EFFECTS OF EXOGENOUS NITRIC OXIDE ON THE PHYSIOLOGICAL CHARACTERISTICS OF *INDOCALAMUS BARBATUS* MCCLURE SEEDLINGS UNDER ACID RAIN STRESS

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Abstract

The effects of four concentrations (100, 400, 700, and 1000 mg/L) of sodium nitroprusside (SNP, adonor of NO) on physiological characteristics were investigated in the leaves of bamboo (*Indocalamus barbatus* McClure) seedlings exposed to simulated acid rain (SAR; pH3.0) stress.The results showed that a foliar application of 100 - 400 mg/L SNP pretreatment mitigates the SAR-inflicted decrease in net photosynthetic rate (Pn), chlorophyll (SPAD) content, soluble protein (SP) content, and superoxide dismutase (SOD) activity, and the increase in peroxidase (POD) activity as well as in preventing an increase in membrane permeability (MP) and superoxide anion radical generation rate (O_2^-). This promoting effect was most pronounced at 400 mg/L SNP treatment, which also exhibited a time-dependent effect. However, seedlings subjected to higher concentrations of SNP such as 700 or 1000 mg/L showed little recovery from damage, and even showed signs of toxic damage, demonstrating the concentration-dependent effect of NO against acid rain. Further analysis showed that acid rain exposure caused oxidative stress by elevating MP and O_2^- in *I. barbatus* seedlings. Treatment with 400 mg/L SNP partly alleviated the acid rain toxicity by reducing O_2^- and stimulating SOD and POD activities. The recovery of Pn, SPAD, and SP was also significantly correlated with oxidative status in the seedlings. Moreover, the changes in the physiological indicators mentioned above, were consistent with the morphological observations. Based on these results, it can be concluded that SNP exerted an advantageous effect on alleviating the inhibitory effect of acid rain by regulating the balance of ROS metabolism and reducing the accumulation of ROS.

Key words: *Indocalamus barbatus,* Acid rain stress. Sodium nitroprusside, SAR-inflicted.

Introduction

Acid rain is a critical global environmental issue that is becoming increasingly prevalent as a result of rapid economic development (Lv *et al.*, 2014). China now constitutes the third most seriously affected region in the world following Northeast America and Europe(Cao *et al.*, 2009), and has attracted much attention in recent years(Wang *et al.*, 2012; Larssen *et al.*, 2006). Acid precipitation has occurred in about 40% of the entire territory of China (Ling *et al.*, 2010), especially in economically developed regions, such as the Jiangsu province. The annual average pH of precipitation in China ranges from 3.0 to 4.5 (State environmental protection administration of China, 2005; Wen *et al.*, 2011). Acid rain exerts deleterious effects on aquatic and terrestrial ecosystems, subsequently threatening human health (González & Aristizábal, 2012). In plants, acid rain causes extensive damage including chlorosis and necrosis of the leaf surface, fruit yield decline, decrease in photosynthetic capacity, damage to membrane functioning, physiological process disorders, accumulation of reactive oxygen species (ROS), and variation in several enzyme activities. Moreover, the extent of injury caused by acid rain is also closely related to pH and the stress duration. (Shukla *et al.*, 2013; Wang *et al.*, 2013; Sun *et al.*, 2013; Choi *et al.*, 2010; Wyrwicka & Skłodowska, 2014; Shaukat & Khan, 2008) In recent years, the effects of protons, ions, photosynthesis, and free radicals have been used to explain the mechanism by which acid rain deleteriously impacts plants (Sun *et al.*, 2013). Furthermore, some experimental observations have reported on this from a proteomic and transcriptional perspective (Liu *et al.*, 2013; Liu *et al.*, 2014). All these studies can help us understand the mechanisms that plants use to adapt to acid rain stress.

Nitric oxide (NO) is well recognized as a major signaling molecule in plants that participates in a diverse range of physiological processes, including seed germination, stomatal movement, photomorphogenesis, maturation and senescence, and programmed cell death (Baudouin & Hancock, 2013; Crawford & Guo, 2005; Gupta *et al.*, 2011; Shi *et al.*, 2014; Chen *et al.*, 2014). NO also regulates multiple plant responses to a variety of environmental stresses including salinity, drought, heavy metals, heat and chilling, and UV-B irradiation (Corpasa *et al.*, 2011; Saxena & Shekhawat, 2013; Manzer *et al.*, 2011). Exogenous NO alleviates the adverse effects caused by abiotic stresses to enhance tolerance in plants, and this improvement in tolerance is the result of NO regulating ROS levels and their toxicity in plants (He *et al.*, 2014). NO plays an important role in enhancing the salt tolerance of *Aegiceras corniculatum* primarily through regulating its antioxidant system (Chen *et al.*, 2014). Boogar *et al.* (2014) proposed that the enhancing effect of NO donors on antioxidant enzyme activity in three grass species is influenced by the application of different concentrations of NO during drought stress. NO also acts as an antioxidant by protecting citrus leaves from DNA strand cleavage caused by the hydroxyl radicals produced following NaCl application, thereby avoiding oxidative damage induced by most harmful ROS (Molassiotis *et al.*, 2016). However, there is no available information regarding the effects of exogenous NO on acid rain stress in plants.

