Defence responses of tomato fruit to exogenous nitric oxide during postharvest storage

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A B S T R A C T
Nitric oxide (NO), an important signalling molecule, has shown diverse physiological functions in plants. We investigated physiological responses of harvested tomato fruit (Solanum lycopersicum cv. Ailsa Craig, AC) to NO treatment. NO released by 1 mM sodium nitroprusside (SNP) aqueous solution could effectively retard pericarp reddening of tomato fruit, suppress ethylene production, and influence quality parameters during storage. The activity of antioxidant enzymes in NO-treated tomato fruit was higher in the late storage period compared to the control. RT-PCR analysis showed that expression of six genes related to fruit ripening was regulated by NO treatment, resulting in an increase in resistance of tomato fruit to gray mold rot caused by Botrytis cinerea. Our results demonstrated that application of NO could be a potential method for treating harvested fruit in order to delay ripening, maintain quality and enhance resistance of fruit to fungal pathogens.

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1. Introduction

Fruit lose quality after harvest because of environmental stress and pathogen infection. Application of some exogenous chemical compounds, such as salicylic acid (Tian et al., 2007; Chan et al., 2008), jasmonic acid (Yao and Tian, 2005; Han and Tian, 2006) and oxalic acid (Zheng et al., 2007; Wang et al., 2009), has been shown to effectively delay fruit senescence, enhance resistance and maintain quality of various fruit crops. NO is a bioactive molecule that exerts a number of diverse signal functions in phylogenetically distant species (Belligni and Lamattina, 2001). As a gaseous free radical, it is one of the smallest diatomic molecules with a high diffusivity (4.8 × 10⁻⁵ cm² s⁻¹ in H₂O), exhibiting hydrophobic properties (Thomas et al., 2008). Thus, NO not only easily migrates in the hydrophilic regions of the cell, such as the cytoplasm, but also freely diffuses through the lipid phase of membranes (Aramiswicz and Floryszak-Wieczorek, 2007). Leshem et al. (1998) provided evidence for the function of NO as an endogenous maturation and senescence regulating factor in higher plants. In recent years, many research reports have shown that NO at a low concentration can effectively extend postharvest life of various fruit, such as strawberry (Soegiarto and Wills, 2006), peach (Zhu et al., 2006), longan (Duan et al., 2007), a number of horticultural crops (Wills et al., 2007), plum (Singh et al., 2009) and winter jujube (Zhu et al., 2009).

However, most results on postharvest NO effects have come from research using biochemical and physiological approaches. Little information focused on NO function with regard to regulating fruit physiology at the molecular level has been reported.

Tomato fruit characteristically follow a climacteric ripening pattern controlled by ethylene (Carrari and Fernie, 2006). A wide range of physical, biochemical and physiological changes occur relatively fast in tomato fruit after harvest (Guillén et al., 2006). In addition, substantial tomato genome sequence is now available, which is beneficial for exploring mechanisms by which exogenous factors regulate fruit ripening and resistance at the molecular level in relation to postharvest storage techniques.

Tomato fruit ripening is usually accompanied by a burst in ethylene production, peel color reddening and flesh softening. Wang et al. (2002) indicated that the genes LeACS2, LeACS4, and LeACO1 from the relevant gene families were most responsible for the massive ethylene production. In addition, the change in pigmentation is caused by accumulation of lycopene within the plastids (Fraser et al., 1994), and polygalacturonase (PG) activity is largely related to pectin depolymerization and solubilization (Villarreal et al., 2008), although PG-mediated pectin depolymerization requires pectin to be de-methyl-esterified by pectin methylesterase (PME) (Pelloux et al., 2007). Therefore, exploring changes in expression of such genes is beneficial to an understanding of responses of fruit to exogenous factors during ripening.

In this study, we determined the potential effects of NO in regulating fruit ripening, evaluated possible mechanisms based on
mRNA information of the above genes, and characterized changes in quality parameters of tomato fruit with or without NO treatment during storage.

2. Materials and methods

2.1. Plant material and pathogen

Tomato (Solanum lycopersicum cv. Ailsa Craig, AC) fruit at the mature green (MG) stage were harvested from a greenhouse in Beijing, China. Fruit without any physical injuries or decay were washed in sodium hypochlorite solution 2% (v/v) for 2 min, then rinsed with tap water and air-dried prior to use.

