*American Journal of Plant Sciences*, **2011, 2, 511-519**
doi:10.4236/ajps.2011.23060 Published Online September 2011 (http://www.SciRP.org/journal/ajps)

# The Benefits of Exogenous NO: Enhancing *Arabidopsis* to Resist *Botrytis cinerea*

**Hongyu Yang[1*], Xiaodan Zhao[2*], Jia Wu[3], Min Hu[1], Shaolei Xia[2]**

[1]College of Life Sciences and Technology, Kunming University, Kunming, China; [2]College of Life Sciences, Yunnan Normal University, Kunming, China; [3]College of Basic Medical Sciences, Kunming Medical University, Kunming, China.
Email: [*]yanghongyu@kmu.edu.cn

Received June 23rd, 2011; revised July 26th, 2011; accepted August 17nd, 2011.

## ABSTRACT

*Botrytis cinerea is a necrotrophic fungal pathogen that impacts a wide range of hosts, including Arabidopsis. Pretreatment with nitric oxide (NO) donor sodium nitroprusside (SNP) on Arabidopsis leaves suppressed Botrytis cinerea infection development. Additionally, in this study the dosage levels of SNP applied to the leaves had no direct, toxic impact on the development of the pathogen. The relationship between NO and reactive oxidant species (ROS) was studied by using both spectrophotometrical methods and staining leaf material with fluorescent dyes, nitro blue tetrazolium (NBT), and with 3,3-diaminobenzidine (DAB). The results showed that exogenous NO restrained the generation of ROS, especially $H_2O_2$, as the pathogen interacted with the Arabidopsis plant. And this inhibition of reactive oxidant burst coincided with delay infection development in NO-supplied leaves. The influence of elevated level of NO on antioxidant enzymes was investigated in this study. The activities of catalase (CAT) and guaiacol peroxidase (POD) were increased to different degrees in both: 1) SNP treated only leaves, and 2) SNP pretreated leaves that were subsequently inoculateted with pathogens. However, the activity of superoxide dismutase (SOD) was unchanged in the leaves studied. The decrease in $H_2O_2$ content probably resulted from the increase in activities of POD and CAT. Our study suggests that NO might trigger some metabolic reactions, i.e. enhanced enzyme activity that restrains $H_2O_2$ which ultimately results in resistance to B. cinerea infection.*

***Keywords*:** *Arabidopsis*, *Botrytis cinerea*, *Nitric Oxide*, *Antioxidant Enzyme*, *Disease Resistance*

## 1. Introduction

Nitric oxide (NO) has been involved in the responses of plants to abiotic and biotic stresses like drought, salt, heat stresses, disease resistance, and apoptosis [1-4]. Most studies that established the role of NO in the defense responses of plants to disease were obtained from analyzing various plant-biotrophic pathogen systems [5]. The data obtained from these studies show that oxidative burst, a transient and rapid accumulation of reactive oxygen species (ROS), is a widespread defense mechanism of plants against the attack of pathogens. ROS, including superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$), was generated following the recognition of a variety of pathogens and has been identified as a threshold trigger for the hypersensitive response (HR) [6]. The death of plant cells surrounding the pathogenic penetration site caused by HR deprived the pathogen of a nutrient source. This has been considered a successful defense strategy of the plant against biotrophic pathogens [7].

In contrast to biotrophic pathogens, *Botrytis cinerea*, a necrotrophic pathogen, uses different strategies to avoid incompatibility. This kind of pathogen is able to colonize dead tissue. The pathogen induces the death of host cells in order to facilitate the infection. Thus HR is not an efficient weapon against the infection of necrotrophic pathogens [8]. Although the death of host cells enables necrotrophic pathogens to spread and develop in the host, enhanced ROS generation in the plants was still observed [9,10]. According to Ediich, ROS contribute to overcoming the defense of a host plant against *B. cinerea* and facilitate infection [11]. This illustrates the different ways that biotrophic and necrotrophic phytopathogenic fungi deal with ROS as they interact with plants. Thus it can be concluded that an efficient response of a plant to necrotrophs like *B. cinerea* should include the suppression of ROS and the stimulation of the antioxidant system responsible for the control of the redox state in the cell. This conclusion is supported by a study [12] that showed that radical scavengers were able to reduce the

severity of diseases induced by *B. cinerea*.

Many studies indicate that NO is involved in modifying post-infection plant metabolism, often leading to a HR of infected cells. However, only a few of these studies examined the role played by NO in the interaction between the plant and the necrotrophic pathogen [13-15]. Baarlen reported endogenous NO generation during the interaction between lilies and *Botrytis elliptica* [13]. Floryszak-Wieczorek also found that NO plays a crucial role in the initiation of a fast, defensive response of pelargonium leaves to a necrotrophic pathogen [15]. The mechanisms of antioxidant action of NO in plant defense reactions were viewed differently. One reckoned that the decrease in $H_2O_2$ concentration in NO-supplied tomato leaves was probably due to a direct NO-$H_2O_2$ interaction [14]. Another study proposed that in the nonspecific resistance of pelargonium to *B. cinerea*, an early NO burst served as a signal to enhance the accumulation of ascorbic acid and the pool of other 'fast antioxidants', and secondary NO emissions provoked a noncell-death-associated defense, following the rule that the concentration of NO is linked to its action [15]. This present study attempts to elucidate whether NO is involved in the resistance reactions of *Arabidopsis* to *B. cinerea* infection and to determine how NO inactivates ROS in the defense responses of *Arabidopsis* plants.

## 2. Materials and Methods

### 2.1. Plant Materials and Growth Conditions

The *Arabidopsis thaliana* plants used in this study were ecotype Columbia. The seeds were sterilized for 1 minute in 95% ethanol and for 5 minutes in 1% bleach, washed 5 times in sterile water, and then pre-germinated on MS salt agar plates at 22˚C ± 2˚C under a light cycle of 12-h light/12-h dark for 1 week. Then the plants were transplanted into soil and grown at 22˚C ± 2˚C under the same light cycle.

### 2.2. Pathogen Culture

*Botrytis cinerea* was isolated from infected tomato plants growing in a greenhouse and was maintained in stock culture on potato sucrose agar in the dark at 22˚C ± 2˚C. The *B. cinerea* spores were cultured in a potato sucrose agar medium without exposure to light at 22˚C ± 2˚C for 8 days. The spores were collected in sterile water and resuspended at $10^5$ spore·$ml^{-1}$.