Bamboo, a tropical and subtropical gramineous plant, is one of the most important forestry and potential bioenergy resources in China (Gao *et al.*, 2012). It is widely used for industrial purposes, such as in paper and building materials, and is also consumed fresh as an edible shoot and is even used in medicine. Bamboo is known for its resistance to a wide range of stress factors, as well as its high growth rate and biomass production, and potential use in phytoremediation (Blanche *et al.*, 2012). *Indocalamus barbatus* McClure is a rhizomatous, fast growing perennial evergreen plant with immense medicinal and ornamental value. The genus *Indocalamus* belongs to the family Poaceae and subfamily Bambusoideae.In recent years, *I. barbatus* has become an important forest resource not only for its ornamental value, but also for soil and water conservation (Tian *et al.*, 2006). Bamboo plants improve the environment and help balance ecosystems. However, acid precipitation has become a frequent event that causes severe damage to bamboo plants, yet little information exists on how to alleviate these damages. Therefore, the main objective of this study was to determine if NO ameliorates the effects of acid rain in plants and elucidate the relief mechanisms from the perspective of ROS metabolism. To our knowledge, this is the first report on using sodium nitroprusside (SNP) to improve acid rain stress tolerance in plants.

Materials and Methods

Plant material and growth conditions: Uniform and healthy *I. barbatus* specimens were collected from the bamboo field of the Bamboo Research Institute in Nanjing Forestry University, Jiangsu Province, China. The rhizomes were collected in December 2006 and transplanted separately in individual plastic pots (13 cm tall × 15 cm diameter), with 20-25 culms per pot and 10 repeats for each treatment. The pots were filled with prepared soil and transferred to a greenhouse located at the Nanjing Forestry University for culturing and future experiments. The soil used in this experiment was collected from the arboretum of the Nanjing Forestry University and was homogenized, air-dried, and sieved through a 4.0 mm sieve. The soil was yellow-brown soil, mixed with suitable organic fertilizer. The soil used was neutral (pH = 6.68), with 6.79 g organic matter, 1.86 g nitrogen, 127 mg available phosphorus, 295 mg available potassium per kg soil, and less than 1 mg mercury per kg soil.

At the end of August 2007, potted seedlings with similar ages, heights, and growth condition were selected and divided following a completely randomized design for the subsequent treatments. Fully expanded, healthy leaves from the third branch (from top to bottom) of the seedlings were marked for the determination of each index and morphological observation. We set up six treatments for the SNP and simulated acid rain (SAR) treatments. These included: S0 (CK), S (pH3.0 SAR treatment), S1 (100 mg/L SNP pretreatment + pH 3.0 SAR treatment), S2 (400 mg/L SNP pretreatment + pH 3.0 SAR treatment), S3 (700 mg/L SNP pretreatment + pH 3.0 SAR treatment), S4 (1000 mg/L SNP pretreatment + pH 3.0 SAR treatment). Six pots were prepared separately for each treatment.

Treatment: SNP, as an NO donor, was freshly prepared at concentrations of 100, 400, 700, 1000 mg/L (pH6.2-6.4) for foliar spraying. Deionized water was used as the solvent and control treatment. The design of the SNP concentration gradient followed the method of Wang *et al.* (2005) with minor modification, and the concentration of SNP used here was proven to be appropriate based on preliminary experiments. During the period of August 25 to 27, 50 mL SNP solutions of different concentrations were applied on the leaves of the plants in each pot at 5:00 pm every day, while 50 mL distilled water was sprayed on the CK plants at the same time. All these treatments were administered prior to the acid rain stress treatments.

The acid rain stock solution (pH 3.0) was prepared with a solution of concentrated H_2SO_4 and HNO_3 in a ratio of 5:1 (v/v, by chemical equivalents) followed by diluting with deionized water to pH 3.0. The method and the selection of the pH of the SAR were according to the general anion composition of rainfall in Jiangsu province, China (Tong *et al.*, 2005). The SAR solution was sprayed on the leaves of the plants in each pot at 5:00 pm every day from August 28 to September 2, until the solution was dripping from the leaves. The same amount of distilled water (pH 7.0) was applied to the CK potted seedlings.

Determination of net photosynthesis and chlorophyll content: For each treatment, Pn was measured on September 3, September 12, and October 3, on sunny days from 9:00 to 11:00 am with an LI-6400 portable gas exchange system (Li-Cor, USA) equipped with a red-blue LED light source (6400-02B). Twelve marked leaves in each treatment were measured and repeated in triplicate per leaf. The light intensity was set to natural light (750 $\mu mol/m^{-2}s^{-1}$) by LI-6400. The temperature control of the LI-6400 was set to track the ambient air temperature. A constant CO_2 concentration of 380 ± 5 mM (CO_2) mol^{-1} in the sample chamber was provided with a CO_2 injection system.

Chlorophyll content was quantified using SPAD-502 (Xu *et al.*, 2007) after the determination of Pn. Under each treatment, 36 marked leaves were randomly selected and three equidistant spots from the base to the tip of the leaf blade were measured and the mean of the three readings was calculated and used for data analyses. After the determination of chlorophyll content, adequate marked leaves were collected at 5:00 pm for the following experiments.

Determination of ROS and soluble protein contents as well as plasma membrane permeability: O_2^- was quantified using the hydroxylamine oxidation method (Li & Gong, 2005). Membrane permeability (MP) was determined according to Tan *et al.* (1985). Soluble protein (SP) content was measured using the method of Bradford (1976); and the standard curve was prepared using bovine serum albumin.