Botrytis cinerea was isolated from naturally infected tomato fruit and cultured on potato dextrose agar (PDA) at 25 °C for 2 weeks. Fungal spores were obtained by flooding the surface of the culture with sterile distilled water containing 0.05% (v/v) Tween-20. The suspension was filtered through four layers of sterile cheesecloth and adjusted to a concentration of 1 × 10^4 spores mL^-1 using a hemocytometer.

2.2. Treatment with SNP

Sodium nitroprusside (SNP) was purchased from Sigma–Aldrich (St Louis, MO, USA). In the preliminary experiment, we tested a series of SNP concentrations and found that a concentration of 1 mM, which could maintain about 5 μM NO in solution for 10 h, significantly limited pericarp reddening. Therefore, SNP at 1 mM concentration was used in the following experiments. Tomato fruit were immersed in 1 mM SNP aqueous solution, or water as a control, for 30 min, dried in air at 25 °C for 2 h, and then divided into two groups. For the first group, the fruit were wounded (4 mm deep and 3 mm wide) at the equator using a sterile nail and inoculated with Fungal spores were obtained by flooding the surface of the culture with sterile distilled water containing 0.05% (v/v) Tween-20. The suspension was filtered through four layers of sterile cheesecloth and adjusted to a concentration of 1 × 10^4 spores mL^-1 using a hemocytometer.

For the second group, the fruit without pathogen inoculation were directly put into plastic trays as the control. At regular intervals, quality parameters were evaluated under the same environmental conditions. The entire experiment was repeated twice.

2.3. Effect of NO at different concentrations on B. cinerea

Aliquots of a spore suspension of B. cinerea were added to 100 mL potato dextrose broths (PDB) to obtain a final concentration of 1.0 × 10^6 spores mL^-1. The culture media were supplemented with different concentrations (0, 0.5, 1, 2, and 4 mM) of SNP, and the flasks were incubated at 25 °C on a rotary shaker at 200 rpm. Spore germination and germ tube length were assayed microscopically after 5 and 9 h of incubation.

2.4. Determination of ethylene production and fruit quality parameters

Fifteen fruit (about 500 g) were placed in 5 L air-tight chamber for each treatment at 25 °C. After 2 h, 1 mL of head space gas was removed from the container using a syringe, then injected into a gas chromatograph (SQ-206, Beijing, China), equipped with an activated alumina column and a flame ionization detector (FID) for ethylene determination.

Flesh firmness was determined on opposite peeled cheeks of fruit using a hand-held firmness tester (FT-327, Italy), equipped with a cylindrical plunger 8 mm in diameter. Soluble solids content (SSC) was determined using an Abbe Refractometer (10481 S/N, USA). Titratable acidity (TA) was analyzed by titration with 0.01 M NaOH up to pH 8.3 in 25 mL of diluted juice from 5 g flesh.

Relative electric conductivity was measured using a conductivity meter (Model EC 215, Italy) as described by Wang et al. (2005). Ten discs, 10 mm diameter and 3 mm thick, from 10 fruit were washed three times in deionized water, and put together in 50 mL conical flasks containing 30 mL of deionized water. After 3 h of incubation at 25 °C, the initial electrolyte leakage was determined (D1). The solution was then placed in a water bath (95 °C) for 30 min before the final conductivity (total electrolyte leakage, D2) was measured. Relative electric conductivity = [(D2 – D1)/D2] × 100%.

2.5. Measurement of antioxidant enzyme activity

Samples of 10 g flesh were obtained from 15 fruit per treatment and homogenized in 25 mL of ice-cold extraction buffer and 0.5 g polyvinyl polypyrrolidone (PVPP) with a Kinematica tissue grinder (Crl-6010, Kriens-LU, Switzerland). For superoxide dismutase (SOD) and catalase (CAT) assays, the extraction buffer was 50 mM sodium phosphate (pH 7.8). For peroxidase (POD), 100 mM sodium phosphate buffer (pH 6.4) was used. The homogenate was centrifuged at 27,000 × g for 50 min at 4 °C, and the supernatants were used for the assay. Activity of the enzymes was determined by a spectrophotometer (Shimadzu, Japan) according to previous reports (Wang et al., 2005; Ding et al., 2006).

