



RESEARCH PAPER

Nitric oxide is involved in abscisic acid-induced antioxidant activities in *Stylosanthes guianensis*

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Abstract

Previous studies suggest that abscisic acid (ABA) stimulates the activities of antioxidant enzymes under normal and chilling temperature and enhanced chilling resistance in *Stylosanthes guianensis*. The objective of this study was to test whether nitric oxide (NO) is involved in the ABA-induced activities of the antioxidant enzymes in *Stylosanthes guianensis* due to its nature as a second messenger in stress responses. Plants were treated with NO donors, ABA, ABA in combination with NO scavengers or the nitric oxide synthase (NOS) inhibitor and their effects on the activity of antioxidant enzymes and NO production were compared. The results showed that ABA increased the activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). The effect of ABA on antioxidant enzyme activities was suppressed by the NOS inhibitor, N^ω-nitro-L-arginine (L-NNA), and the NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl3-oxide (PTIO). NO content increased after 5 h of ABA treatment. The NO-scavenger, PTIO, and the NOS-inhibitor, L-NNA, inhibited the accumulation of NO in ABA-treated *Stylosanthes guianensis*. NO donor treatment enhanced the activities of SOD, CAT, and APX. The results suggested that NO was involved in the ABA-induced activities of SOD, CAT, and APX in *Stylosanthes guianensis*. ABA triggered NO production that may lead to the stimulation of antioxidant enzyme activities.

Key words: Abscisic acid, antioxidant enzyme, nitric oxide, *Stylosanthes guianensis*.

Introduction

Accumulating evidence suggests that nitric oxide (NO) is an important signal molecule involved in the plant response to biotic and abiotic stresses (Delledonne *et al.*, 1998; García-Mata and Lamattina, 2001; Uchida *et al.*, 2002). Tobacco mosaic virus infection of resistant tobacco (*Nicotiana tabacum*) and bacterial infection of soybean (*Glycine max*) induced the rapid production of NO. The NO donor induced the expression of disease-defence genes (Delledonne *et al.*, 1998; Durner *et al.*, 1998; Wendehenne *et al.*, 2004). NO production increased in tobacco plants in response to abiotic stresses such as salinity, hyperosmotic stress, and high temperature (Gould *et al.*, 2003). NO produced in plants at low concentration may rapidly eliminate lipid peroxyl radicals, and alter the species and component of reactive oxygen species (ROS), block the injury from ROS, induce the expression of antioxidant genes, and the activity of antioxidant enzymes (Cheng *et al.*, 2002; De Pinto *et al.*, 2002; Huang *et al.*, 2002; Lamattina *et al.*, 2003), and protect plants from abiotic stress (Neill *et al.*, 2003). Tolerance to drought, salt, and heat stress is enhanced in wheat (*Triticum aestivum*) and rice (*Oryza sativa*) seedlings when the plants were treated with the NO donor, sodium nitroprusside (SNP) (García-Mata and Lamattina, 2001, 2002; Uchida *et al.*, 2002). SNP pretreatment increased the resistance of potato (*Solanum tuberosum*) and tobacco to methylviologen- and bipyridinium-induced oxidative stress and to the infection by *Phytophthora infestans* (Beligni and Lamattina, 1999, 2002).

NO is also involved in plant hormone signalling pathways (Neill *et al.*, 2003). ABA induced significant NO production in guard cells. cGMP and cyclic ADP-ribose

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(cADPR) mediate the NO effect on stomatal closure (Neill *et al.*, 2002a; Wendehenne *et al.*, 2004). Two pathways have been proposed to be responsible for ABA-induced NO production in guard cells. Nitrate reductase (NR) is required for ABA-induced NO generation (Desikan *et al.*, 2002; García-Mata and Lamattina, 2002). Either ABA or nitrite failed to induce stomatal closure in the epidermal peels of NR-deficient mutants of *Arabidopsis thaliana* (Desikan *et al.*, 2002). Guo *et al.* (2003) reported that a NOS-like enzyme, AtNOS1, is also required for ABA-triggered NO synthesis and stomatal closure in *Arabidopsis*. Auxin-induced adventitious root development is also dependent on NO. IAA treatment induces a transient increase in endogenous NO level in the basal region of hypocotyls where new adventitious roots are developed (Pagnussat *et al.*, 2002).