Determination of antioxidant system: Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) (Dhindsa *et al.*, 1981). Peroxidase (POD) activity was determined using the method of Koghba *et al.* (1977).

Statistical analysis: All the physiological measurements above were performed in six replicates in two independent experiments (n = 12). The results were expressed as mean ± SE. Multiple comparison analyses were performed with SPSS17.0 software using one-way ANOVA (p<0.05) with Duncan's test. In some cases, correlation analyses were performed, also using SPSS 17.0 (*p≤0.05, **p≤0.01).

Results

Effects of SNP on Pn and chlorophyll content: We assayed the net photosynthetic rate and the chlorophyll contents to investigate the NO-mediated acid rain stress response on photosynthesis of *I. barbatus* plants. Our results showed that acid rain stress inhibited Pn in *I. barbatus* by 41.92% compared with the S0 treatment, whereas Pn in the S1 and S2 treatments increased by 13.69% and 32.22% respectively, in comparison with the S treatment (Fig. 1). These results indicated that low-dose SNP pretreatment could partly restrain the decrease in photosynthetic rate in *I. Barbatus* under acid rain stress, and a particularly good effect was observed at 400 mg/L. However, the S3 and S4 treatments decreased Pn by 24.85% and 24.46% respectively in comparison to the S treatment indicating that high levels of exogenous NO accelerated Pn decrease in *I. barbatus*. No significant changes were observed among the different treatments during the one-month recovery, suggesting that Pn could not be recovered further following acid rain stress regardless of the exogenous NO pretreatment level.

The changes in chlorophyll content were similar to that observed in Pn but to different extents (Fig. 2). Acid rain significantly decreased the chlorophyll concentration by 20.45% (indicated by a SPAD reading) compared with the S0 treatment. In comparison with the S treatments, the S1 treatment tended to mitigate the decrease, but the result was not statistically significant. The chlorophyll contents of the S2 treatment were higher than the S treatments by 14.00%, suggesting that this treatment markedly inhibited the decrease of chlorophyll concentration. However, the toxic effect on seedlings was aggravated under the S3 or S4 treatment (compared to the S treatment). A tiny and statistically insignificant decrease was observed in the S3 treatment, while the S4 treatment showed a decrease of 12.54% in comparison with S. The data recorded in the SPAD reading did not show significant changes during one month of recovery, which was similar to that observed in Pn. This indicated that the changes in Pn were probably caused by the changes in chlorophyll content. Conversely, NO-mediated effects on Pn or chlorophyll contents could maintain this stability for a long time.

Effects of SNP on SP: The S treatment decreased SP by 32.26% relative to S0 (Fig. 3). In comparison with the S treatment, the S1 and S2 treatments increased SP by 8.43% and 16.37%, respectively, but no difference was observed between S and S1 or S1 and S2. The S3 and S4 treatments decreased SP further by 13.39% and 12.40%, respectively. These results indicated that 400mg/L SNP could effectively restrain the decrease in SP, while 700 mg/L or 1000 mg/L SNP further decreased SP in *I.*

barbatus seedlings under pH 3.0 acid rain. Surprisingly, the change in SP differed from that of Pn or SPAD among the different treatments during one month of recovery. SP was significantly elevated in response to the S, S2 and S3 treatments during the first 11 days. Treatment S2 showed a 17.55% increase in SP compared to the S treatment. Furthermore, the S1 and S4 treatments caused a small but insignificant increase in SP. On day 31, a small but insignificant increase was observed in SP in response to all of the treatments except S1, which increased SP by 10.06% compared with that on day11. SP showed stronger recovery than SPAD and Pn during the experimental period, indicating that the triggering of physiological processes by NO in response to acid rain in *I. barbatus* plants was unique. Among foliar applications of these SNP treatments, Pn, SPAD, and SP exhibited the best results under the S2 treatment.

Effects of SNP on plasma membrane permeability: MP was determined to elucidate the metabolic processes of ROS in *I. barbatus* under acid rain stress (Fig. 4).According to the results, we found that treatment S increased MP by 15.98% compared with S0, indicating that acid rain caused an increase in MP in *I. barbatus* seedlings. The S1 treatment did not significantly decrease the MP, whereas S2 decreased MP by 7.07% as compared with the S treatment, and the other treatments did not inhibit this trend. This indicated that 400mg/L SNP partly restrained the increase in MP under acid rain stress. On day11, we observed that the S1 and S2 treatments decreased MP remarkably by 7.58% and 9.21% compared to S, but the effect of S3 and S4 was not significantly different from S. This indicated that 100mg/L or 400mg/L SNP had positive effects on mitigating membrane damage. On day 31, treatments S, S3 and S4 showed a small but insignificant decrease. S1 and S2 caused little changes; therefore, S1, S2, S3, and S4 did not significantly differ from S. This indicated that S1 and S2 had positive but limited effects on inhibiting the increase in MP.

