm, respectively. The Zeiss 2012 software was used to analyze the images.

2.5 Assay of NR activity

NR activity was measured as described by Scheible *et al.* (1997) with slight modifications. Leaf samples (0.5 g) were homogenized in extraction buffer, including 100 mmol/L HEPES-KOH (pH 7.5), 5 mmol/L dithiothreitol, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.1% (1 g/L) Triton X-100, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 μmol/L leupeptin, 20 μmol/L flavin adenine dinucleotide, 5 μmol/L Na_2MoO_4, and 10 g/L polyvinylpolypyrrolidone (PVP). The homogenates were centrifuged at 4 °C and 10000*g* for 20 min, and then the resulted supernatant was used for NR analysis. The nitrite produced was determined by absorbance at 520 nm.

2.6 Assay of NOS-like activity

The NOS-like activity was measured with an NOS colorimetric assay kit (Nanjing Jiancheng Bioengineering Institute, China). Leaf samples (0.5 g) were homogenized with 2 ml of 50 mmol/L potassium phosphate buffer (pH 7.4, 1 mmol/L leupeptin, 1 mmol/L EDTA, 10 mmol/L ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 g/L PVP) and centrifuged at 15000*g* for 20 min; the supernatant was incubated in the assay reagent at 37 °C for 15 min, which was then terminated by a stop buffer. The absorbance was recorded at 530 nm.

2.7 Determination of H_2O_2 content

H_2O_2 content was quantified by the method of Patterson *et al.* (1984) with some modifications. Leaf samples (0.5 g) were homogenized with 3 ml ice-cold acetone. Titanium reagent (20% titanium tetrachloride in concentrated HCl) was added to extract the supernatant. An ammonia solution (0.2 ml at 17 mol/L) was added in Ti-H_2O_2, centrifuged at 4 °C and 30000*g* for 10 min, and the supernatant was discarded. The pellet was washed three times with ice-cold acetone, then drained, and dissolved in 3 ml 1 mol/L H_2SO_4. The absorbance of the solution was measured at 410 nm.

2.8 Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured with a Dual-PAM 100 chlorophyll fluorometer (Walz, Effeltrich, Germany) at room temperature according to the method of Song *et al.* (2015).

2.9 Determination of antioxidative enzyme activity

For the extraction of antioxidative enzymes, leaf samples (0.5 g) were homogenized with 50 mmol/L Na_2HPO_4-NaH_2PO_4 buffer (pH 7.8) including 0.2 mmol/L EDTA and 20 g/L PVP, and then centrifuged for 20 min at 12000*g*, and the resulting supernatant was used for the assay of enzyme activity. All operations were carried out at the temperature of 0–4 °C. All spectrophotometric analyses were conducted on a UV-vis spectrophotometer (UV-2401, Shimadzu Co., Ltd., Japan).

SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), following the method of Giannopolitis and Ries (1977). POD activity was measured as described by Thomas *et al.* (1982). The reaction mixture contained 3 ml of phosphate buffer (pH 7.0), 1.0 ml of 0.18% H_2O_2, 1.0 ml of enzyme extract, and 1.0 ml of 0.1% guaiacol. CAT activity was assayed by the method of Cakmak and Marschner (1992). The decomposition of H_2O_2 was observed as a decrease in absorbance at 240 nm. APX activity was measured following the description of Nakano and Asada (1981) by measuring the rate of ascorbate oxidation at 290 nm.

In all enzyme preparations, protein concentration was determined according to the method of Bradford (1976); bovine serum albumin (BSA; Sigma) was used as standard.

2.10 Total RNA extraction and gene expression analysis

Total RNA was extracted with the RNAprep pure plant total RNA extraction kit (Kangwei, Beijing, China). The complementary DNA (cDNA) synthesis was carried out according to the manufacturer's instructions (Tiangen, China). Primer 3.0 was used for primer design. The polymerase chain reaction (PCR) primer sequences are listed in Table 1. Real-time PCR

Table 1 Accession numbers and primer sequences of the analyzed genes in this study

Category	Accession No.	Encode corresponding enzyme	Primer sequence
Cu/Zn-SOD	AF034411.1	SOD	F: 5'-ACCACAACCAGCACTACCAAT-3' R: 5'-GTCCAGGAGCAAGTCCAGTTA-3'
cat1	M93719.1	CAT	F: 5'-GATGAGCACACTTTGGAGCA-3' R: 5'-TGCCCTTCTATTGTGGTTCC-3'
APX	NM_001247702	APX	F: 5'-CCACTTGAGGGACGTGTTTG-3' R: 5'-CCACTTGAGGGACGTGTTTG-3'
cevi16	NM_001247041.2	POD	F: 5'-ACAGCTCCTCCGAATTCCAA-3' R: 5'-GGAATCACGAGCAGCAAGAG-3'
TPX1	NM_001247242.1	POD	F: 5'-GAGATGCAGTTGTGGCTACG-3' R: 5'-GCGAAGGATTGTTGCAGTCT-3'
TPX2	NM_001247715.2	POD	F: 5'-AGCGGGTTCTAGCTATGGTC-3' R: 5'-AAGAGATGGAGCGTTTGGGA-3'
Actin	Q96483	Reference gene	F: 5'-TGTCCCTATTTACGAGGGTTATGC-3' R: 5'-AGTTAAATCACGACCAGCAAGAT-3'

F: forward; R: reverse

analysis was performed on ABI 7500 (Applied Biosystems, USA) by using the SYBR Green PCR Real Master Mix (Tiangen, China). The $2^{-\Delta\Delta C_T}$ method was used to measure relative expression of gene, and the threshold cycle (C_T) value was normalized to *actin*.