Abcisic acid plays important roles in plant tolerance to stresses (Davies and Zhang, 1991). Among the mechanisms is the involvement of antioxidative system. ABA treatments enhanced the activities of the antioxidant enzymes in various species such as maize (*Zea mays*) (Gong *et al.*, 1998; Jiang and Zhang, 2001) and rice (Lin *et al.*, 2001). Previous studies showed that ABA increased the activities of antioxidant enzymes in bermudagrass and a forage legume, *Stylosanthes guianensis*, and enhanced the drought resistance of bermudagrass and the chilling resistance in *Stylosanthes guianensis* (Lu *et al.*, 2003; Zhou *et al.*, 2005). Ca^{2+} and H_2O_2 were demonstrated to be signal molecules involved in the ABA-induced activities of antioxidant enzyme (Gong *et al.*, 1998; Jiang and Zhang, 2002). H_2O_2 and NO are signal molecules involved in the responses to pathogen-induced hypersensitivities, abiotic stress, programmed cell death, and ABA-induced stomata closure (Delledonne *et al.*, 1998; Durner *et al.*, 1998; Neill *et al.*, 2002; Lamattina *et al.*, 2003). It is unknown, however, whether NO is involved in controlling ABA-induced antioxidant enzymes in plants.

The objective of this work was to investigate the relationship between NO and ABA-induced activities of antioxidant enzymes in *Stylosanthes guianensis*. Plants were treated with ABA, the nitric oxide synthase (NOS) inhibitor, N^{ω} -nitro-L-arginine (L-NNA), and the NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (PTIO). Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) activity, and NO content were determined in *S. guianensis* plants treated with ABA alone or in combination with the NO scavenger or the NO synthase inhibitor.

Materials and methods

Plant material

Seeds of *Stylosanthes guianensis* (cv. CIAT184) were sown in 15 cm diameter plastic pots containing a mixture of peat and perlite (3:1, v/v) for germination. Seedlings were grown under natural light in a greenhouse with the temperature from 25–30 °C from March to

June, 2004. Plants were irrigated daily and fertilized once a week by irrigating with 0.3% N-P-K fertilizer (15-15-15 by vol.) solution. Eight-week-old plants of similar size were used for the study.

Experimental procedures

Shoots (stems and leaves) that were 6 cm long were cut off a plant. To investigate the role of NO on the ABA-induced antioxidant enzymes, detached shoots were immediately placed in different solutions containing the following chemicals: (1) water (control); (2) 37.9 μM S-ABA (90% powder, Lomon Bio Technology Co, Ltd., Sichuan Province, China); (3) 37.9 μM ABA+400 μM PTIO (Sigma); (4) 400 μM PTIO; (5) 37.9 μM ABA+200 μM L-NNA (Sigma); and (6) 200 μM L-NNA. To investigate the effect of the NO donor on the antioxidant enzymes in *Stylosanthes guianensis*, the cut-end of detached shoots was placed in solutions containing the following chemicals: (1) water (control); (2) 200 μM of sodium nitroprusside (SNP) (Shanghai Chemical Reagent Co, Ltd., Shanghai, China); (3) 200 μM SNP+400 μM PTIO; (4) 100 μM ascorbic acid (AsA)+200 μM NaNO_2 ; and (5) 100 μM AsA+200 μM NaNO_2 +400 μM PTIO. All the treated shoot cuttings were placed in a growth chamber at 160 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density at 28 °C for 36 h. Treatments were arranged in a completely randomized design with three or four replications. The second fully expanded leaflets were selected for measurements. All data were subjected to analysis of variances according to the model for a completely randomized design using a SPSS program (SPSS Inc.). The differences among treatment means were evaluated by Duncan's multiple range test at the 0.05 probability level.