Effects of SNP on SOD and and O2⁻generation rate: In the antioxidant defense system, SOD detoxifies O_2 by forming H_2O_2 while POD and other relative enzymes catalyze the breakdown of H_2O_2. Our study shows that SOD activity declined 32.70% in response to pH 3.0 acid rain. Spraying SNP solutions on leaves at different concentrations before acid rain treatment could inhibit this decline in different extents. Among the foliar applications of S1, S2, S3, and S4 treatments, the best alleviation was observed in the S1 and S2 treatments, which were higher than S by 29.25% and 37.59% respectively (Fig. 5). Interestingly, we detected a significant increase in SOD activities in the S, S3 and S4 treatments, but this was not observed in S1 and S2 during the first 11 days of the experimental period. Moreover, from day11 to day31, all of the treatments showed a drastic increase, among which, S1, S2, S3, and S4 were higher than S0 by 3.23%, 5.63%, 8.03%, and 6.46%. The above results indicated that *I. barbatus* seedlings display a great resistance response under acid rain stress, but require a long time to recover the SOD activity. Alternatively, different concentrations of SNP could effectively inhibit the decrease in SOD activity, especially under low dosages. In addition, acid rain resistance was improved by the increased SOD activity.
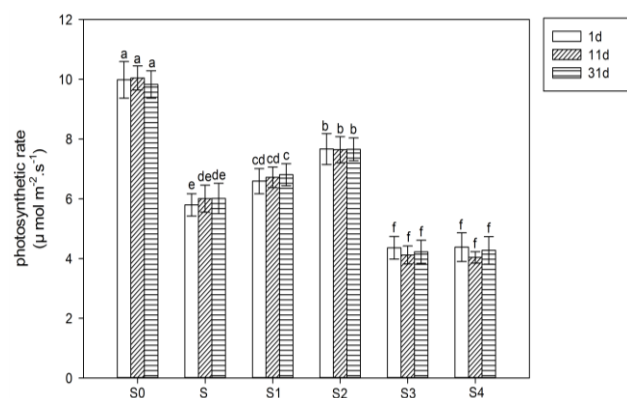
Fig. 1. Effects of exogenous NO under acid rain stress on net photosynthetic rate in *I. barbatus*. Bars represent SD (n = 12). Different lowercase letters indicate a significant difference at the 0.05 level between different treatments. S0, S, S1, S2, S3, and S4 represent CK, pH 3.0 SAR, 100 mg/L SNP + pH 3.0 SAR, 400 mg/L SNP + pH 3.0 SAR, 700 mg/L SNP + pH 3.0 SAR, 1000 mg/L SNP + pH 3.0 SAR treatments, respectively.
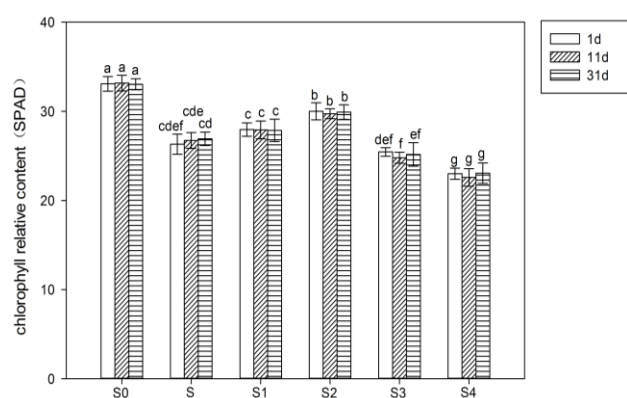

Fig. 2. Effects of exogenous NO under acid rain stress on the relative chlorophyll content in *I. barbatus*. Bars represent SD (n = 36). Different lowercase letters indicate a significant difference at the 0.05 level between different treatments. S0, S, S1, S2, S3, and S4 represent CK, pH3.0 SAR, 100mg/L SNP + pH3.0 SAR, 400 mg/L SNP + pH3.0 SAR, 700 mg/L SNP + pH3.0 SAR, 1000 mg/L SNP + pH3.0 SAR treatments, respectively.
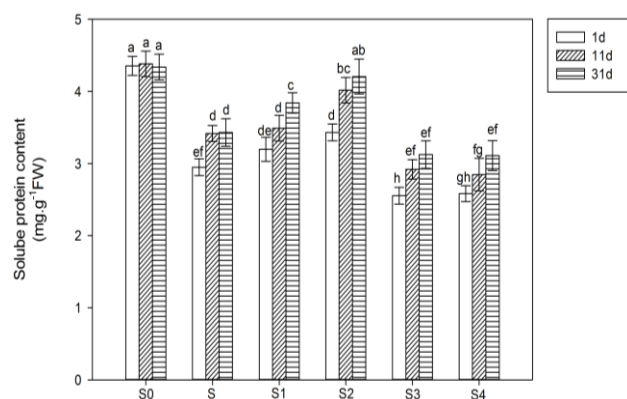

Fig. 3. Effects of exogenous NO under acid rain stress on the soluble protein content in *I. barbatus*. Bars represent SD (n = 12). Different lowercase letters indicate a significant difference at the 0.05 level between different treatments. S0, S, S1, S2, S3, and S4 represent CK, pH 3.0 SAR, 100mg/L SNP + pH 3.0 SAR, 400 mg/L SNP + pH 3.0 SAR, 700 mg/L SNP + pH 3.0 SAR, 1000 mg/L SNP + pH 3.0 SAR treatments, respectively.