2.6. RNA isolation and semi-quantitative RT-PCR analysis

The total RNA was extracted from 5 g of pericarp tissue in liquid nitrogen and extracted with phenol as described by Moore et al. (2005). The residual DNA was digested by RNase-Free DNase (Promega, USA), and 2 μg of RNA was used to synthesize the first-strand cDNA with M-MLV Reverse Transcriptase (Promega, USA) according to the manual protocol. Semi-quantitative RT-PCR was performed as previously described by Spencer and Cistensen (1999). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from tomato was used as internal control to normalize the cDNA stock of each sample (Eum et al., 2009).

To amplify sequences across intron regions, specific primers targeting different genes (LeGAPDH, LeACS2, LeACS4, LeACO1, LePG, LePME and LePhy1) were designed from deposited cDNA sequences in Nucleotide Database of National Center for Biotechnology Information using the Primer 5.0 Software (listed in Table 1) and synthesized by the Beijing Genomics Institute (Beijing, China).

PCR reactions were conducted in a total volume of 25 μL containing 0.5 μL cDNA, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 12.5 μL 2 × Taq PCR Master Mix (Tiangen, China) and 11 μL deionized water. PCR conditions for amplification were 94 °C for 3 min followed by 25 or 40 cycles (25 for LeACS2, LeACO1, LePG, LePhy1, LeGAPDH; 40 for LeACS4 and LePME) of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min. PCR products were separated on 1.2% agarose gels. The density of each band was quantified using Scion Image software (Scion Corporation, Frederick, MD, USA).

2.7. Statistical analysis

Data were pooled across three independent repeated experiments and were performed with SPSS software (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to compare more than two means, and Duncan’s multiple range test was used for means separations. Differences at P=0.05 were considered to be significant.
3. Results

3.1. Effects of NO on peel color and ethylene production

After 20 days at 25 °C, comparing to the controls, the change of pericarp color had been significantly delayed in the tomato fruit with NO treatment (Fig. 1A). In addition, ethylene production of tomato fruit was also suppressed by NO. The rate of ethylene release in the fruit without the NO treatment initially increased and reached a peak value of 5.7 μmol kg\(^{-1}\) h\(^{-1}\) after 12 days, then gradually decreased. However, the ethylene release rate in NO-treated fruit was still lower compared to control fruit during storage, and reached a peak value of 4.9 μmol kg\(^{-1}\) h\(^{-1}\) after 16 days (Fig. 1B). The results indicate that NO has a significant effect on limiting pericarp color reddening and ethylene production in tomato fruit, which leads to a delay in fruit ripening. (For interpretation of the references to color in this paragraph, the reader is referred to the web version of the article.)

3.2. Effect of NO on fruit quality

NO treatment markedly affected fruit quality. SSC in all treatments showed an increasing trend during storage. However, since NO effectively limited fruit ripening (Fig. 1A), there were lower levels of SSC in NO-treated fruit compared to the controls (Fig. 2A). Although TA, firmness and relative electrical conductivity in all fruit decreased gradually with increasing storage duration, NO treatment significantly delayed the rate of decrease of these parameters (Fig. 2B–D).

3.3. Effects of NO on resistance of tomato fruit

As shown in Fig. 3, the activities of SOD in all fruit increased early in storage, then decreased gradually. However, NO maintained relatively higher activities of SOD in treated fruit after 12 days \((P=0.001)\) than those in the control (Fig. 3A). Although CAT activity gradually decreased in all fruit during storage, it was at a higher level in NO-treated fruit compared to the control at 8 and 12 days \((P=0.049\) and \(P=0.044\), Fig. 3B). POD activity in control fruit initially decreased, then increased to a peak at 12 days, and constantly decreased afterwards. A similar trend was found in NO-treated fruit with a higher activity than that of the control at 12 and 16 days \((P=0.041\) and \(P=0.035\), Fig. 3C).