Antioxidant enzyme assay

Fresh leaves (0.5 g) were ground in a mortar and pestle in 5 ml of 50 mM cool phosphate buffer (pH 7.8), containing 2% (w/v) polyvinylpyrrolidone (PVP). The homogenates were centrifuged at 13 000 g for 20 min at 4 °C. The supernatants were used for assays of enzyme activity.

Superoxide dismutase activity was determined according to the method of Giannopolitis and Ries (1977). The 3 ml reaction solution contained 13 μM methionine, 63 μM ρ -nitro blue tetrazolium chloride (NBT), 1.3 μM riboflavin, 50 mM phosphate buffer (pH 7.8), and enzyme extract. The reaction solution was incubated for 10 min under fluorescent light with 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Absorbance was determined at 560 nm with a spectrophotometer (Model UV-2010, Hitachi, Japan). One unit of SOD activity was defined as the amount of enzyme required for the inhibition of the photochemical reduction of NBT by 50%.

Catalase activity was determined spectrophotometrically by following the decrease of absorbance of H_2O_2 (extinction coefficient 0.0394 $\text{mM}^{-1} \text{cm}^{-1}$) within 1 min at 240 nm according to the method of Aebi (1984). The 3 ml reaction solution contained 15 mM H_2O_2 , 50 mM phosphate buffer (pH 7.0), and 50 μl of enzyme extract. The reaction was initiated by adding enzyme extract.

Ascorbate peroxidase activity was determined spectrophotometrically according to the method of Nakano and Asada (1981). Leaves (0.3 g) were ground in a mortar and pestle in 3 ml of 50 mM cool phosphate buffer (pH 7.0), containing 1 mM AsA, 1 mM EDTA. The homogenates were centrifuged at 13 000 g for 20 min at 4 °C. The supernatants were used for assays of enzyme activity. The 3 ml reaction solution contained 50 mM phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2 , and 0.1 ml enzyme extract. APX activity was calculated by following the decrease in absorbance of AsA (extinction coefficient 2.8 $\text{mM}^{-1} \text{cm}^{-1}$) within 1 min at 290 nm.

Protein content

Protein content in enzyme extracts was determined according to the method of Bradford (1976). 25 μl of enzyme extract was added to

3 ml of 0.01% (w/v) Coomassie Brilliant Blue G-250 solution (4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid). The absorbance at 595 nm was spectrophotometrically recorded after protein-dye binding for 5 min. The protein content was calculated by comparison with a standard curve using bovine serum albumin as a standard.

Nitric oxide content

Nitric oxide content was determined using the method described by Ding *et al.* (1998) and Hu *et al.* (2003) with slight modifications. Leaves (0.6 g) were ground in a mortar and pestle in 3 ml of 50 mM cool acetic acid buffer (pH 3.6, containing 4% zinc diacetate). The homogenates were centrifuged at 10 000 g for 15 min at 4 °C. The supernatant was collected. The pellet was washed by 1 ml of extraction buffer and centrifuged as before. The two supernatants were combined and 0.1 g of charcoal was added. After vortex and filtration, the filtrate was leached and collected. The mixture of 1 ml of filtrate and 1 ml of the Greiss reagent was incubated at room temperature for 30 min. Absorbance was determined at 540 nm. NO content was calculated by comparison to a standard curve of NaNO₂.