Fig. 4. Effects of exogenous NO under acid rain stress on plasma membrane permeability in *I. barbatus*. Bars represent SD (n = 12). Different lowercase letters indicate a significant difference at the 0.05 level between the different treatments. S0, S, S1, S2, S3, and S4 represent CK, pH 3.0 SAR, 100 mg/L SNP + pH 3.0 SAR, 400 mg/L SNP + pH 3.0 SAR, 700 mg/L SNP + pH 3.0 SAR, 1000 mg/L SNP + pH 3.0 SAR treatments, respectively.
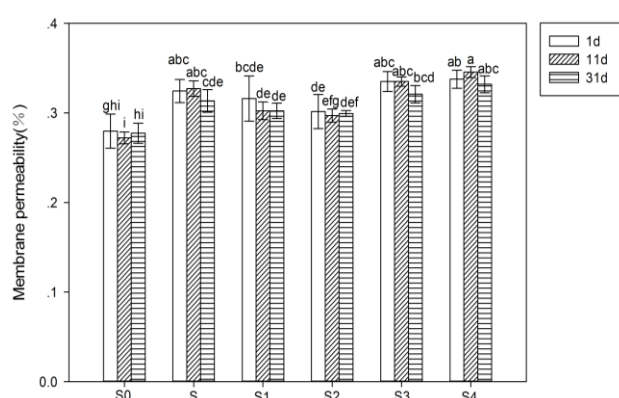

Fig. 5. Effects of exogenous NO under acid rain stress on SOD activity in *I. barbatus*. Bars represent SD (n = 12). Different lowercase letters indicate significant difference at the 0.05 level between the different treatments. S0, S, S1, S2, S3, and S4 represent CK, pH 3.0 SAR, 100 mg/L SNP + pH 3.0 SAR, 400 mg/L SNP + pH 3.0 SAR, 700 mg/L SNP + pH 3.0 SAR, 1000 mg/L SNP + pH 3.0 SAR treatments, respectively.
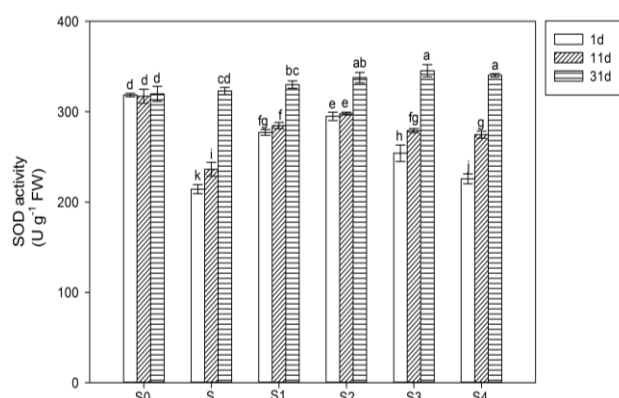

Fig. 6. Effects of exogenous NO under the acid rain stress on the O_2content in *I. barbatus*. Bars represent SD (n = 12). Different lowercase letters indicate a significant difference at the 0.05 level between the different treatments. S0, S, S1, S2, S3, and S4 represent CK, pH 3.0 SAR, 100 mg/L SNP + pH 3.0 SAR, 400 mg/L SNP + pH 3.0 SAR, 700 mg/L SNP + pH 3.0 SAR, 1000 mg/L SNP + pH 3.0 SAR treatments, respectively.
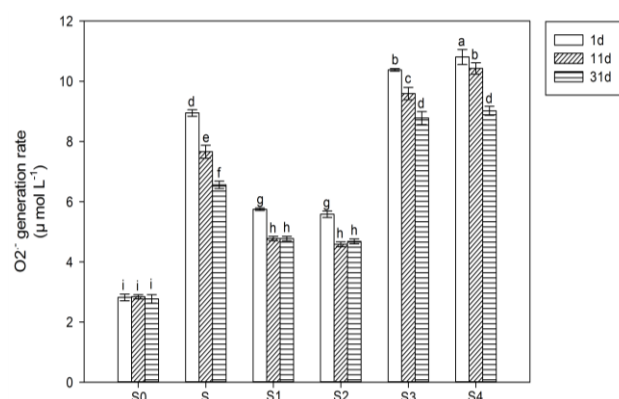
Fig. 7. Effects of exogenous NO under acid rain stress on POD activity in *I. barbatus*. Bars represent SD (n = 12). Different lowercase letters indicate significant difference at the 0.05 level between the different treatments. S0, S, S1, S2, S3, and S4 represent CK, pH 3.0 SAR, 100 mg/L SNP + pH 3.0 SAR, 400 mg/L SNP + pH 3.0 SAR, 700 mg/L SNP + pH 3.0 SAR, 1000 mg/L SNP + pH 3.0 SAR treatments, respectively.
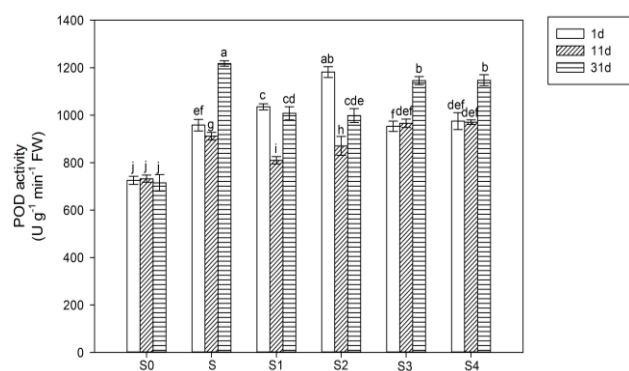
We determined the O_2 generation rate under the same conditions at the same time to clarify the SOD results (Fig. 6). The results showed that the S treatment dramatically increased O_2 generation rate by 32.03% more than S0, which indicated that acid rain caused a massive accumulation of ROS, especially O_2. The S1 and S2 treatments showed a decrease of 35.73% and 37.59%, respectively, compared with S, whereas S3 and S4 showed an increase of 16.00% and 20.80% respectively, relative to the S treatment. This demonstrated that the S1 and S2 treatments effectively mitigated this rising trend of O_2 while S3 or S4 further increased the O_2 content of seedlings, leading to further deterioration. During the recovery period, all of the treatments decreased O_2 of the seedlings during the first 11 days; particularly the S1 and S2 treatments, which showed a 37.67% and 40.18% decrease, respectively, compared with S. No notable changes were observed in the S1 and S2 treatments during the last 20 days until day 31, whereas the seedlings subjected to the other treatments exhibited further deterioration. However, O_2 generation rate in these treatments far exceeded S0. Together with the results of the SOD activity, this indicated that a low dose of NO had an effective and constant roles in decreasing O_2 generation rate and cleaning up O_2 content. Moreover, it maintained the seedlings in a stable condition for a long time, while a high concentration of NO had little effect on the declining O_2 generation rate and influenced the capacity of the seedlings to recover. Surprisingly, the S1 and S2 treatments exerted almost the same reducing effects on the O_2 generation rate at the end of the recovery period, which differed from that observed in Pn, SPAD or SP (S2 was more favorable). This indicated that recovery after O_2 generation was stronger in the S1 and S2 treatments as compared with Pn, SPAD or SP. Moreover, based on the amplitude of variation observed in all the indicators above and the results of multiple comparative analyses, O_2 generation rate was more sensitive to the metabolic process of ROS.