### 2.3. Effect of NO on Conidial Germination and Mycelial Growth of *B. cinerea*

50 mM of NO donor (sodium nitroprusside, SNP) stock solution was added to the potato sucrose medium containing $10^5$ $ml^{-1}$ fungal spore suspension in order to produce final concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 mM [16,17]. The spores were further cultured at 22˚C ± 2˚C in the dark for 24 hours. The medium containing sterile water served as a control. Then the germinating spores were counted under a light microscope [15]. A spore was recognized as a germinating spore when a tube was longer than the spore diameter. Each experiment was replicated three times and the germination rate was calculated as follows: rate of spore germination = the number of germinated spore/total number of spore × 100%.

In order to study the effect of SNP on the mycelial growth of *B. cinerea*, we added 50 mM of the SNP stock solution to the potato sucrose agar medium which was cooling after having been sterilized. We added various amounts of the SNP stock to produce final concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 mM. 10 ml of the medium was poured into a plate. We then placed 10 ml ($10^5$ spores/ml spore suspension) of *B. cinerea* into the center of the medium and the diameter (cm) of the area occupied by *B. cinerea* mycelium was measured after being cultured at 22˚C ± 2˚C in the dark for 3 days.

### 2.4. Inoculation

5 leaves were selected from one 5-week-old plant and marked. 10 μl drops of *B. cinerea* conidial suspension ($10^5$ $ml^{-1}$) or sterile water were transferred onto the upper surface of each leaf blade using a 1 ml syringe without a needle. After inoculation, the plants were placed in a growth chamber in the dark at 22˚C ± 2˚C and 100% relative humidity for 24 hours. After 24 hours, the growth chamber conditions were changed to a light cycle of 12-h light/12-h dark and the humidity was maintained at 70%.

### 2.5. Assessment of Disease Development

In order to quantify the severity of the symptoms, individual leaves were examined each day for ten days after inoculation with the pathogen and rated on a scale of 0 to 5 on the basis of the Murray method with modifications [18] where 0 = no visible symptoms; 1 = very few (less than 10% of leaf area) flecks on leaf area lamina; 2 = chlorotic flecks covered 10% - 25% of leaf area lamina; 3 = 26% - 50% of leaf area shows symptoms; 4 = disease spots covered 51% - 70% of leaf areas; and 5 = most of leaf lamina (71% - 100%) are covered with chlorotic flecks. Plants were assigned a disease index (DI) as follows:

$$\mathrm{DI} = \left(\sum i \times j / n \times k\right) \times 100\%$$

in which $i$ = infection class, $j$ = the number of plants scored for that infection class, $n$ = the total number of plants in the replicate, and $k$ = the highest infection class.

### 2.6. Assays of Hydrogen Peroxide

A concentration of hydrogen peroxide ($H_2O_2$) was measured spectrophotometrically using the titanium ($Ti^{4+}$) method [19]. $H_2O_2$ was extracted from the plant leaf tissues at various time intervals after the leaves were sprayed with SNP. Some samples were then inoculated with *B. cinerea*, while some samples were not. Leaf material (0.2 g) from the plants was ground with a mortar and pestle in 1.5 ml of pre-chilled acetone. The homogenate was centrifuged at 15,000 × g for 10 min. The supernatant was collected and 1 ml of the supernatant was mixed with 0.1 ml 5% sodium titanium and 0.2 ml hartshorn. The reaction mixture was centrifuged for 10 min at 15,000 × g after precipitation occurred. The precipitate was collected and washed 5 times with acetone. Then 5 ml of 2 M HCl was added to dissolve the precipitate. After the precipitate was dissolved, the reaction solution was made with a final volume of 10 ml. This solution was used for the assay of $H_2O_2$ at 415 nm in a spectrophotometer. The experiment was repeated three times with similar results, and each experiment had three replications.

### 2.7. $H_2O_2$ Detection by the DAB Uptake Method

The formation of hydrogen peroxide was detected by using a fluorescent dye, 3,3-diaminobenzidine (DAB), following Orozco-Cardenas with minor modifications [20]. Some of the sample leaves from the pathogen inoculated plants were pretreated with SNP and some were not. These sample leaves were excised and immersed in 2 $mg \cdot ml^{-1}$ DAB solution (pH 3.8) at specific time intervals. The samples were then incubated in the dark at 24˚C for 48 hours. After reacting, the leaves were placed in a boiling, decolorizing solution for 5 - 10 minutes. The decolorizing solution contained acetic acid, glycerol, and ethanol at a ratio of 1:1:3. After cooling, the decolorized leaf was mounted in 60% glycerol and examined under a light microscope. $H_2O_2$ appeared reddish-brown in colour.

### 2.8. Histochemical Localization of $O_2^-$ Using NBT

In order to detect $O_2^-$ formation and accumulation in the leaves, nitro blue tetrazolium (NBT) staining was performed according to Brodersen with slight modifications [21]. Leaves inoculated with the pathogen, some of which had been treated with SNP, were excised from the plants and submerged in 0.1% NBT ($w \cdot v^{-1}$) (in 10 mM $NaN_3$ and 10 mM potassium phosphate buffer, pH 7.8) and shaken lightly for 24 hours. After staining, the leaves were washed with water and photographed.

### 2.9. Antioxidant Enzyme Assays

A small amount (0.2 g) of the leaf tissue was homogenized at 0˚C - 4˚C in 1.5 ml of 50 mM phosphate buffer, pH 7.8. The homogenate was centrifuged at 15000 × g for 15 minutes and the supernatant obtained was used as enzyme extract.

Catalase (CAT) activity was assayed by monitoring the consumption of $H_2O_2$ at 240 nm in a spectrophotometer. The 3 ml reaction mixture contained 1 ml of 0.3% $H_2O_2$, 1.9 ml of $H_2O$ and 0.1 ml of enzyme extract. The reduction rate of OD at 240 nm was recorded [16, 22]. The activity was expressed in U $g^{-1} \cdot FW^{-1}$ where one unit of catalase = 0.01 reduction in OD per minute.