2.11 Statistical analysis

Two independent experiments were performed with three replicates in each treatment. Data used Duncan's multiple range tests at the 0.05 level of significance. The charts were made by using Origin 8.0.

3 Results and discussion

3.1 Exogenous Spd-induced NO production in tomato leaves under chilling stress

NO as a signaling molecule in plant was found as late as 1998 (Delledonne *et al*., 1998). A growing number of studies have indicated that NO is involved in plant's stress response (Siddiqui *et al*., 2011). PAs have also been known to increase NO generation. However, the actual reaction mechanism has not been solved. Yamasaki and Cohen (2006) have indicated that PAs can induce NO generation through an uncertain pathway. Here, we describe the correlation of NO production with PAs in tomato leaves under chilling stress.

Under chilling stress, exogenous Spd treatment greatly increased the NO content at 12 and 24 h compared to control. However, compared to control, there was no change in the NO content with Put treatment (Figs. 1a and 1b). To further confirm whether Spd had this effect, we analyzed the levels of NO with MGBG and PTIO pretreatments (Bais and Ravishankar, 2002; He *et al.*, 2002; Arasimowicz-Jelonek *et al.*, 2009; Gong *et al.*, 2014) before Spd application. Both MGBG and PTIO reduced the NO content induced by Spd under chilling stress (Figs. 1c and 1d). Hence, we concluded that Spd could induce NO production in tomato leaves under chilling stress. In accordance with our results, Tun *et al.* (2006) reported a correlation between PAs and NO biosynthesis in *Arabidopsis* seedlings, in which Spd induced NO production, whereas Put had very little effect. However, Silveira *et al.* (2006) reported that Put enhanced NO production in the embryogenic culture of *Araucaria angustifolia.* Therefore, the effect of PAs on NO may depend on the species, types, and stress conditions.

3.2 NOS and NR pathways involved in Spd-induced NO synthesis in tomato leaves under chilling stress

In plants, NOS-like and NR enzymes have been suggested to be the two major sources of NO accumulation (Guan *et al.*, 2014). Since Ninnemann and Maier (1996) first identified the existence of NOS-like enzyme activity in plants, a growing number of studies have indicated that NOS-like activity is detectable in different plant species (del Río *et al.*, 2004). NOS-like enzyme could produce NO through the oxidation of L-arginine (Guo *et al.*, 2003; Neill *et al.*, 2003). With the exception of NOS-like enzyme, the well documented route for NO in plants is the NR pathway, which is located in the cytosol and catalyzes the reduction of nitrate to nitrite by NADH (Gupta *et al*., 2011). In our study, the NOS-like and NR activities were both increased by Spd treatment,