Effects of SNP on POD: POD activity increased to different extents among all the treatments as compared with S0, particularly in the S2 treatment where it reached its maximum (Fig. 7). This indicated that acid rain alone or combined with NO, especially a low dose of NO, could notably increase the POD activity of *I. barbatus* seedlings. The S1 or S2 treatment caused a significant decrease in POD activity from day 1 to day 11, while an elevation was observed from day11 to day 31. Conversely, the other treatments exhibited a highly abnormal raise in POD activity at the end of the experiment. POD activity in S, S3 and S4 were higher than in S0 by 70.28%, 60.26% and 60.49%, and POD activity even increased by 41.03% or 39.63% in S1 and S2 compared with S0. Based on the above results as well as the MP and SOD activity findings, we concluded that both the S1 and S2 treatment improved the adaptive resistance of *I. barbatus* plants to oxidative stress by regulating SOD and POD activities. The oxidative status of plants under all the treatments might result from antioxidant defense system that protects seedlings from ROS toxicity.

Analysis of correlation: We measured the correlations of different physiological indicators to identify any possible associations (Tables 1-3). Our results showed that Pn exhibited a significant positive linear correlation with SPAD and SP, and had a significant negative correlation with MP and O_2 at different times of recovery. Furthermore, we also found significant negative correlations between SPAD and MP or SPAD and O_2, as well as between SP and MP or SP and O_2 during different recovery times within one month. However, we observed different correlations among SOD, POD, and Pn during the recovery period. Among them, SOD was significantly negatively correlated with MP or O_2, and was significantly positively correlated with Pn, SPAD, or SP on Sep 3 and Sep 13. Nevertheless, on Oct. 3, the degree of correlation between SOD and Pn, or SOD and SPAD was weak and we did not observe any correlation between SOD and SP. Moreover, SOD and MP as well as SOD and O_2 were significantly positively correlated. On the contrary, on Sep. 3 no correlation was observed between POD and any other indicators. However, on Sep. 13 or Oct. 3, our results showed a significant positive correlation between POD and MP or O_2 and a significant negative correlation between POD and Pn, SPAD, or SP. Thus, SOD or POD are often used to explain the balance of ROS metabolism, but are unable to reflect the condition of oxidative stress directly.

Table 1. Correlation analysis of net photosynthetic rate (P$_n$) and physiological characters of *I. barbatus* on Sep. 3.

Indicators	Pn	SPAD	SP	MP	O_2	SOD	POD
Pn	1	0.972**	0.993**	-0.699**	-0.946**	0.839**	-0.320
SPAD		1	0.959**	-0.681**	-0.936**	0.885**	-0.262
SP			1	-0.707**	-0.938**	0.833**	-0.416
MP				1	0.808**	-0.658**	0.340
$O_2 \bullet^{-}$					1	-0.876**	0.249
SOD						1	-0.161
POD							1

*Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level

Table 2. Correlation between net photosynthetic rate (P_n) and physiological characters of *I. barbatus* on Sep. 13.

Indicators	Pn	SPAD	SP	MP	O_2	SOD	POD
Pn	1	0.980**	0.978**	-0.905**	-0.940**	0.627**	-0.884**
SPAD		1	0.978**	-0.888**	-0.931**	0.635**	-0.849**
SP			1	-0.848**	-0.907**	0.615**	-0.790**
MP				1	0.962**	-0.654**	0.950**
$O_2 \bullet^-$					1	-0.604**	0.933**
SOD						1	-0.584*
POD							1

*Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level

Table 3. Correlation between net photosynthetic rate (P_n) and physiological characters of *I. barbatus* on Oct. 3.