Guaiacol peroxidase was measured colorimetrically with guaiacol as a substrate. The 4 ml reaction mixture contained 1ml of 50 mM phosphate buffer (pH 7.0), 2 ml of 0.3% $H_2O_2$ and 0.95 ml of 0.2% guaiacol. Then 0.05 ml enzyme extract was added to start the reaction. The linear increases in absorbance at 480 nm were monitored [14]. The activity was expressed in U $g^{-1} \cdot FW^{-1}$ where one unit = 0.01 increase in OD per minute.

The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of NBT following Beauchamp with minor modifications [23]. A volume of 3 ml of our reaction mixture contained 2.5 ml of 13 μM methionine, 0.15 ml of 13 μM riboflavin, 0.25ml of 75 μM NBT and 0.05 ml of 50 mM phosphate buffer (pH 7.8), and 0.05 ml of enzyme extract. Instead of NBT, the mixture contained a phosphate buffer to serve as control. Riboflavin was added last and the reaction was initiated by placing the tubes under 4000 lx light for 20 minutes. The absorbances at 560 nm were read. The volume of enzyme extract corresponding to 50% inhibition of the reaction was considered to be one enzyme unit.

## 3. Results

### 3.1. The Effect of SNP on Conidia Germination and Mycelial Growth of *B. cinerea*

Germination of the conidia was examined 24 hours after having been treated with 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 mM of SNP respectively. Germination of the conidia was obviously inhibited in those samples that had been treated with 5 or 10 mM of SNP. However, the lower concentrations of SNP treatment did not impact the germination of the conidia in the other samples (**Figure 1**). The mycelial growth of *B. cinerea* was not affected by the 0.01, 0.05, 0.1, 0.5 and 1mM SNP treatments, but was significantly restrained by the 5 and 10 mM SNP treatments (**Table 1**). Therefore, we chose 0.01, 0.05, 0.1 0.5, and 1 mM of SNP, levels which did not directly affect the development of *B. cinerea*, to optimize the following experiments.

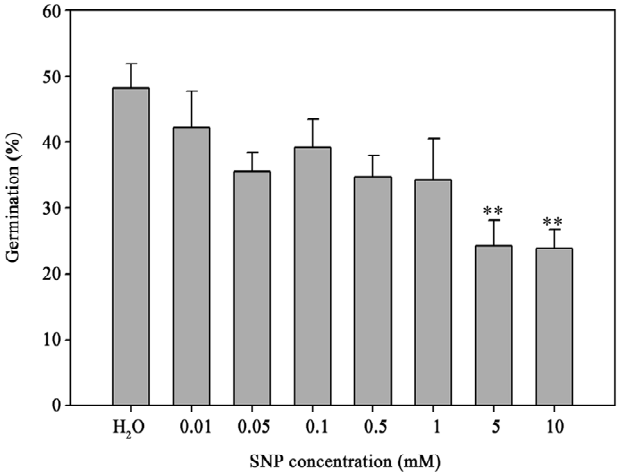



**Figure 1. Effect of NO generator—sodium nitroprusside (SNP) on conidia germination of *Botrytis cinerea* in vitro. A variety of SNP concentrations were supplied to medium containing $10^5$ $ml^{-1}$ fungal spore suspension (sterile water served as a control) and cultured at 22˚C ± 2˚C for 24 hours. Then the germinating spores were counted and the germination rate was calculated (desciption as method ). Values in this figure represent the means and SE from three independent experiments with three replicates each, *n* = 9. ** indicates values that differ significantly from the control at *P* < 0.01.**

**Table 1. Effect of SNP on the mycelial growth of *B. cinerea*.**

| SNPcontent (mmol/L) | Diameter of the fungal colony (cm/day) | suppression (%) |
|---|---|---|
| control | 5.10 ± 0.31 | - |
| 0.01 | 5.03 ± 0.39 | 1.52 |
| 0.05 | 4.93 ± 0.38 | 3.72 |
| 0.10 | 4.95 ± 0.39 | 3.30 |
| 0.50 | 4.44 ± 0.42 | 13.41 |
| 1.00 | 4.19 ± 0.40 | 18.34 |
| 5.00 | 1.62 ± 0.03** | 67.97 |
| 10.00 | 0.39 ± 0.05** | 92.60 |

Values represent the means and SE from three independent experiments with three replicates each, *n* = 9. ** indicates values that differ significantly from the control at *P* < 0.01.

## 3.2. Optimum Concentration and Interval of SNP Treatment

In order to test whether SNP has a toxic effect on *Arabidopsis* plants, we used various SNP concentrations that did not directly affect the development of *B. cinerea*, namely, 0.01, 0.05, 0.1, 0.5 and 1 mM, sprayed on the leaves. The leaves were examined each day for ten days after having been sprayed and the results showed that 0.01 to 0.5 mM had no significant effect on the leaves. However, the leaves treated by 1 mM of SNP turned yellow after 5 - 7 days.

We then sprayed 0.01, 0.05, 0.1 and 0.5 mM of SNP on the surface of the leaves. Three days later, we inoculated the leaves with *B. cinerea*. The symptoms of infection on the leaves were checked and compared each day from the first day to the seventh day after their inoculation (dpi). The results indicated that the optimum concentration of SNP was 0.5 and 0.1 mM (**Figure 2**).

The *Arabidopsis* leaves that had been pre-sprayed with 0.1 or 0.5 mM SNP (sprayed sterile water as a control) over a four-day period were then inoculated with the conidial suspension of *B. cinerea.* Twenty-four hours after being inoculated, the disease index was determined. The results showed that challenging with the pathogen 1 - 4 days after 0.5 mM SNP pretreatment and 1 day and 3 - 4 days 0.1 mM SNP pretreatment had an obvious effect in limiting the disease symptoms (**Table 2**). We chose 3 days pretreatment with both 0.5 and 0.1 mM SNP followed by inoculation with the pathogen as the optimum interval.