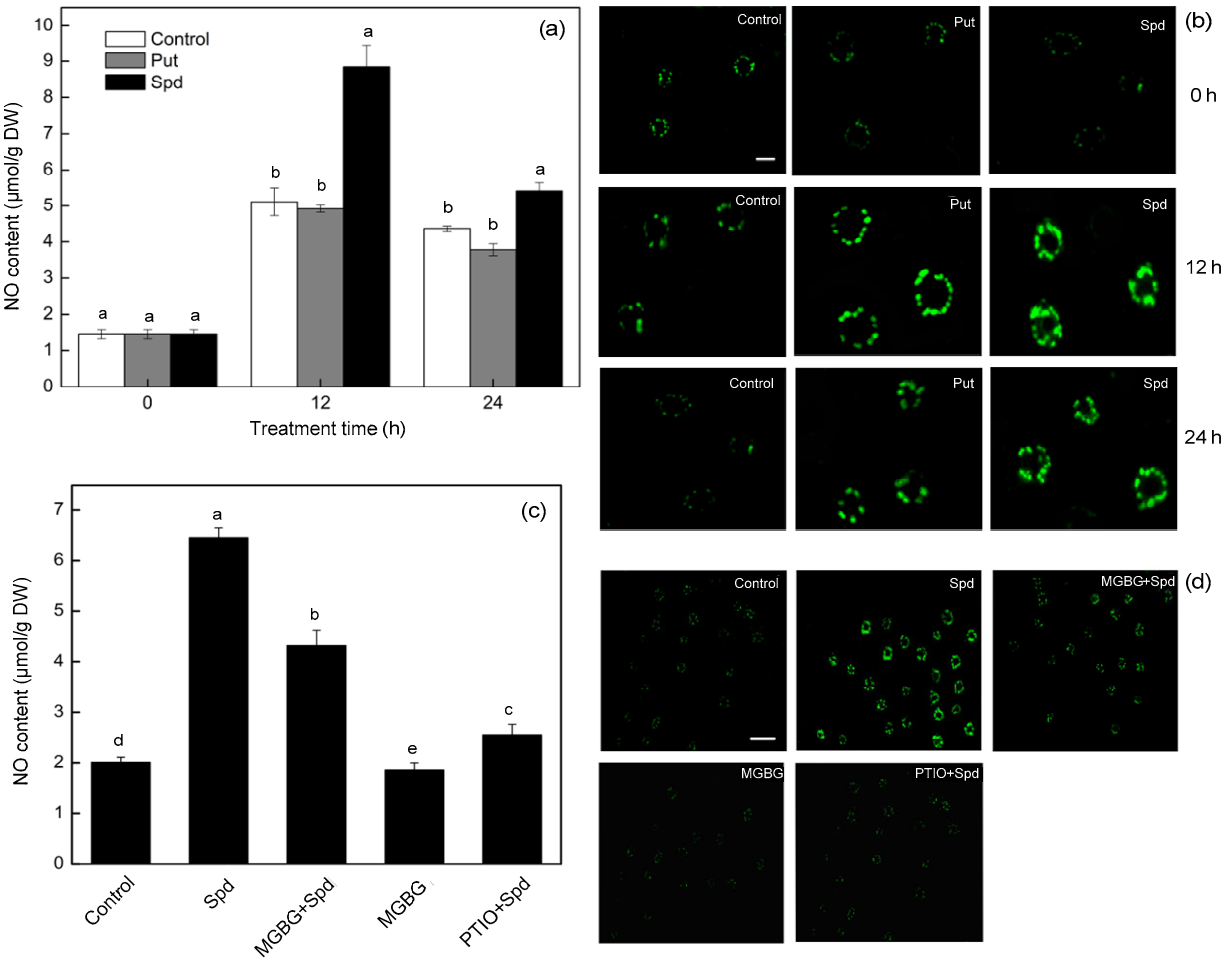

Fig. 1 NO accumulation induced by PAs

(a) Tomato seedlings were applied with PAs (1 mmol/L Put and 1 mmol/L Spd) or distilled water (control). The samples were harvested for analysis of NO content during chilling stress (4 °C; 0, 12, and 24 h). (b) Fluorescence images of NO in tomato leaves by the NO-selective fluorochrome DAF-FM DA. Scale bar for NO accumulation represents 25 μm. (c) To further investigate the effect of Spd on NO content, some seedlings were pretreated with 1 mmol/L MGBG (an inhibitor of Spd synthesis) 12 h before chilling treatment; other seedlings were pretreated with 1 mmol/L MGBG, 200 μmol/L PTIO (a scavenger of NO), or distilled water, and then sprayed with Spd 12 h later. The seedling leaves for NO analysis with various treatments were harvested at 24 h under chilling stress. (d) Fluorescence images of NO in tomato leaves by the NO-selective fluorochrome DAF-FM DA. Scale bar for NO accumulation represents 60 μm. Data are expressed as mean±standard error (SE), with n=3. Different letters denote significant differences at $P \leq 0.05$ according to Duncan's multiple range tests

compared to control. However, the application of Put did not have this effect (Figs. 2a and 2b). Rosales *et al.* (2012) found that PAs can regulate the combination of 14-3-3 proteins with the H^+-ATPase, thereby inducing NR activity. Tanou *et al.* (2014) indicated that Spm can increase the relative expression of *leNR* in citrus under salinity stress. In addition, compared to control, treatment with Spd increased transcript levels of *leNR* in tomato, but exogenous Put did not alter *leNR* expression under chilling stress. Application of Put and Spd reduced *leNOS1* relative expression compared to control (Fig. S1 and Table S1). The *leNOS1* expression was inconsistent with NOS-like activity, most probably due to an uncertain gene, but in most cases the genes were predicted. Therefore, it still needs to be clarified whether they did encode relative genes or affect enzyme synthesis. Furthermore, we also examined the contents of NO in tomato leaves after treatment with tungstate, an inhibitor of NR, and L-NAME, an inhibitor of NOS (Besson-Bard *et al.*, 2009; Xiong *et al.*, 2012; Alemayehu *et al.*, 2015; Sun *et al.*, 2015). In this experiment, the two inhibitors