Results

Effects of ABA, NO scavenger, and NOS inhibitor on antioxidant enzymes

Figure 1A shows that ABA significantly increased SOD activity at 12 h and 36 h of treatment. The combination of ABA with either the NO-scavenger, PTIO, or the NOS-inhibitor, L-NNA, reduced SOD activity to below the level of ABA treatment alone at 12 h and 36 h of treatment. Plants treated with the NO-scavenger or the NOS-inhibitor had lower SOD activity than the control at 12 h, but increased to the level of the control at 36 h of treatment. CAT activity was also increased by ABA treatment. At 12 h and 36 h of treatment with ABA, CAT activity increased by 18.2% and 38.2%, respectively. ABA and the NO-scavenger or the NOS-inhibitor reduced CAT activity to a lower level than ABA-treated plants at 12 h or 36 h of treatment (Fig. 1B). Ascorbate peroxidase activity increased by 41.7% with 12 h of treatment with ABA, but the induction effect decreased at 36 h. ABA in combination with either the NO-scavenger or the NOS-inhibitor had lower APX activity than ABA treatment alone (Fig. 1C).

Effects of ABA on NO contents

Figure 2 shows the time-course of NO levels as affected by ABA treatment. NO content reached a peak level between 5–7 h of ABA treatment. The NO content in ABA-treated leaves was 38.2% higher than that in the control at 6 h of ABA treatment. In order to test whether the increase in NO content is attributed to ABA treatment, shoot cuttings were treated for 6 h with the NO-scavenger and the NOS-inhibitor in combination with ABA on the same experimental system and the NO contents of the leaves were analysed. The result showed that ABA-treated leaves had a higher NO content, while the other treatments had similar NO contents to the control leaves. The increase of