## 3.3. Effect of NO on *B. cinerea* Infection Development in *Arabidopsis* Leaves

Symptoms of infection in leaves inoculated with *B. cinerea* conidial suspension appeared on the surface of the leaves 2 days post inoculation. First, the edge and/or tip of the leaf started to curl. Then water-soaked spots appeared on the inoculation site of the leaf surface. These small spots developed into maceration or necrosis lesions (3 dpi). The lesions expanded and became dark and chlorotic within 5 days. On the seventh day after inoculation (7 dpi), the necrotic lesions had significantly expanded and covered about 90% of the leaf surface. However, on the leaves that were sprayed with 0.1 or 0.5 mM SNP three days prior to their inoculation with the

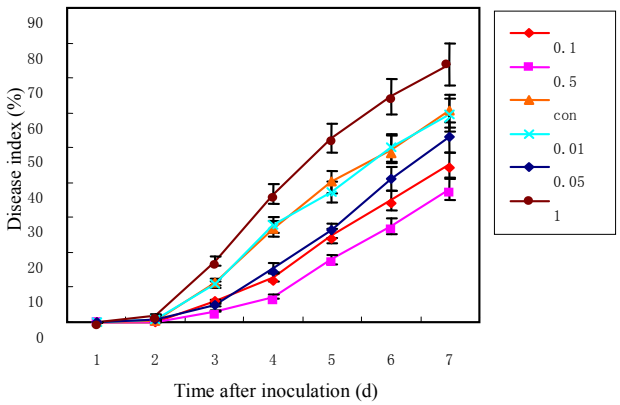



**Figure 2. Effect of SNP pretreatment on the disease index of *Arabidopsis*. Values represent the means and SE from three independent experiments with three replicates each, *n* = 9.**

**Table 2. Influence of interval SNP pretreatment on the rate of disease in *Arabidopsis*.**

| [w]SNP concentration （mmol/L） | [v]Rate of Disease | | |
|---|---|---|---|
| | 3th day | 4th day | 5th day |
| control | 30.33 ± 4.91 | 54.00 ± 5.51 | 71.67 ± 4.63 |
| 0.10 - 1 | 12.67 ± 1.86* | 29.33 ± 3.76** | 56.67 ± 5.36* |
| 0.10 - 2 | 24.00 ± 5.03 | 40.00 ± 4.36** | 65.33 ± 4.06* |
| 0.10 - 3 | 19.33 ± 3.53* | 37.67 ± 5.90** | 58.33 ± 5.49* |
| 0.10 - 4 | 27.00 ± 5.20 | 47.33 ± 5.78** | 63.67 ± 5.81* |
| 0.50 - 1 | 10.67 ± 2.19* | 28.00 ± 4.04** | 48.00 ± 4.36* |
| 0.50 - 2 | 10.33 ± 1.76* | 27.00 ± 1.53** | 52.67 ± 7.31* |
| 0.50 - 3 | 10.67 ± 3.84* | 23.33 ± 2.60** | 43.00 ± 5.86* |
| 0.50 - 4 | 18.33 ± 3.53* | 36.00 ± 4.73** | 57.67 ± 6.33* |

[w]0.1 - 1, 0.1 - 2, 0.1 - 3, 0.1 - 4: Leaves were sprayed with 0.1 mM of SNP and subsequently inoculation with pathogen interval 1, 2, 3 or 4 days respectively. 0.5 - 1, 0.5 - 2, 0.5 - 3, 0.5 - 4: Leaves were sprayed with 0.5 mM of SNP, subsequently inoculation with pathogen interval 1, 2, 3 or 4 days respectively. [v]days after inoculation with pathogen, the rate of disease of leaves was evaluated. Values represent the means and SE from three independent experiments with three replicates each, $n = 9$. * indicates values that differ significantly from the control at $P < 0.05$. ** indicates values that differ significantly from the control at $P < 0.01$.

fungus, the development of the infection was signifycantly restrained. The necrotic lesion appeared one day later than those in the control group, the lesions only affected about half of the leaf surface by the seventh day after inoculation (7 dpi), and the disease index was dramatically lowered from 3 to 7 dpi (**Figure 2**).

### 3.4. ROS Generation and Accumulation in SNP Treated Leaf Tissues

In our examination of the hydrogen peroxide ($H_2O_2$) content in *Arabidopsis* leaves, we compared those leaves that had been inoculated with *B. cinerea* with those leaves that were pretreated with SNP and then inoculated with *B. cinere*a (**Figure 3**). The $H_2O_2$ content in the pathogen-inoculated leaves was significantly increased 4 - 24 hours post inoculation (hpi) and reached its peak 24 hpi. The $H_2O_2$ content in the pathogen-inoculated leaves decreased a little and remained largely unchanged from 2 - 7 days post inoculation (dpi). The $H_2O_2$ content in the SNP pretreated and pathogen-inoculated leaves also increased 4 - 24 hpi. However, the increase was lower than that of the pathogen-inoculated leaves. Furthermore, the $H_2O_2$ content in the SNP pretreated and pathogen-inoculated leaves reached its peak at 48 hpi, one day later than the pathogen-inoculated leaves, and it decreased sharply after reaching this peak (**Figure 3**).

The cytochemical analyses detected the presence of $H_2O_2$ in the inoculated leaves that were pretreated with SNP and those that were not. The reddish-brown colouring of the leaves indicated a considerable accumulation of $H_2O_2$ (**Figure 4**). On those leaves without the SNP pretreatment, the reddish-brown colouring covered the whole surface on which the conidial suspension was transferred, starting from 2 dpi. On the leaves sprayed with 0.1 or 0.5 mM of SNP and inoculated with the fungus, the portions that were coloured were smaller and lighter. $H_2O_2$ was present only at the site of the necrosis formation. As the disease developed, the number and size of the spots on the leaves marking $H_2O_2$ synthesis increased. This was especially evident at the boundary between healthy and diseased tissue. In both experiments of $H_2O_2$ content assay and cytochemical detection, we got the same results. This suggests that NO retards the accumulation of $H_2O_2$ in leaves during the process of plant and pathogen interaction.