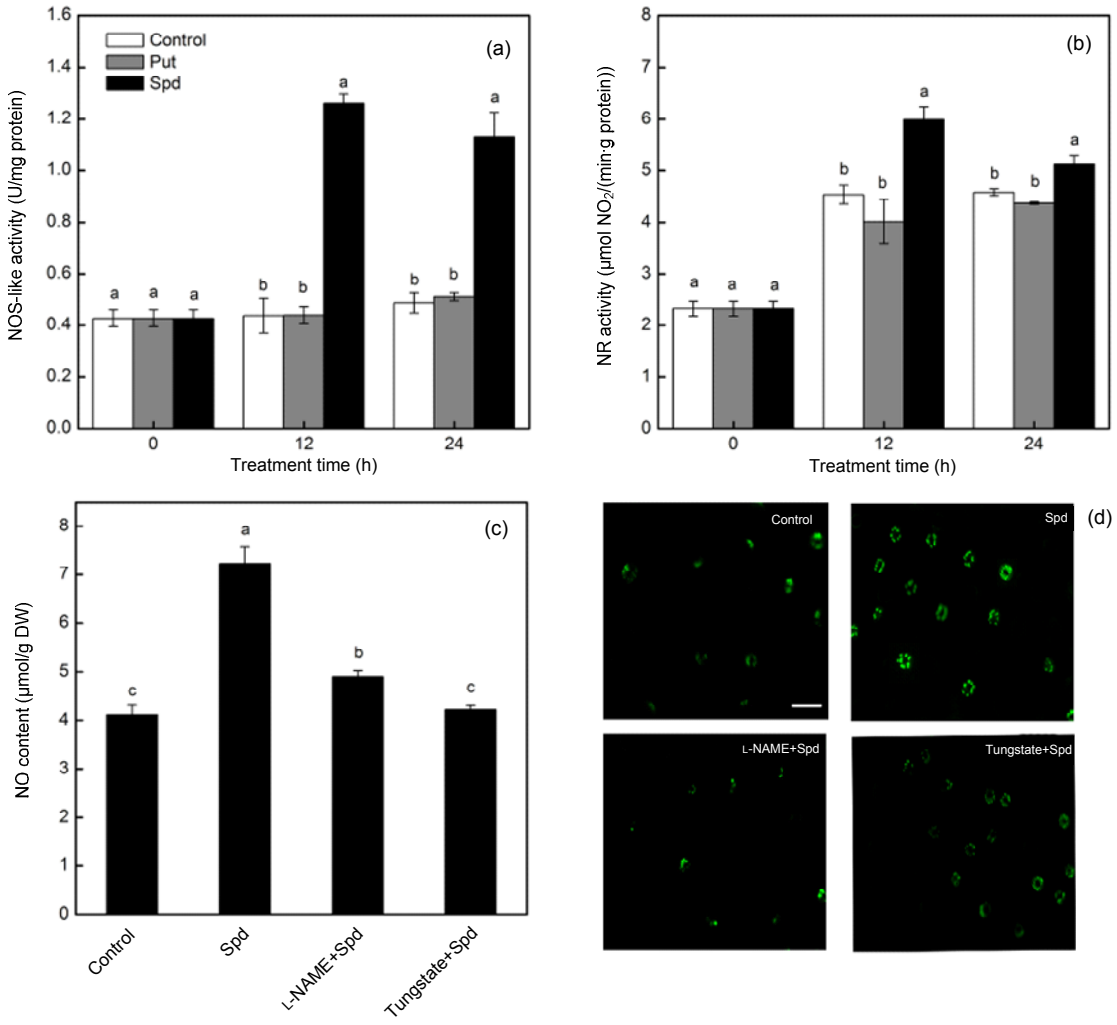

Fig. 2 Involvement of NOS-like and NR in PA-induced NO generation

(a) Tomato seedlings were treated with PAs (1 mmol/L Put and 1 mmol/L Spd) and distilled water (control). The samples were harvested for analysis of NOS-like activity during chilling stress (4 °C; 0, 12, and 24 h). (b) Analysis of NR activity during chilling stress. (c) Some seedlings with distilled water treatment at 4 °C were used as control. Other seedlings were pretreated with distilled water, 200 μmol/L L-NAME (an inhibitor of NOS), or 200 μmol/L tungstate (an inhibitor of NR). After 12 h, the seedlings were treated with 1 mmol/L Spd, and then exposed to chilling stress at 4 °C for 24 h. The seedlings' leaves for NO analysis were harvested at 24 h during chilling stress. (d) Fluorescence images of NO in tomato leaves by the NO selective fluorochrome DAF-FM DA. Scale bar for NO accumulation represents 60 μm. Data are expressed as mean±SE, with n=3. Different letters denote significant difference at $P \leq 0.05$ according to Duncan's multiple range tests

both abolished the effect of Spd on the NO content (Figs. 2c and 2d). Arasimowicz-Jelonek *et al.* (2009) indicated that the NOS-like and NR pathways are associated with PA-induced NO generation in cucumber leaves during drought stress. The present study suggests that Spd induced NO production through both the NOS-like and NR pathways in tomato leaves under chilling stress.

PAs can enhance H_2O_2 production via Put, Spd, or Spm catabolism (Su *et al.*, 2006; Alcázar *et al.*, 2010; Wimalasekera *et al.*, 2011; Moschou *et al.*, 2012; Pál *et al.*, 2015). H_2O_2 plays a dual role in plants: at low concentrations, it serves as a signal molecule, playing a pivotal role in signal transduction network under various stress conditions (Tanou *et al.*, 2009; Jiang *et al.*, 2012; Lizárraga-Paulín *et al.*, 2013);