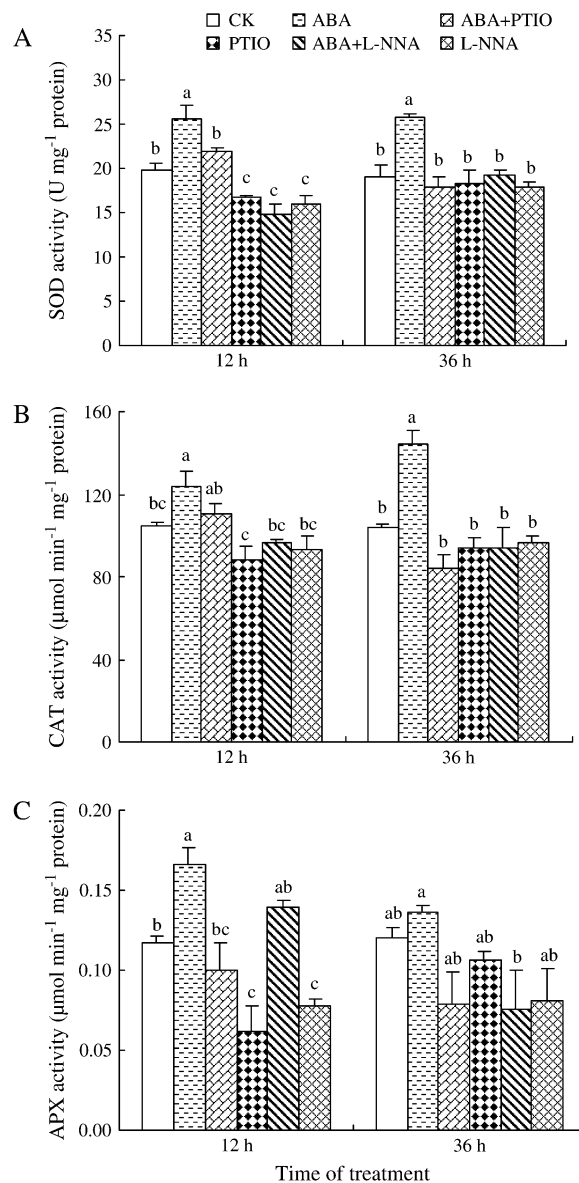


Fig. 1. SOD (A), CAT (B), and APX (C) activities in 12 h and 36 h of ABA treatment in the presence or absence of NO scavenger (PTIO) or NOS inhibitor (L-NNA). Shoots that were 6 cm long were cut off a plant and were immediately placed in water as a control (CK) or in solutions containing 37.9 μM ABA, 37.9 μM ABA+400 μM PTIO, 400 μM PTIO, 37.9 μM ABA+200 μM L-NNA, or 200 μM L-NNA. All the treated shoot cuttings were placed in a growth chamber at 160 μmol m⁻² s⁻¹ photosynthetic photon flux density at 28 °C. SOD, CAT, and APX activities in leaves were assayed at 12 h and 36 h of treatment. Values represent the means of three replicates and bars indicate SE. Different letters indicate a statistical difference at the $P \leq 0.05$ level among a given treatment time according to Duncan's multiple range test.

NO contents in ABA-treated leaves was reduced by the NO-scavenger or the NOS-inhibitor (Fig. 3).

Effects of the NO donor on antioxidant enzymes

Based on the observation that exogenous ABA induced NO accumulation, it was hypothesized that ABA might trigger NO production which leads to the induction of antioxidant

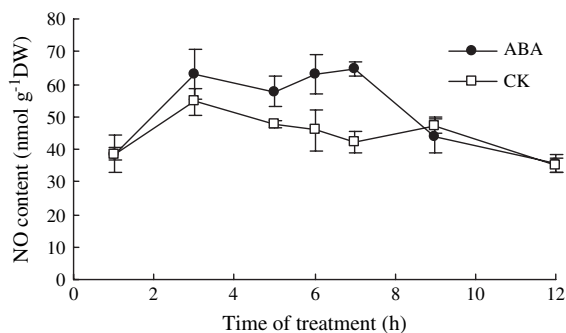


Fig. 2. Effects of ABA on NO content during 12 h of treatment. Shoots that were 6 cm long were cut off a plant and were immediately placed in water as a control (CK) or in a solution containing 37.9 μM ABA. All the treated shoot cuttings were placed in a growth chamber at 160 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density at 28 °C. NO contents in leaves were assayed at the times indicated. Values represent the means of three replicates and bars indicate SE.

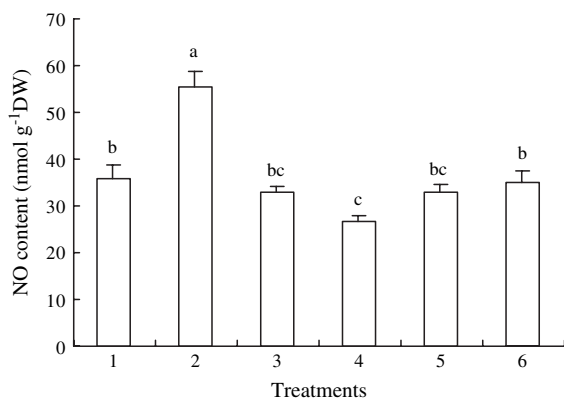


Fig. 3. NO contents in 6 h of ABA treatment in the presence or absence of the NO scavenger (PTIO) or the NOS inhibitor (L-NNA). Shoots that were 6 cm long were cut off a plant and were immediately placed in water as a control or in solutions containing 37.9 μM ABA, 37.9 μM ABA+400 μM PTIO, 400 μM PTIO, 37.9 μM ABA+200 μM L-NNA, or 200 μM L-NNA. All the treated shoot cuttings were placed in a growth chamber at 160 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density at 28 °C. NO contents in leaves were assayed at 6 h of treatment. Values represent the means of four replicates and bars indicate SE. Different letters indicate the statistical difference at the $P \leq 0.05$ level among treatments according to Duncan's multiple range test. 1, Control treatment; 2, ABA treatment; 3, ABA plus PTIO treatment; 4, PTIO treatment; 5, ABA plus L-NNA treatment; 6, L-NNA treatment.