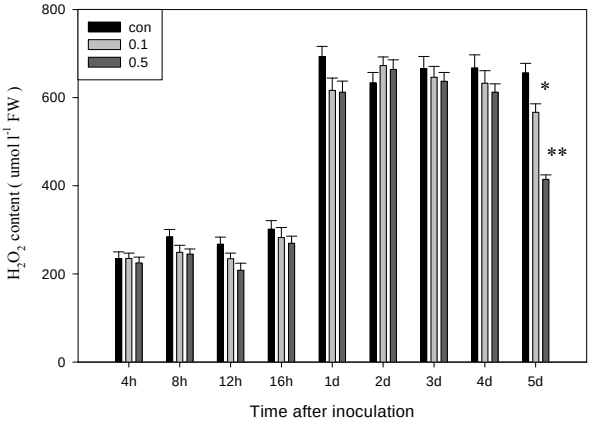



**Figure 3. Time course of changes in $H_2O_2$ accumulation in *Arabidopsis* leaves pretreatment with SNP and infection with *B. cinerea*. Values represent the means and SE from three independent experiments. * indicates values that differ significantly from the control at $P < 0.05$. ** indicates values that differ significantly from the control at $P < 0.01$.**


**Figure 4. A comparison of $H_2O_2$ accumulation revealed by 3,3-diaminobenzidine (DAB) staining in *Arabidopsis* leaves. (a) Control, sprayed with water and inoculation with *B. cinerea*; (b) sprayed with 0.1 mM SNP and inoculation with *B. cinerea*; (c) supplied 0.5 mM SNP and inoculation with *B. cinerea*. Photos were taken 2 dpi and shown the typical ones.**

The NBT staining of leaves was performed on the second day after the plants were inoculated with the *B. cinerea* conidial suspension. The dark blue coloring that appeared indicated the accumulation of $O_2^-$. In the pathogen-inoculated leaves, intensely dark blue spots appeared and were scattered across the whole surface of the leaves. However, in the leaves which were treated with SNP prior to being inoculated with the pathogen, the areas of dark blue coloring were smaller and confined (**Figure 5**).

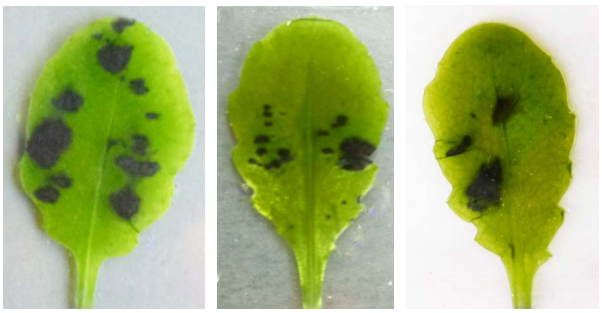
### 3.5. Effect of NO on the Antioxidant Enzymes

We tested four groups of *Arabidopsis* leaves: leaves treated with SNP; leaves sprayed with sterile water as a control; leaves inoculated with *B. cinerea*; and leaves pretreated with SNP which were then inoculated with *B. cinerea*. CAT and guaiacol peroxidase (POD) showed an increase in activity during the 7 day study period. This increase was especially significant in the leaves treated with SNP and then inoculated with pathogen (3 dpi) when compared with the increase in leaves without the SNP treatment [**Figures 6(a)**, **(b)**]. The CAT seemed more sensitive to 0.1 mM of SNP, and 0.5 mM worked a little better than the 0.1 mM in elevating POD activity.

Although SOD activities were slightly increased by the seventh day only in those leaves treated with SNP [about 7.79% in 0.1 mM and 10% in 0.5 mM SNP treatment was observed **Figure 6(c)**], no significant change in SOD activity in all of the leaves studied was observed.

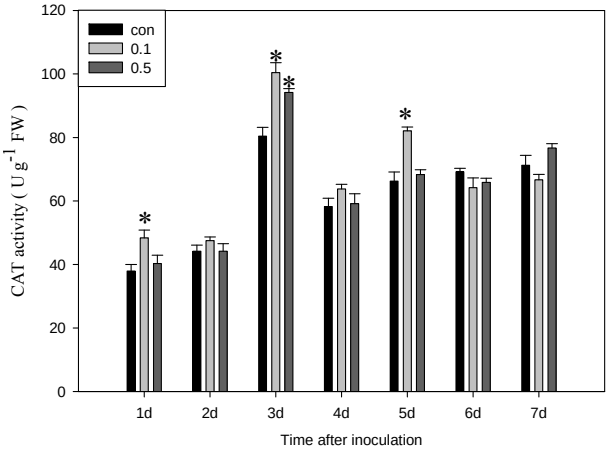
## 4. Discussion

The results of this study showed that NO enhanced the resistance of *Arabidopsis* to *B. cinerea* infection. The treatment of NO donor SNP on *Arabidopsis* leaves three

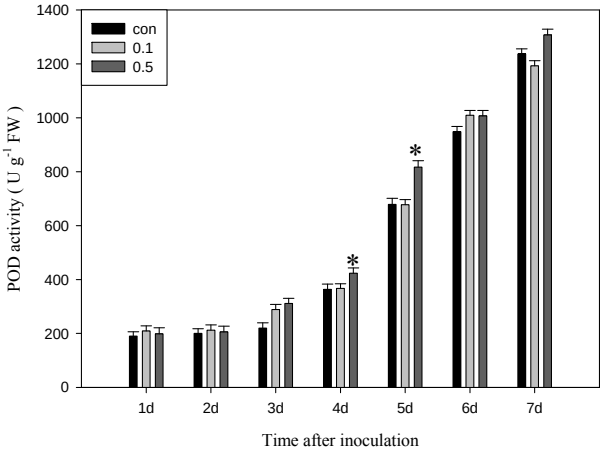


(a) (b) (c)

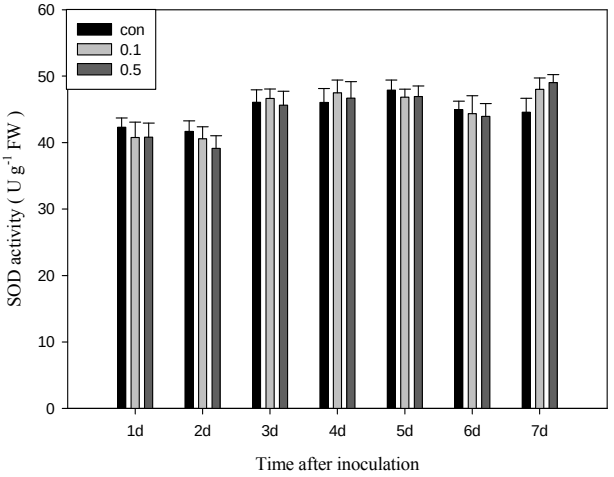
**Figure 5. Superoxide anion accumulation in *Arabidopsis* leaves. Shown is a comparison of typical photos of superoxide accumulation no treated or pretreated with SNP and inoculation with pathogen revealed by NBT. (a) Treated with water and inoculation with *B. cinerea*; (b) 0.1 mM SNP pretreatment and inoculation with *B. cinerea*; (c) 0.5 mM SNP pretreatment and inoculation with *B. cinerea*. Photos were taken 2 dpi.**




(a)




(b)




(c)

**Figure 6. CAT (a) POD (b) and SOD (c) activities in *Arabidopsis* leaves treated with 0.1 mM or 0.5 mM SNP, sprayed sterile water served as control (con) and inoculation with *B. cinerea*. Values represent the means and SE from three independent experiments. * indicates values that differ significantly from the control at $P < 0.05$.**

days prior to inoculation with fungus significantly hampered the development of symptoms of disease. The effective concentration (0.1 and 0.5 mM) of SNP did not have any direct role in reducing pathogen viability or inhibiting their development (**Figure 1** and **Table 1**). This suggests that NO induces the intrinsic resistance of *Arabidopsis* plants to *B. cinerea*.