at high concentrations, it can lead to extensive cell injury or death (Quan *et al.*, 2008). H_2O_2 and NO are two types of signaling molecules, the generation of which often occurs in short succession or in parallel, and they can act both synergistically and independently (Bright *et al.*, 2006; Pasqualini *et al.*, 2009). To determine whether PAs increase NO production by inducing H_2O_2 under chilling stress, we studied the effect of PAs on the H_2O_2 content under chilling stress and the NO content in tomato leaves pretreated with CAT (an H_2O_2 scavenger) before the application of PAs. As shown in Fig. 3a, exogenous Spd resulted in an increased H_2O_2 content in tomato leaves compared to control. However, compared to control, there was no obvious change in H_2O_2 production with Put treatment. In accordance with our results, Iannone *et al.* (2013) indicated that Spd and Spm increased H_2O_2 content by enhancing PAO activity in tobacco tissues, whereas Put had little effect on H_2O_2 formation. Moschou *et al.* (2008) and Yoda *et al.* (2003) have suggested that Spd is catabolized by PAO to produce H_2O_2. The results reported here suggest that H_2O_2 can be generated via PA catabolic pathways induced by Spd treatment. However, the application of Put has no such effect. NO production induced by Spd was markedly reduced by CAT, but CAT did not affect the NO production with Put treatment (Fig. 3b). It was observed that H_2O_2 can activate calcium channels (Pei *et al*., 2000; Kwak *et al.*, 2003); in turn, calcium transients could induce NO accumulation (Besson-Bard *et al.*, 2008; Courtois *et al*., 2008). Taken together, our results suggest that H_2O_2 acts upstream of NO to enhance its production in tomato. Some studies have also shown that H_2O_2 can induce NO production (Bright *et al.*, 2006; Zhang *et al.*, 2007). However, opposite results were reported in other studies.

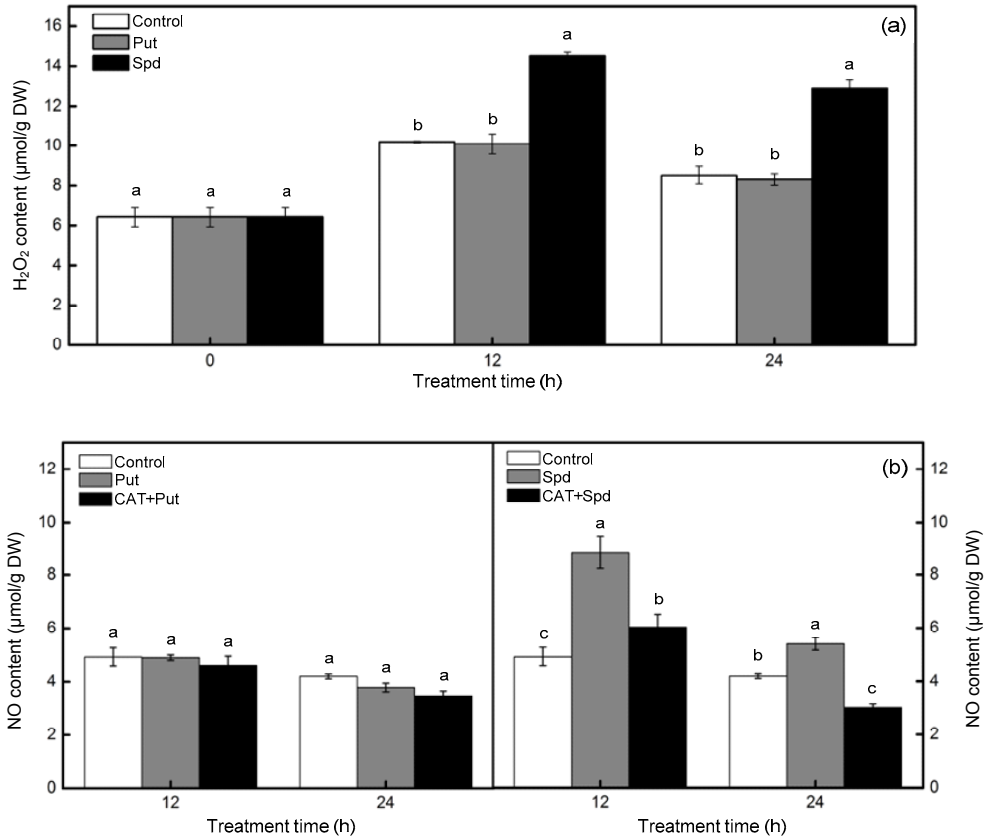

Fig. 3 Involvement of H_2O_2 in PA-induced NO generation

(a) Tomato seedlings were treated with PAs (1 mmol/L Put and 1 mmol/L Spd) and distilled water (control). The samples were harvested for analysis of H_2O_2 content during chilling stress (4 °C; 0, 12, and 24 h). (b) To further investigate the effect of Spd on NO content, some seedlings were treated with CAT (100 U/ml, H_2O_2 scavenger), and then sprayed with Put and Spd 12 h later, respectively. The seedlings' leaves for NO analysis were harvested at 12 and 24 h during chilling stress. Data are expressed as mean±SE, with n=3. Different letters denote significant difference at $P \leq 0.05$ according to Duncan's multiple range tests

In tobacco leaves, NO treatment caused rapid H_2O_2 accumulation, but H_2O_2 treatment had no effect on NO generation (Pasqualini *et al.*, 2009). This emphasizes that the linkage between NO and H_2O_2 in plants is a complicated issue to elucidate, due to the differences found in different species, the types of stress, or the experimental conditions used. Recent reports have indicated that exogenous NO induces PA generation (Fan *et al.*, 2013; Li *et al.*, 2014). As observed by Filippou *et al.* (2013), SNP treatment led to an enhancement in Put levels in *Medicago truncatula* plants. Similarly, in our previous studies, applying SNP increased the Put and Spm contents in tomato seedlings under chilling stress (data not shown). Hence, there may be a potential link between PAs and NO under environmental stresses.