enzymes. Therefore, the effect of NO on SOD, CAT, and APX activity in *Stylosanthes guianensis* was also determined, although there was evidence in other plant species that NO enhanced the activities of antioxidant enzymes (Ruan *et al.*, 2002; Cheng *et al.*, 2002). SNP and NaNO_2 plus AsA were used in this experimental system as the NO donors in order to test whether NO could induce SOD, CAT, and APX activity. NaNO_2 can be reduced to produce NO in the presence of AsA and 0.5 mM of SNP can produce 2 μM of NO (Delledonne *et al.*, 1998). As shown in Fig. 4, the NO donors increased SOD activity in *Stylosanthes guianensis*. For example, when shoot cuttings were treated with the NO donors at 12 h and 36 h, SOD activity in

SNP-treated leaves was 39.4% and 26.8% higher than that in control leaves, respectively. NaNO_2 plus AsA (NaNO_2 -AsA)-treated leaves had 44.1% and 23.8% higher SOD activity than that in control leaves at 12 h and 36 h, respectively. The promotive effect of the NO donors was reversed in the presence of the NO scavenger. CAT and APX activities were also increased by treatments with two NO-donors, SNP and NaNO_2 plus AsA. However, the promotive effect of the NO-donors on antioxidant enzymes was reversed in the presence of the NO-scavenger (Fig. 4).

Discussion

This study demonstrated that ABA increased the activities of SOD, CAT, and APX in *Stylosanthes guianensis*, but it was reversed in the presence of the NO-scavenger (Fig. 1). The result is consistent with the observations that ABA induced the expression of SOD, CAT, and APX genes in maize and *Chlamydomonas reinhardtii* (Zhu and Scandalios, 1994, Guan and Scandalios, 2000, Yoshida *et al.*, 2004). NO content increased 5–7 h after ABA treatment (Fig. 2). Moreover, the increase in NO content in ABA-treated shoot cuttings was suppressed by the NO-scavenger (Fig. 3), indicating that ABA triggered NO production in *Stylosanthes guianensis* leaves. These data supported the hypothesis that NO may mediate ABA-induced antioxidant enzymes.

Two enzymatic NO-generation pathways were proposed in plants, NR (Desikan *et al.*, 2002; García-Mata and Lamattina, 2002, 2003) and NOS-like enzymes (Guo *et al.*, 2003). In *Stylosanthes guianensis*, both ABA-induced antioxidant enzyme activity and ABA-triggered NO production were reversed in the presence of the NOS-inhibitor (Figs 1, 3), indicating that NOS may play an important role in NO-mediated ABA-induced antioxidant enzyme activity. Because the NR activity and NO production after ABA treatment in the presence of NR inhibitor were not measured, the present data can not rule out the possible involvement of NR in ABA-induced NO production in *Stylosanthes guianensis* leaves.

ABA might first induce NO synthesis and the ABA-induced NO then stimulates the antioxidant enzymes. Figures 1–3 supported this hypothesis. It has been observed in many other plant species that NO stimulates antioxidant enzymes. The inducible effect of the NO donor on the activity of SOD, CAT, and APX was observed in rice seedlings (Uchida *et al.*, 2002). The NO donor increased SOD activity in rice under osmotic stress (Cheng *et al.*, 2002) and increased the activities of SOD and CAT in wheat under oxidized stress by paraquat treatment (Hung *et al.*, 2002). The plant species used in this experimental system, *Stylosanthes guianensis* has not been reported before. So the activities of antioxidant enzymes in *Stylosanthes guianensis* under NO donor treatment were