Production of ROS during oxidative burst is one of the earliest and most effective defensive responses of plants [24-28]. The process of ROS generation in plant leaves during the interaction between *Arabidopsis* and *B. cinerea* has been previously elucidated [8,9,11,12,29,30]. *B. cinerea* can induce an oxidative burst and hypersensitive cell death in *Arabidopsis*. Additionally, levels of ROS, especially $H_2O_2$ levels during hypersensitive response, correlate positively with *B. cinerea* growth in plant tissues [8,31]. In this study we have examined the generation of ROS and the actions of antioxidant enzymes in an attempt to determine their roles in the NO treatment induced resistance reactions of *Arabidopsis* plants to *B. cinerea* infection.

The results show that the concentration of $H_2O_2$ increased rapidly during the early stage of pathogen infection both in plant leaves treated with SNP three days prior to inoculation with the pathogen and in those leaves not treated prior to inoculation. However, the time when the $H_2O_2$ concentration reached its peak was different between the two groups (those leaves treated with SNP and those not treated). The time of maximal $H_2O_2$ concentration was delayed one day in the leaves that were treated with SNP (**Figure 3**). $H_2O_2$ production in the SNP pretreated and pathogen-inoculated plants was lower than in the pathogen inoculated and SNP non-treated plants during the entire study period. Similar results were obtained from the histochemical determination of $H_2O_2$ and superoxide, which showed less accumulation of $H_2O_2$ and superoxide in SNP pretreated and pathogen-inoculated leaves. In conclusion, it is evident that the lower levels of $H_2O_2$ concentration induced by NO treatment in *Arabidopsis* plants enhanced their resistance to infection caused by *B. cinerea*. Thus, there appears to be a correlation between the induction and accumulation of $H_2O_2$ in host plants during compatible interactions with *B. cinerea* [8,9,11,12]. In many plant-pathogen systems, $H_2O_2$ also plays a prominent role in host cell death during infection by necrotrophic pathogens, and in oxidative burst [32]. But some studies noted that pretreatment with *o*-hydroxyethylorutin enhanced $H_2O_2$ generation in tomato leaves during the interaction of the tomato plants with *B. cinerea*. This higher level of $H_2O_2$ may act as a direct antimicrobial agent limiting the germination of pathogen spores, and enhancing the tomato plant's resistance [17]. A dual role for $H_2O_2$ in the process of plant-pathogen interaction is either protective or toxic, probably depending on its concentration and the plant species.

Early studies revealed that secretion of alcohol oxidases by *B. cinerea* can release $H_2O_2$ into the extracellular medium [33]. Additionally, superoxide dismutase (SOD), which is an $H_2O_2$-generating enzyme of *B. cinerea,* contributed to pathogenesis during the interaction of the pathogen with beans [34]. $H_2O_2$ could be produced by the fungus in order to enable *Botrytis* to colonize plant tissues [35]. However, Rij indicated that toxic levels of extracellular $H_2O_2$ were likely to be more harmful to the fungus than the plant [36]. Thus, it would be much more advantageous for the fungus if the $H_2O_2$ generation were to take place inside the plant cell compartment and thus be separated from sensitive fungal hyphae. A recent study showed that diffuse $H_2O_2$ probe signals were not observed in lily cells in the presence of *Botrytis elliptica* (a necrotrophic pathogen) mycelium, demonstrating that intracellular $H_2O_2$ was exclusively produced by live plant cells [13]. Although the sources of $H_2O_2$ still need to be clarified during the process of plant-necrotrophic interaction, the levels of ROS, mostly $H_2O_2$, in plants are increased and the accumulation of these compounds is concomitant with disease progression and *Arabidopsis* cell death. A significant reduction in $H_2O_2$ and superoxide content and/or the delay of the advent of the highest $H_2O_2$ content in *Arabidopsis* leaves as a result of NO donor treatment may suggest that NO can act as an antioxidant that inhibits $H_2O_2$ from signaling pathways that lead to cell death.

Data about the relationship between NO and antioxidant enzymes are also a source of controversy. SOD, CAT, ascorbate (APX) and guaiacol peroxidases (POD) have been shown to be inhibited by NO in tobacco and zinnia plants [16,37]. But different results also have been observed. The activities of SOD, CAT and APX in tomato cells remain unchanged in NO donor SNP pretreated plants [17]. Our results indicated that the activeity of the enzyme (SOD) generating $H_2O_2$ in *Arabidopsis* plant cells remained unchanged in the plants studied. However, the activity of CAT and guaiacol peroxidase (POD), enzymes that reduce $H_2O_2$, was significantly increased in *Arabidopsis* leaves through the treatment of SNP prior to inoculation with the pathogen. This suggests that the decrease in $H_2O_2$ content is due to the work of the antioxidant enzyme system. It also indicates that NO may play a role through regulating metabolic activity in the protection of cells against the destructive action of ROS. In the present study, we found that in the leaves pretreated with SNP, POD activity rapidly and signifycantly increased 24 hours after the leaves were inoculated with *B. cinerea* and this activity kept increasing during the entire study period (**Figure 6(b)**). Tiedemann

reported that *B. cinerea* suppressed the activity of plant peroxidase in bean leaf discs [9]. His findings suggested that peroxidases play a role as scavengers of harmful $H_2O_2$ in plant resistance. POD can not only scavenge $H_2O_2$ in the initial stage of infection, but can also be active in further resistance reactions (e.g., the cross-linking of cell wall proteins and the polymerization of lignin precursors), thus providing protection from pathogen invasion. Our results offer evidence that NO might stimilate antioxidant enzymes which destroy ROS in plant cells. This triggers subsequent defense reactions in the plant and enhances the resistance of the *Arabidopsis* plant to infection by *B. cinerea*.