Indicators	Pn	SPAD	SP	MP	O_2	SOD	POD
Pn	1	0.974**	0.954**	-0.836**	-0.953**	-0.578*	-0.844**
SPAD		1	0.948**	-0.806**	-0.914**	-0.485*	-0.801**
SP			1	-0.732**	-0.905**	-0.361	-0.757**
MP				1	0.915**	0.687**	0.859**
$O_2 \bullet^-$					1	0.673**	0.830**
SOD						1	0.493*
POD							1

*Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level

Discussion

Acid rain toxicity adversely affects plants by reducing the total biomass (Lee *et al.*, 2006), causing a decline in photosynthetic rate and chlorophyll content (Liu *et al.*, 2007), damaging the membrane system (Anna & Maria, 2006), altering antioxidant enzyme activities (Chen *et al.*, 2013), and adversely affecting the leaf nutrient balance, reflected by visible injury symptoms. In our study, acid rain exposure significantly decreased Pn, chlorophyll content and soluble protein, and significantly increased MP, which was accompanied by leaf chlorosis and the appearance of necrotic spots on the leaf surface of *I. barbatus*. Moreover, our data showed that the physiological and biochemical indicators could not be restored in a recovery time of one month indicating that acid rain causes irreversible damage to *I. barbatus.* NO has long been recognized as an activating and multi-functional signaling molecule. It is associated with a broad range of abiotic stress. Previous studies showed that exogenously applied SNP alleviates a wide range of abiotic stress factors such as extreme temperature, salinity, drought, heavy metals, UV-B radiation (Corpasa *et al.*, 2011; Liu *et al.*, 2010; Li *et al.*, 2013). These reports suggest that a low-dose of NO provides protection against abiotic stresses while high NO-donor concentrations have a detrimental effect on plants. To the best of our knowledge, no previous reports on the use of SNP, as an NO donor, in regulating the physiological response of plants to acid rain stress. Our data showed that supplementation with 100-400 mg/L SNP reversed the decrease in Pn, SPAD, SP, and the increase in MP, inflicted by acid rain stress; and 400 mg/L SNP treatment

in particular was most effective on all the indicators above. Higher concentrations of 700 or 1000 mg/L SNP had insignificant alleviation; for some indicators, it even exhibited a detrimental effect. The results of the morphological observations also indicated that 100-400 mg/L SNP pre-treatment apparently alleviated leaf chlorosis and decreased necrotic spots in the leaves of *I. barbatus*, especially at 400mg/L (barely any injury symptom were observed in the leaves pre-treat with 400mg/L SNP). On the contrary, 700 or 1000 mg/L SNP supplement accelerated leaf chlorosis and increased necrotic spots in the stressed leaves of *I. barbatus* seedlings, and these injury symptoms became more severe with increasing SNP concentration. During the recovery period, all the indicators above (except SP) varied significantly under NO treatment at different concentrations in comparison with that on day1; and only the 400 mg/L SNP treatment restored SP to the CK level. In summary, the NO-mediated acid rain stress response in *I. barbatus* was basically similar to that of other abiotic stress from previous studies, especially regarding the harmful and beneficial effects of NO. Moreover, we selected Pn and SPAD as the major physiological indicators reflecting the extent of damage in *I. barbatus* in response to acid rain. This selection was based on the similar trends in variations observed between Pn and SPAD as well as morphological observations.

Many environmental stresses induce metabolic processes in plants to generate ROS, which in turn results in cellular oxidative damage (Gill & Tute, 2010; Laspina *et al.*, 2005; Kazemi *et al.*, 2010). In this study, we measured MP, O_2 generation rate, and SOD and POD activities to reflect the metabolic processes of ROS in *I.*

barbatus when subjected to acid rain. As expected, MP and O_2^- generation rate significantly increased to different extents suggesting that acid rain caused cellular oxidative damage in *I. barbatus* plants. The striking and substantial rise in POD activity and the significant decline in SOD activity under acid rain exposure implied that ROS accumulation exceeds the scavenging activity of antioxidant enzymes and contributed towards the injury of the plasma membrane. After a recovery period of one month, we observed a slight but insignificant decrease in MP, which further indicated that acid rain caused severe and irreversible oxidative injury. Previous studies showed that low concentrations of NO act as a direct scavenger, or activate antioxidant enzymes, e.g., SOD or POD, to eliminate ROS (Beligni *et al.*, 2002; Arasimowicz & Floryszak-Wieczorek, 2007; Neill *et al.*, 2008; Li *et al.*, 2013; Bai *et al.*, 2015; Groß *et al.*, 2013; Dinler &Aksoy; Weng *et al.*). Our results show that the foliar addition of 100 mg/L or 400 mg/L SNP pretreatment effectively relieved the rise in O_2^- generation rate, alleviated the decrease in SOD activity, and elevated the activity of POD. During the recovery period, 100 mg/L or 400 mg/L SNP showed a notable reduction in O_2^- generation rate for the first 11 days, and this was stably maintained until day 31. Both SOD and POD were significantly promoted at the end of recovery compared to S0. All these results suggested that a low dose of NO confers a protective role in alleviating cellular oxidative damage in *I. barbatus* plants by activating SOD and POD, as well as by preventing the O_2^- generation rate from augmenting and reducing the accumulation of O_2^- under acid rain stress, especially at 400mg/L. Meanwhile, the results may at least partially account for the improved tolerance to acid rain stress conferred upon *I. barbatus* by a low dose of NO. However, only treatment with 400 mg/L SNP showed a significant relief for MP on day 1; while the S, S1 and S2 treatments did not show a significant difference to MP after the recovery period indicating that severe oxidative damage still existed.The increase in SOD and POD activities as well as the decrease of O_2^- accumulation appeared to hardly alleviate the damage to the plasma membrane, and this may be closely related to the multiple physiological functions of POD (Huang & Xiao, 2002; Tong *et al.*, 2005). A high dose of SNP (700 mg/L or 1000 mg/L) notably elevated the O_2^- generation rate more than acid rain treatment alone during the one-month recovery period. Moreover, at the end of the recovery period, we also observed higher MP and POD activities under 700 mg/L or 1000 mg/L SNP treatments than under the S0 treatment. Thus, high concentrations of NO have a damaging rather than ameliorating effect on preventing oxidative damage in *I. barbatus*. The possible mechanism for this situation is that high NO-donor concentrations cause O_2^- to react with NO to form damaging peroxynitrite (ONOO-) and peroxynitrous acid, which are deleterious to lipids, proteins, and DNA (Beligni & Lamattina, 2001; Frank *et al.*, 2000; Yamasaki *et al.*, 1999).