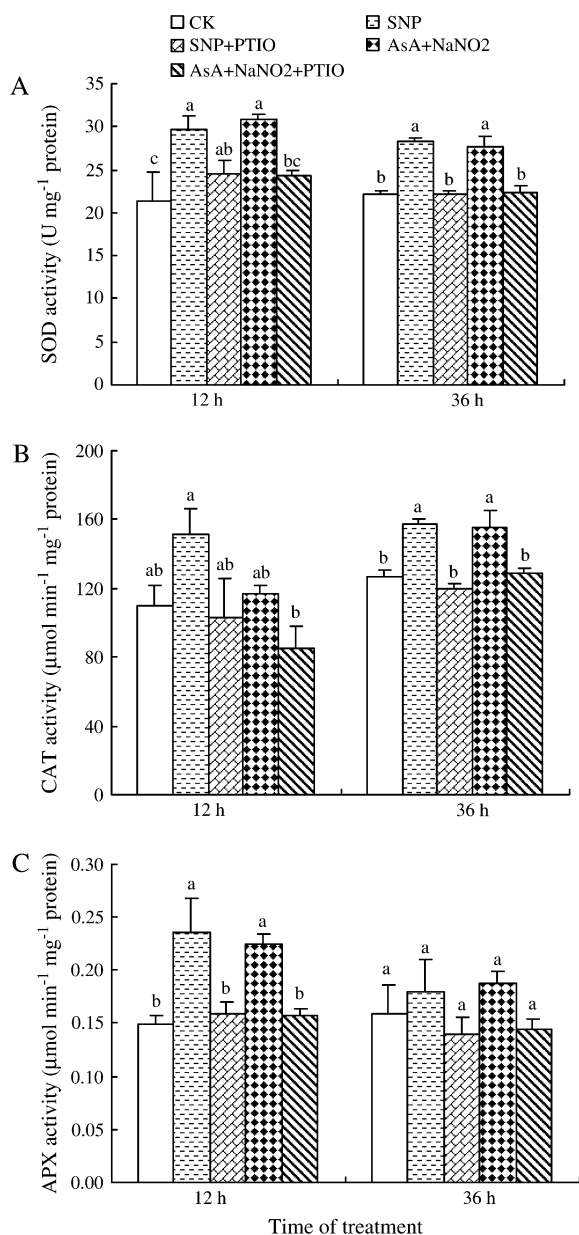


Fig. 4. SOD (A), CAT (B), and APX (C) activities in 12 h and 36 h of the NO donor of SNP or AsA plus NaNO₂ treatments in the presence or absence of PTIO. Shoots that were 6 cm long were cut off a plant and were immediately placed in water as a control (CK) or in solutions containing 200 μM of SNP, 200 μM SNP+400 μM PTIO, 100 μM AsA+200 μM NaNO₂, 100 μM AsA+200 μM NaNO₂+400 μM PTIO. All the treated shoot cuttings were placed in a growth chamber at 160 μmol m⁻² s⁻¹ photosynthetic photon flux density at 28 °C. SOD, CAT, and APX activities in leaves were assayed at 12 h and 36 h of treatment. Values represent the means of three replicates and bars indicate SE. Different letters indicate a statistical difference at the $P \leq 0.05$ level among treatments at a given treatment time according to Duncan's multiple range test.

determined. Figure 4 shows that two types of NO donors increased the activity of SOD, CAT, and APX in *Stylosanthes guianensis*, which was suppressed by the NO scavenger. The result suggested that NO stimulated antioxidant enzyme activity in *Stylosanthes guianensis*.

NO and H₂O₂ are signals involved in plant growth and development (Neill *et al.*, 2002b), however, their relationship is not well known. Jiang and Zhang (2003) demonstrated signalling crosstalk of ROS and calcium in ABA-induced antioxidant enzyme activity. In the ABA-induced stomatal closure signal pathway, signalling components positioned downstream of H₂O₂ and NO are calcium, protein kinases, and cyclic GMP (Desikan *et al.*, 2004). The relationship between NO and other signalling molecules, such as H₂O₂ and calcium, involved in ABA-induced antioxidant enzyme activity in *Stylosanthes guianensis*, need to be studied further.

In summary, the present results demonstrate that NO is involved in ABA-induced antioxidant enzymes activity in *Stylosanthes guianensis*. ABA-triggered NO production could be associated with NOS-like enzyme.

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