3.3 NO involved in Spd-induced chilling tolerance in tomato

We have shown that Spd can induce increased production of NO; however, whether the NO induced by Spd is involved in Spd-enhanced stress tolerance remains unclear. The diamine Put protects against cell death and membrane damage; however, the higher PAs, Spd and Spm, are documented to be detrimental to cell viability, relying on the concentration and exposure time (Iannone *et al.*, 2013). In our study, we found that the increase in the F_v/F_m of tomatoes treated with Spd was reduced by NO synthesis inhibitors and scavengers, but SNP (NO donor) pretreatment could increase F_v/F_m compared to control (Fig. 4). It is well known that the chlorophyll fluorescence parameter is used to detect and quantify chilling stress by means of changes induced in PSII. F_v/F_m, as a kind of chlorophyll fluorescence parameter, can be used as an important indicator of injury to PSII (Rizza *et al.*, 2001; Lu *et al.*, 2003; Tambussi *et al.*, 2004; Baker, 2008; Liu *et al.*, 2013; Zhou *et al.*, 2015). Additionally, the present study showed that the application of Spd and SNP significantly decreased chilling-induced electrolyte leakage, compared to the chilling treatment alone. NO synthesis inhibitors and the scavenger both decreased the function of Spd (Fig. 5). These results showed that Spd can enhance chilling tolerance by inducing NO accumulation in tomato leaves. However, according to Groppa *et al.* (2008), NO induced by Spm is involved in wheat root growth inhibition. These contrasting results concerning whether PAs induce NO production or whether NO is induced by the physiological effects of PAs can be attributed to the use of different species, plant parts, and conditions.

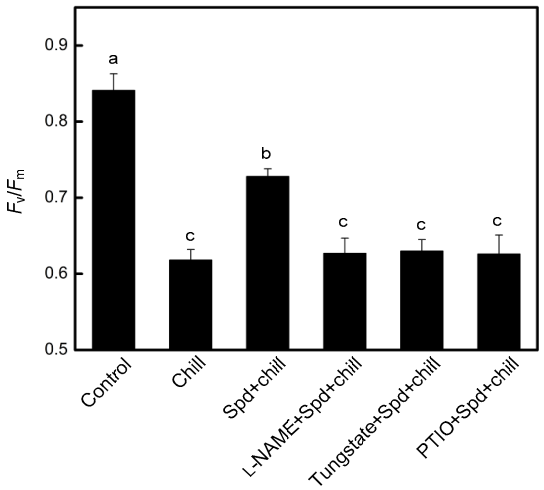

Fig. 4 F_v/F_m of different treatments in tomato leaves during chilling stress

Some seedlings were treated with 200 μmol/L SNP, and after 12 h exposed to chilling stress at 4 °C for 24 h. Other seedlings were treated with distilled water, 200 μmol/L L-NAME, 200 μmol/L tungstate, or 200 μmol/L PTIO. After 12 h, the seedlings were sprayed with 1 mmol/L Spd, and then exposed to chilling stress at 4 °C for 24 h. The seedlings subjected to 25 or 4 °C for 24 h in phytotron were used as control and chilling treatment, respectively. F_v/F_m was measured with a Dual-PAM 100 chlorophyll fluorometer at 24 h during chilling stress. Data are expressed as mean±SE, with n=3. Different letters denote significant difference at $P \leq 0.05$ according to Duncan's multiple range tests

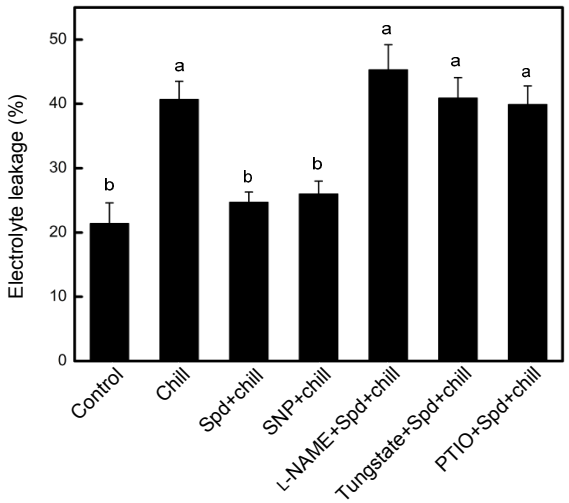

Fig. 5 Electrolyte leakage of different treatments in tomato leaves under chilling stress

The treatment details are as in the Fig. 4. Data are expressed as mean±SE, with n=3. Different letters denote significant difference at $P \leq 0.05$ according to Duncan's multiple range tests