## 5. Conclusions

1) Low concentrations (0.1 and 0.5 mM) of nitric oxide donor sodium nitroprusside (SNP) had neither a direct, toxic effect on conidial germination and mycelial growth of *B. cinerea*, nor a toxic impact on development of *Arabipdopsis*. But high concentrations of SNP, such as 5 mM and 10 mM, were harmful to both pathogen and plant.

2) Applying 0.1 and 0.5 mM SNP to *Arabidopaia* leaves could induce disease resistance of *Arabidopsis* to *B. cinerea*. The exogenous NO could delay ROS burst and restrain the rapid accumlation of $H_2O_2$ and $O_2^-$ in plants thereby delaying progression of disease.

3) The biochemical mechanism of ROS reduction might be related to exogenous NO stimulation of the antioxidase POD and CAT activities. The changes of SOD (generating $H_2O_2$) activities were not obvious in plants which were pretreated with SNP and subsequently inoculated with a pathogen. Therefore NO might play an important role in scavenging $H_2O_2$ and enhancing resistance of *Arabidopsis* to *B cinerea*.

## 6. Acknowledgements

This work was funded by the National Natural Science Foundation of China (grant no. 30860121) and the Open Program of State Key Laboratory of Plant Physiology and Biochemistry, China Agriculture University (PPB08010).

## REFERENCES

[1] J. Durner and D. F. Klessig, "Nitric Oxide as a Signal in Plants," *Current Opinion in Plant Biology*, Vol. 2, No. 5, 1999, pp. 369-374.

[2] M. Delledonne, Y. Xia, R. A. Dixon and C. Lamb, "Nitric Oxide Functions as a Signal in Plant Disease Resistance," *Nature*, Vol. 394, No. 6, 1998, pp. 585-588.

[3] M. Arasimowicz and W. J. Floryszak, "Nitric Oxide as a Bioactive Signalling Molecule in Plant Stress Responses," *Plant Science*, Vol. 172, No. 5, 2007, pp. 876-887.

[4] M. G. Zhao, Q. Y. Tian and W. H. Zhang, "Nitric Oxide Synthase-Dependent Nitric Oxide Production Is Associated with Salt Tolerance in *Arabidopsis*," *Plant Physiology*, Vol. 144, No. 1, 2007, pp. 206-217.

[5] M. Delledonne, "NO News Is Good News for Plants," *Current Option in Plant Biology*, Vol. 8, No. 4, 2005, pp. 390-396.

[6] P. Wojtaszek, "Oxidative Burst, an Early Plant Response to Pathogen Infection," *Biochemical Journal*, Vol. 322, No. 3, 1997, pp. 681-692.

[7] D. G. Gilchrist, "Programmed Cell Death in Plant Disease, the Purpose and Promise of Cellular Suicide," *Annual Review of Phytopathology*, Vol. 36, 1998, pp. 393-414.

[8] E. M. Govrin and A. Levine, "The Hypersensitive Response Facilitates Plant Infection by the Necrotrophic Pathogen *Botrytis cinerea*," *Current Biology*, Vol. 10, No. 13, 2000, pp. 751-757.

[9] A. V. Tiedemann, "Evidence for a Primary Role of Active Oxygen Species in Induction of Host Cell Death during Infection of Bean Leaves with *Botrytis cinerea*," *Physiological and Molecular Plant Pathology*, Vol. 50, No. 3, 1997, pp. 151-166.

[10] A. J. Able, "Role of Reactive Oxygen Species in the Response of Barley to Necrotrophic Pathogens," *Protoplasma*, Vol. 221, No. 1-2, 2003, pp. 137-143.

[11] W. Edlich, G. Lorenz, H. Lyr, E. Nega and E. H. Pommer, "New Aspects on the Infection Mechanism of *Botrytis cinerea* Pers," *European Journal of Plant Pathology*, Vol. 95, Supplement 1, 1989, pp. 53-62.

[12] Y. Elad, "The Use of Antioxidants (Free Radical Scavengers) to Control Grey Mold (*Botrytis cinerea*) and White Mold (*Sclerotinia sclerotiorum*) in Various Crops," *Plant Pathology*, Vol. 41, No. 4, 1992, pp. 417-426.

[13] P. V. Barrlen, M. Staats and J. A. L. V. Kan, "Induction of Programmed Cell Death in Lily by the Fungal Pathogen *Botrytis elliptica*," *Molecular Plant Pathology*, Vol. 5, No. 6, 2004, pp. 559-574.

[14] U. Małolepsza and H. Urbanek, "The Oxidants and Antioxidant Enzymes in Tomato Leaves Treated with O-Hydroxyethylorutin and Infected with *Botrytis cinerea*," *European Journal of Plant Pathology*, Vol. 106, No. 7, 2000, pp. 657-665.

[15] J. Floryszak-Wieczorek, M. Arasimowicz, G. Milczarek, H. Jelen and H. Jackowiak, "Only an Early Nitric Oxide Burst and the Following Wave of Secondary Nitric Oxide Generation Enhanced Effective Defence Responses of Pelargonium to a Necrotrophic Pathogen," *New Phytologist*, Vol. 175, No. 4, 2007, pp. 718-730.

[16] D. Clark, J. Durner, D. A. Navarre and D. F. Klessig, "Nitric Oxide Inhibition of Tobacco Catalase and Ascorbate Peroxidase," *Molecular Plant Microbe Interaction*, Vol. 13, No. 12, 2000, pp. 1380-1384.

[17] U. Małolepsza and S. Rózalska, "Nitric Oxide and Hydrogen Peroxide in Tomato Resistance, Nitric Oxide Modulates Hydrogen Peroxide Level in O-Hydroxyethylorutin-Induced Resistance to *Botrytis cinerea* in Tomato," *Plant Physiology and Biochemistry*, Vol. 43, No. 6, 2005, pp.

623-635.