We used Pearson's correlation analysis to access the relationships among oxidative stress and relative physiological processes including photosynthesis. Our results showed that O_2^- generation rate was negatively correlated with Pn, SPAD and SP under different treatments, and MP in the different treatments was also negatively correlated with Pn, SPAD and SP, during the experimental period (Tables 1-3). Thus, NO probably participates in the modulation of the antioxidant defense system against the acid rain-induced decline in photosynthesis and inhibition of relative physiological processes in *I. barbatus* seedlings. Therefore, the underlying mechanism for the protective role of NO against acid rain could mainly depend on its role as a direct ROS scavenger and as a signaling molecule involved in the activation of plant antioxidant enzymes. Notably, the O_2^- generation rate in the presence of 100 mg/L or 400 mg/L SNP pretreatment exhibited a clear variation from day 1 to day 11, whereas it exhibited little difference from day11 to day31. By comparison, SP showed a constant increase during the recovery period under the same treatments. On the contrary, Pn or SPAD showed unremarkable changes under the same conditions. The observations above suggested that the recovery capacity under different metabolic processes conferred by a low concentration of NO were unique. Evidence indicates that under abiotic stresses NO performs its signaling function through protein post-translational modifications (PTMs) (Ziogas *et al.*, 2015; Tanou *et al.*, 2014). These protein redox modifications alter the function of a broad spectrum of proteins and operate different metabolic processes. Thus, low concentration of NO may have a concentration-dependent as well as a time-dependent effect on different metabolic processes in the response to acid rain stress. Alternatively, we hypothesize that the alteration of O_2^- generation rate was associated with metabolic processes of ROS in *I. barbatus*. Therefore, O_2^- generation rate is proposed as a sensitive indicator reflecting the injury caused by oxidative stress in *I. barbatus*.

It is noteworthy that O_2^- generation rate, MP, SOD, or POD activity in the presence of 100 mg/L and 400 mg/L NO on day 31 did not exhibit any noticeable differences, which was not in agreement with the results of the morphological observation. However, Pn or SPAD of exogenously applied SNP treatments in the same concentrations showed marked differences, especially at 400 mg/L SNP supplementation, which was consistent with the morphological observations. Based on the above results, we conclude that the NO-induced alleviation of oxidative stress is an important and unique step in the regulation of the response of *I. barbatus* seedlings to acid rain stress. However, this effect was not totally compatible with the recovery of damage symptoms in the leaves. Therefore, other mechanisms of alleviation possibly exist that regulate NO against acid rain stress, and are related to the recovery of photosynthetic capacity. Previous studies have implicated NO in the modulation of chlorophyll synthesis (Beligni & Lamattina, 2000; Leshem & Haramaty, 1996), while improvement of chlorophyll content was found to be accompanied by the recovery of photosynthetic rate. This indicated that the NO-mediated improvement in photosynthesis, partly due to increasing chlorophyll synthesis, was important for improving plant acid rain tolerance. Moreover, it has been well documented that the response of NO is a dynamic, photosynthetic activity-demanding process. Treatment

with NO prior to the application of various abiotic stresses that cause proteome reprogramming was associated with photosynthesis- and Calvin cycle-responsive proteins (Molassiotis *et al.*, 2016). These proteins such as glutamine synthase, NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Rubisco, Rubisco activase, Photosystem II reaction center proteins D1 and D2, and proteins of the energy transduction system in chloroplast thylakoids are targets for NO (Misra *et al.*, 2014). This further reflects the multiple functional and complex pathways of NO in modulating photosynthetic performance against acid rain-induced injury in *I. barbatus* seedlings. Further investigation is necessary for the complete elucidation of the NO alleviation mechanism.

Conclusion

An assessment of the results allows us to conclude that NO treatment (as SNP) can effectively protect *I. barbatus* seedlings from acid rain stress damage by enhancing Pn, chlorophyll content, SP content, SOD and POD activities, reducing MP and O_2^- under pH 3.0 acid rain stress, but has a significant concentration- and a time-dependent effect. The induced effect of NO on antioxidant enzymes and the scavenging of O_2^- plays an important role in alleviating acid rain-induced oxidative damage, and the changes in Pn, SPAD, and SP were significant correlated with the oxidative status of the leaves. Moreover, the changes in physiological indicators mentioned above were consistent with the morphological observations during the experimental period. Therefore, the underlying mechanism for the protective role of NO against acid rain operates through maintaining the balance of ROS metabolism and reducing the accumulation of ROS. A concentration of 400 mg/L SNP was optimal for increasing tolerance to acid rain stress in *I. barbatus* seedlings.

Acknowledgments

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