We also further investigated the effects of NO inhibitor or scavenger treatment on the transcript levels and activities of antioxidant enzymes, including SOD, POD, CAT, and APX. In the present study, chilling treatment reduced both the activities and gene expressions of antioxidant enzymes, compared to those obtained with control. Exogenously applied Spd increased the transcript levels of antioxidant enzymes, as well as the activities of their relevant antioxidant enzymes, compared to chilling treatment. The increases in both transcripts and activities were suppressed by NO scavengers or inhibitors of NO biosynthesis (Figs. 6 and 7). NO has been suggested to increase the activities of antioxidant enzymes and up-regulate the expressions of the antioxidant genes in plants (Zhou *et al*., 2005; Zhang *et al.*, 2007). It is well known that the antioxidant defense system plays vital roles in plants' tolerance to stressful conditions (Guan *et al.*, 2009; Gill and Tuteja, 2010). Therefore, Spd could improve the chilling tolerance in tomato via the antioxidant system that is activated by NO. Moreover, previous studies have demonstrated that PAs could enhance the expressions and activities of antioxidant enzymes (Wi *et al.*, 2006; Hussain *et al*., 2011). This phenomenon is generally attributed to their multifaceted nature (Velikova *et al.*, 2000). Thus, it is concluded that Spd, as a kind of PA, can enhance the expressions and activities of antioxidant enzymes by inducing NO production in tomato.

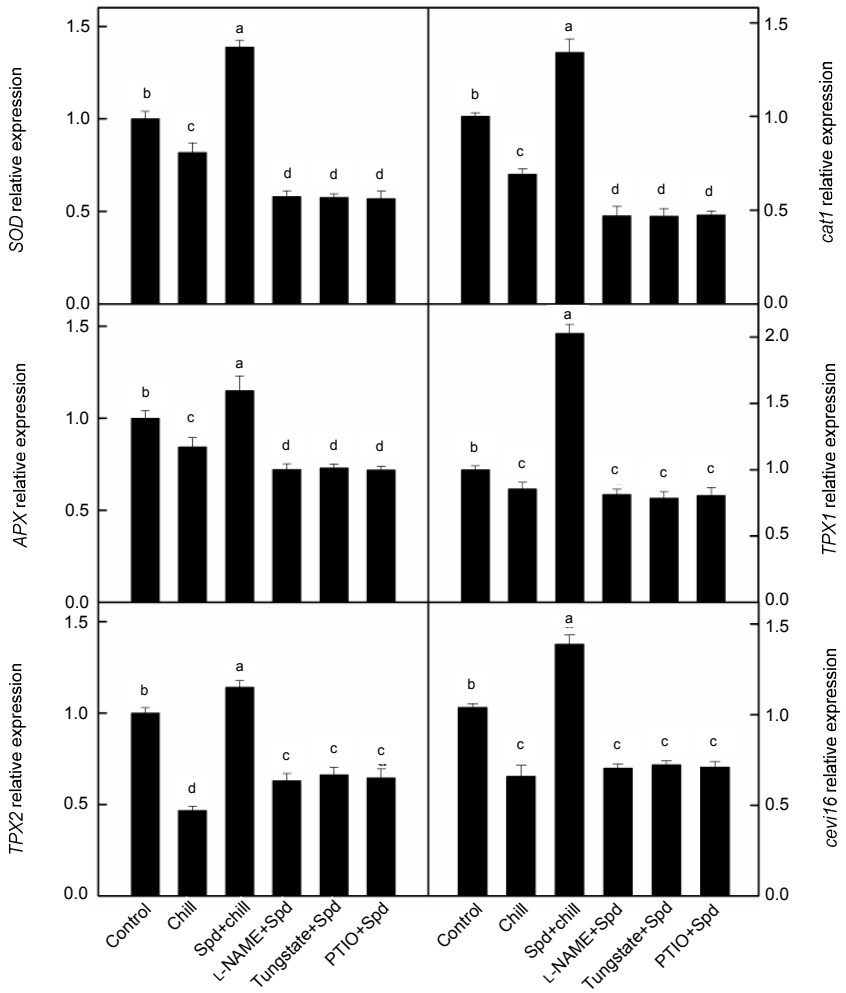

Fig. 6 Involvement of NO in Spd-induced expression of antioxidant genes in tomato leaves during chilling stress
The tomato seedlings were pretreated with distilled water, 200 μmol/L L-NAME, 200 μmol/L tungstate, or 200 μmol/L PTIO. After 12 h, the seedlings were applied with 1 mmol/L Spd and exposed to chilling stress at 4 °C for 24 h. The seedlings subjected to 25 or 4 °C for 24 h were used as control and chilling treatment, respectively. The seedlings' leaves for antioxidant genes with various treatments were harvested at 24 h during chilling stress. Data are expressed as mean±SE, with n=3. Different letters denote significant difference at $P \leq 0.05$ according to Duncan's multiple range tests