[18] S. L. Murray, C. Thomson, A. Chini, N. D. Read and G. J. Loake, "Characterization of a Novel, Defense-Related *Arabidopsis* Mutant, *cir*1, Isolated by Luciferase Imagin," *Molecular Plant—Microbe Interactions*, Vol. 15, No. 6, 2002, pp. 57-566.

[19] M. Becana, P. Aparicio-Tejo, J. J. Irigoyen and D. M. Sanchez, "Some Enzymes of Hydrogen Peroxide Metabolism in Leaves and Root Nodules of *Medicago sativa*," *Plant Physiology*, Vol. 82, No. 4, 1986, pp. 1169-1171.

[20] M. L. Orozco-Cardenas and C. A. Ryan, "Hydrogen Peroxide Is Generated Systemically in Plant Leaves by Wounding and Systemin via the Octadecanoid Pathway," *Proceedings of the National Academy of Sciences in the USA*, Vol. 96, No. 11, 1999, pp. 6553-6557.

[21] P. Brodersen, M. Petersen, H. M. Pike, B. Olszak, S. Skov, N. dum, L. B. Jørgensen, R. E. Brown and J. Mundy, "Knockout of *Arabidopsis ACCELERATED-CELL-DEATH*11 Encoding a Sphingosine Transfer Protein Causes Activation of Programmed Cell Death and Defense," *Genes & Development*, Vol. 16, No. 4, 2002, pp. 490-502.

[22] I. Cakmak and H. Marschner, "Magnesium Deficiency and High Light Intensity Enhance Activities of Superoxide Dismutase, Ascorbate Peroxidase, and Glutathione Rreducatse in Bean Leaves," *Plant Physiology*, Vol. 98, No. 4, 1992, pp. 1222-1227.

[23] C. Beauchamp and I. Fridovich, "Superoxide Dismutase, Improved Assays and Assay Applicable to Acrylamide Gels," *Analytical Biochemistry*, Vol. 44, No. 1, 1971, pp. 276-287.

[24] N. Doke, "Involvement of Superoxide Generation in the Hypersensitive Response of Potato Tuber Tissues to Infection with an Incompatible Race of *Phytophthora infestans* and to Hyphal Wall Components," *Physiological Plant Pathology*, Vol. 23, No. 3, 1983, pp. 345-357.

[25] M. W. Sutherland, "The Generation of Oxygen Radicals during Host Plant Responses to Infection," *Physiological and Molecular Plant Pathology*, Vol. 39, No. 2, 1991, pp. 79-93.

[26] M. C. Mehdy, "Active Oxygen Species in Plant Defense against Pathogens," *Plant Physiology*, Vol. 105, No. 2, 1994, pp. 467-472.

[27] M. C. Mehdy, Y. K. Sharma, K. Sathasivan and N. W. Bays, "The Role of Activated Oxygen Species in Plant Disease Resistance," *Physiology Plant*, Vol. 98, 1996, pp. 365-374.

[28] R. Tenhaken, A. Levine, L. F. Brisson, R. A. Dixon and C. Lamb, "Function of the Oxidative Burst in Hypersensitive Disease Resistance," *Proceedings of the National Academy of Sciences of the USA*, Vol. 92, No. 10, 1995, pp. 4158-4163.

[29] T. Mengiste, X. Chen, J. Salmeron and R. Dietrich, "The *BOTRYTIS SUSCEPTIBLE*1 Gene Encodes an R2R3MYB Transcription Factor Protein That Is Required for Biotic and Abiotic Stress Responses in *Arabidopsis*," *Plant Cell*, Vol. 15, No. 11, 2003, pp. 2551-2565.

[30] P. Veronese, X. Chen, B. Bluhm, J. Salmeron, R. Dietrich and T. Mengiste, "The *BOS* Loci of *Arabidopsis* Are Required for Resistance to *Botrytis cinerea* Infection," *The Plant Journal*, Vol. 40, No. 4, 2004, pp. 558-574.

[31] C. H. Unger, S. Kleta, G. Janell and A. Tiedemann, "Suppression of the Defence-Related Oxidative Burst in Leaf Tissue and Bean Suspension Cells by the Necrotrophic Pathogen *Botrytis cinerea*," *Journal of Phytopathology*, Vol. 153, No. 1, 2005, pp. 15-26.

[32] Y. Tada, T. Mori, T. Shinogi, N. Yao, S. Takahashi, S. Betsuyaku, M. Sakamoto, P. Park, H. Nakayashiki, Y. Tosa and S. Mayama, "Nitric Oxide and Reactive Oxygen Species Do Not Elicit Hypersensitive Cell Death but Induce Apoptosis in the Adjacent Cells during the Defense Response of Oats," *Molecular Plant—Microbe Interact*, Vol. 17, No. 3, 2004, pp. 245-253.

[33] R. P. Pratt, A. E. Brown and P. C. Mercer, "A Role for Hydrogen Peroxide in Degradation of Flax Fibre by *Botrytis cinerea*," *Transactions of the British Mycological Society*, Vol. 91, No. 3, 1988, pp. 481-488.

[34] Y. Rolke, S. liu, T. Quidde, B. Williamson, A. Schouten, K. M Weltring, V. Siewers, K. B. Tenberge, B. Tudzynski and P. Tudzydki, "Functional Analysis of $H_2O_2$-Generating Systems in *Botrytis cinerea*, the Major Cu-Zn-Superoxide Dismutase (BCSOD1) Contributes to Virulence on French bean, Whereas a Glucose Oxidase (BCGOD1) Is Dispensable," *Molecular Plant Pathology*, Vol. 5, No. 1, 2004, pp. 17-27.

[35] M. Weigend and H. Lyr, "The Involvement of Oxidative Stress in the Pathogenesis of *Botrytis cinerea* on *Vicia faba* Leaves," *Journal of Plant Diseases and Protection*, Vol. 103, 1996, pp. 310-320.

[36] R. E. Rij and C. F. Forney, **"**Phytotoxicity of Vapour Phase Hydrogen Peroxide to Thompson Seedless Grapes and *Botrytis cinerea* Spores," *Crop Protection*, Vol. 14, No. 2, 1995, pp. 131-135.

[37] M. A. Ferrer and A. R. Barcelo, "Differential Effects of Nitric Oxide on Peroxidase and $H_2O_2$ Production by the Xylem of *Zinnia elegans*," *Plant*, *Cell & Environment*, Vol. 22, No. 7, 1999, pp. 891-897.