Additionally, NO treatment effectively maintained or enhanced resistance of tomato fruit against gray mold rot caused by B. cinerea, resulting in lower disease incidence and smaller lesion diameters in NO-treated fruit during the storage period of 48 h to 96 h (Fig. 4A and B). The enhanced resistance of fruit to the fungal pathogen caused by NO is indirect because at least 4 mM SNP resulted in a significant inhibitory effect on B. cinerea in vitro (Table 2).

3.4. Effects of NO on expression of six age-related genes

From Fig. 5, it is clear that expression of LeACS2 gradually increased with increased storage time in all fruit samples, but a higher level, about 1.5 times that in NO-treated fruit, was detected in the control fruit after 8 days. However, LeACS4 levels were relatively higher in NO-treated fruit than those in controls. mRNA levels of LeACO1, LePG and LePhy1 increased gradually in fruit with or without NO treatment during the whole storage period, but their abundances were obviously higher in controls than those in NO-treated fruit. Expression of LePME reached a peak after 16 days of storage in control fruit, but this was not obvious in NO-treated fruit.

4. Discussion

Fruit often show physiological responses when exposed to exogenous elicitors (Tian et al., 2006). Defense response of fruit to fungal pathogens, induced by salicylic acid (SA) and oxalic acid (OA), is related to maturity and ripening. SA can enhance fruit resistance by up-regulating antioxidant proteins and relative gene expression in peach (Chan et al., 2007) and sweet cherry fruit (Chan et al., 2008), whereas OA may limit ethylene biosynthesis in mango (Zheng et al., 2007) and jujube fruit (Wang et al., 2009). In our experiments, we observed that NO could effectively retard pericarp color reddening and suppress ethylene production in tomato fruit at the MG stage (Fig. 1), which indicates that NO is beneficial for delaying fruit ripening, resulting in higher resistance to...
the fungal pathogen *B. cinerea* as compared to the controls (Fig. 4). Eum and Lee (2007) reported that NO effectively reduced the rate of ethylene production in strawberries and delayed fruit softening during storage compared to untreated fruit. NO has a potential effect on regulating ethylene biosynthesis in various fruit, which affects physiological changes related to fruit ripening, and leads to enhance resistance to fungal pathogens. Ethylene plays a critical role in fruit ripening in stimulating transcription and translation of ripening-related genes, such as polygalacturonase, pectin methylesterase, and β-galactosidase (Smith et al., 1998). Two key enzymes, 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), effectively regulate ethylene production (Alexander and Grierson, 2002). Barry et al. (1996) considered that LeACO1 and, at a lower level LeACO3, were expressed at the onset of fruit ripening, indicating that the first step in catalytic ethylene biosynthesis was the synthesis of ACO1. Barry et al. (2000) found that LeACS2 expression required ethylene, whereas LeACS1A and LeACS4 exhibited only slightly delayed expression in the never-ripe mutant. Xie et al. (2006) showed that transgenic tomatoes carrying antisense LeACS2 produced less ethylene and failed to ripen, with complete suppression of the LeACS2 and LeACS4 genes during ripening. Zhu et al. (2006) investigated the inhibitory effect of NO on ethylene biosynthesis and LOX activity in peach fruit, and they suggested that NO is bound to ACC oxidase to form an ACC oxidase–NO complex, which is chelated by ACC to produce an ACC–ACC oxidase–NO complex, leading to a decrease in ethylene production. However, the mechanisms by which NO regulates fruit ripening in biological systems were not completely clear. In this study, we provided evidence that NO could regulate expression of ethylene-related genes in tomato fruit (Fig. 5). Our results demonstrated that suppression of ethylene production in NO-treated fruit might be attributed to the suppression of LeACS2, LeACS4, and LeACO1 gene expression (Fig. 5). Eum et al. (2009) recently demonstrated that NO treatment effectively delayed the burst of ethylene production and color development of tomato fruit at the breaker (BR) stage, and regulation of ethylene biosynthesis involved suppression of ACO rather than ACS. The difference between these results and ours may be due to: (1) the different variety (*Solanum lycopersicum* L. Myrock or *Solanum lycopersicum* cv. Ailsa Craig), (2) the different treatment method (NO gas or SNP) and (3) analysis interval and range.