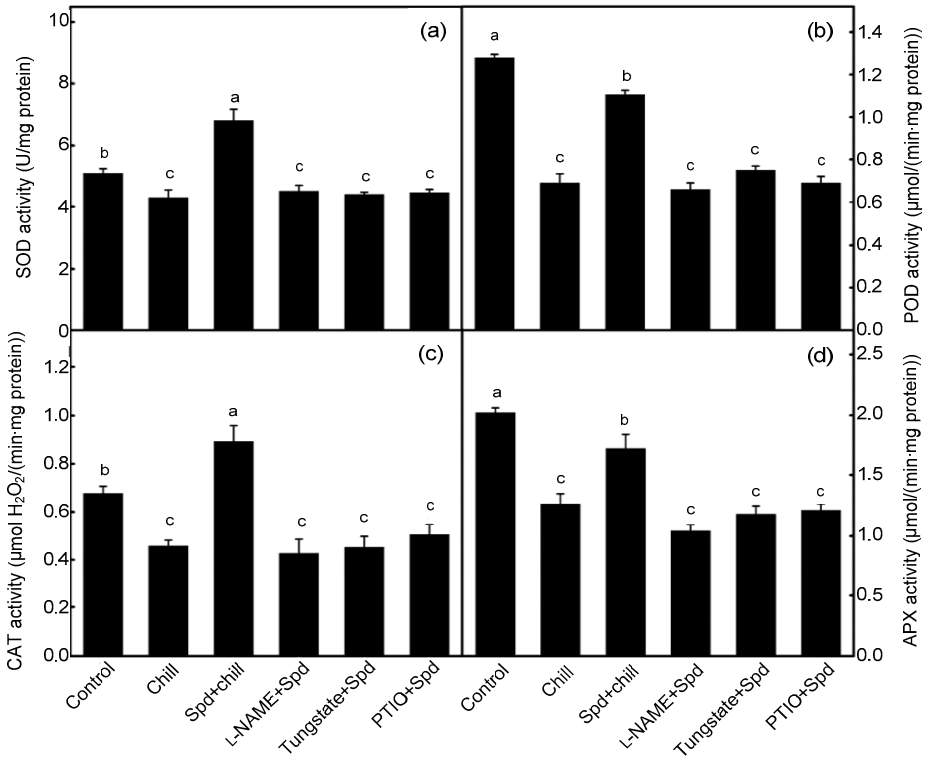

Fig. 7 Involvement of NO in Spd-induced antioxidant enzyme activity in tomato leaves under chilling stress
The treatment details are as in the Fig. 6. Data are expressed as mean±SE, with n=3. Different letters denote significant difference at $P \leq 0.05$ according to Duncan's multiple range tests

4 Conclusions

Based on our results, we suggest that Spd induces NO production directly through enhancing both NOS-like and NR activities or indirectly through inducing H_2O_2, which acts upstream of NO synthesis in tomato leaves under chilling stress. However, Put does not show such an effect. Moreover, NO participates in Spd-induced chilling tolerance in tomato, most probably via regulating the induction of antioxidant genes and enhancing the antioxidant activities.

Compliance with ethics guidelines

Qian-nan DIAO, Yong-jun SONG, Dong-mei SHI, and Hong-yan QI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Fig. S1 Effects of exogenous Put and Spd on *leNR* and *leNOS1* relative expression in the leaves of tomato under chilling stress

Table S1 Gene accession numbers and primer sequences of tomato *NR* and *NOS1* in this study

中文概要

题　目：多胺诱导产生的一氧化氮通过影响番茄幼苗抗氧化系统抵御低温胁迫

目　的：研究多胺（PA）对低温胁迫下番茄幼苗中一氧化氮（NO）产生的影响，并探讨 NO 在 PA 诱导的耐冷性中发挥的作用。

创新点：在番茄幼苗中证明亚精胺（Spd）对 NO 产生的影响及可能的作用途径，且此作用与番茄耐低温性有密切关系。

方　法：通过检测氧合血红蛋白（HbO_2）向高铁血红蛋白（metHb）的转化进行 NO 含量测定；通过与 NO 特异性荧光探针（DAF-FM DA）结合检测 NO 释放量（图 1 和 2）。超氧化物歧化酶（SOD）活性根据其抑制氮蓝四唑（NBT）在光下的还原作用测定；过氧化物酶（POD）活性通过测定酶提取液与愈创木酚、过氧化氢（H_2O_2）的混合物的吸光度确定；过氧化氢酶（CAT）活性根据 H_2O_2 在 240 nm 波长下的降解能力来测定；抗坏血酸过氧化物酶（APX）活性的测定参照 Nakano 和 Asada（1981）的方法在波长 290 nm 下测定（图 6 和 7）。

结　论：本研究的结果显示，Spd 诱导番茄叶片中 NO 的产生可直接通过增加一氧化氮合酶（NOS）和硝酸还原酶（NR）的活性实现（图 2）。H_2O_2 作为上游信号能够刺激 NO 的生成（图 3）。NO 通过增加抗氧化酶活性和相关基因的表达来参与 Spd 诱导的番茄耐冷性（图 6 和 7）。综上所述，Spd 诱导产生的 NO 在番茄响应低温胁迫中发挥重要作用。

关键词：番茄；亚精胺；耐冷性；一氧化氮；抗氧化酶