Fruit softening often occurs during fruit ripening, particularly in the climacteric fruit, because of degradation of cell wall components, a consequence of the coordinated action of cell wall-modifying enzymes and proteins (Brummell and Harpster, 2001). As is well known, PG, as a major cell wall polyuronide degrading enzyme, is transcriptionally activated during ripening, and the PG
Effect of NO on spore germination and germ tube elongation of Botrytis cinerea in potato fruit during 4 days storage at 25 °C. Values in each column followed by a different letter are statistically different according to least significant difference test (P < 0.05).

Table 2
Effect of NO on spore germination and germ tube elongation of Botrytis cinerea in potato dextrose broth.

<table>
<thead>
<tr>
<th>SNP (mM)</th>
<th>5 h</th>
<th>9 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spore germination (%)</td>
<td>Germ tube length (µm)</td>
</tr>
<tr>
<td>0</td>
<td>28.90 ± 2.52a</td>
<td>26.33 ± 3.08a</td>
</tr>
<tr>
<td>0.5</td>
<td>29.26 ± 3.99a</td>
<td>28.03 ± 2.40a</td>
</tr>
<tr>
<td>1</td>
<td>26.81 ± 3.72a</td>
<td>24.57 ± 2.46a</td>
</tr>
<tr>
<td>2</td>
<td>25.29 ± 2.92a</td>
<td>25.53 ± 3.49a</td>
</tr>
<tr>
<td>4</td>
<td>15.75 ± 3.71b</td>
<td>17.43 ± 2.68b</td>
</tr>
</tbody>
</table>

Values in each column followed by a different letter are statistically different according to least significant difference test (P < 0.05).

Fig. 4. Effects of NO on disease incidence (A) and lesion diameter (B) of B. cinerea in tomato fruit during 25 °C. Values in each column followed by a different letter are significant between samples according to Student’s t-test at P < 0.05.

promoter sequence contains ethylene dependent ripening-specific control elements (Nicholass et al., 1995). Analysis of low PG fruit cell walls showed reduced amounts of water-soluble polyuronides, matched by an increase in calcium carbonate-soluble polyuronides suggesting that PG depolymerizes covalently bound pectin allowing it to solubilize into an aqueous fraction (Carrington et al., 1993). In addition, PME has been proved to be responsible for de-esterification of the highly methyl-esterified polygalacturonans in the cell wall during fruit ripening (Koch and Nevins, 1989). Our results further suggest that NO suppressed expression of LePG and LePME genes in tomato fruit, possibly resulting in a delay of cell wall polyuronide solubilization, and thus potentially affecting pectin metabolism and fruit softening directly or indirectly.

Fig. 5. RT-PCR analysis for the expression of LeACS2, LeACS4, LeACO1, LePG, LePME, and LePhy1 in harvested tomato during 20 days storage period at 25 °C. PCR products were separated on 1.2% agarose gels. LeGAPDH was used as a control for normalizing mRNA quantity.

Fruit, in defense response to exogenous elicitors, usually show high activity of PR and antioxidant enzymes. Clark et al. (2000) indicated that NO treatment could directly inhibit CAT and POD activities. But Carreras and Poderoso (2007) pointed out that NO had a competitive relationship with SOD in mitochondrial metabolism. According to our results, there were relatively higher activities of antioxidant enzymes (CAT, SOD and POD) in NO-treated fruit (Fig. 3). We presume that the transitory stimulation by NO can induce expression of genes encoding antioxidant and protective enzymes in tomato fruit. As antioxidant enzymes have been closely correlated with senescence in plants (Lacan and Baccou, 1998), increasing the activities of antioxidant enzymes by NO treatment may be beneficial for extending postharvest life of fruit. In addition, NO treatment can also restrain pathogen infection by B. cinerea in fruit (Fig. 4), indicating that NO is a possible candidate as a key signal in the establishment of systemic acquired resistance (Mur et al., 2006).

Taken together, our results demonstrated that NO treatment has a positive effect on delaying ripening and enhancing resistance of tomato fruit to a postharvest fungal pathogen, because NO can suppress ethylene biosynthesis, stimulate the activity of antioxidant enzymes and regulate the expression of age-related genes. Therefore, NO has potential for delaying fruit ripening and maintaining or enhancing defense responses, favorable for increasing storability and extending postharvest storage time. However, the detailed mechanisms by which NO executes function in fruit defense responses should be investigated in the future.

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References


