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Reduction of postharvest anthracnose and enhancement of disease resistance in ripening mango fruit by nitric oxide treatment

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ABSTRACT

Nitric oxide (NO) acts as an important signal molecule with diverse physiological functions in plants. In this study we investigated the effects and possible mechanisms of exogenous NO on anthracnose caused by *Colletotrichum gloeosporioides* in mango fruit. 'Guifei' mango fruit were treated with NO donor (sodium nitroprusside of 0.1 mM) at 25 °C for 5 min, inoculated with spore suspension of *C. gloeosporioides* after 24 h of NO treatment, and stored at ambient temperature (25 °C). NO treatment effectively suppressed lesion development on mango fruit inoculated with *C. gloeosporioides*, and lesion diameters at 2 through 8 d in NO-treated fruit averaged 30% lower than those in control fruit. Additionally, NO treatment reduced natural anthracnose incidence and severity of mango fruit ripened at ambient temperature, and the values of both parameters from 4 to 10 d of storage in NO-treated fruit averaged 40 and 45% lower, respectively, than those for control fruit. NO did not exhibit *in vitro* antifungal activity against *C. gloeosporioides*. NO treatment enhanced the activities of defense-related enzymes including phenylalanine ammonia-lyase (PAL), cinnamate-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), peroxidase (POD), β-1,3-glucanase (GLU) and chitinase (CHT). NO treatment also promoted the accumulation of total phenolics, flavonoids and lignin that might contribute to inhibition of the pathogen. In addition to antifungal efficacy, NO treatment delayed flesh softening, yellowing, and changes in soluble solids content (SSC) and titratable acidity (TA), and peaks of respiration rate and ethylene production during ripening. These results suggest that the resistance of NO-treated mango to anthracnose may be attributed to activation of defense responses as well as delay of ripening.

1. Introduction

Mango (*Mangifera indica* L.) is considered a functional tropical fruit due to its favorable flavor, superior taste and particular nutritional qualities (Sivakumar et al., 2011). However, the fruit are highly susceptible to various pathogens, leading to quality deterioration and reduced market value. Anthracnose, caused by *Colletotrichum gloeosporioides* (Penz), is one of the most serious postharvest diseases of mango fruit (Zhang et al., 2013). The pathogen infects immature fruit and remains latent until storage and ripening, when lesions progressively appear (Dodd et al., 1989). Traditionally, control of mango postharvest anthracnose has been performed with fungicides such as benomyl and prochloraz. However, because of issues associated with fungicide toxicity, environmental pollution, development of fungicide resistance in pathogens and potential risks on human health, alternative strategies for reducing postharvest disease have been required (Terry and Joyce, 2004).

Induction of resistance to pathogens by biotic or abiotic factors is becoming a promising approach for controlling postharvest diseases (Terry and Joyce, 2004). Previous studies have shown that application of exogenous chemical elicitors including salicylic acid (SA) (Zeng et al., 2006), acibenzolar-S-methyl (Zhu et al., 2008a), oxalic acid (Zheng et al., 2012), and β-aminobutyric acid (Zhang et al., 2013) suppressed anthracnose in harvested mango fruit.

Nitric oxide (NO), a highly reactive free radical gas, is recognized as a multifunctional signal molecule that participates in

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diverse physiological processes in phylogenetically distant species (Leshem et al., 1998; Belligni and Lamattina, 2001; Hong et al., 2008; Shi et al., 2012a). Postharvest application of NO, either through direct gas fumigation or by means of NO releasing agents including 3-morpholino sydnonimine, 2,2-(hydroxynitroso-hydrazine)-bisethanamine and sodium nitroprusside (SNP), has been shown to delay fruit ripening and senescence, as well as enhance tolerance to chilling stress in a number of climacteric and non-climacteric fruits (Leshem and Wills, 1998; Wills et al., 2000; Manjunatha et al., 2010; Zhao et al., 2011; Wang et al., 2013; Singh et al., 2013). Recently, the effect of NO on postharvest disease has also received attention. Treatment of tomato fruit with NO or its precursor (L-arginine) resulted in enhanced resistance against *Botrytis cinerea* and *Rhizopus stolonifer*, increased activities of defense-related enzymes, and promoted reactive oxyen species (ROS) metabolism (Fan et al., 2008; Lai et al., 2011b; Zheng et al., 2011a). Furthermore, Zheng et al. (2011b) reported that a fungal elicitor isolated from *B. cinerea* induced disease resistance of tomato fruit in parallel with increased NO and nitric oxide synthase activity. These findings indicate that NO signaling participates in systemic acquired resistance in fruit. However, although mango fruit is prone to pathogen infection and rapidly loses its commercial value after harvest, there is little information available on the inhibitory effects of postharvest NO application against *C. gloeosporioides* and possible defense mechanisms in mango and other tropical fruit.

The main objective of this study was to investigate the effects of NO on control of anthracnose caused by *C. gloeosporioides* in mango fruit during ripening at ambient temperature (25 °C). Evaluations addressed the antifungal activity against *C. gloeosporioides* *in vivo* and *in vitro*, the responses of defense-related enzymes and antifungal metabolites, and the influence on ripening.

2. Materials and methods

2.1. Plant material

Mature green mango (*M. indica* L. cv. Guifei) fruit were harvested from a commercial orchard located in Dongfang city, Hainan Province of China. Fruit were packed in cartons and transported to the postharvest laboratory within 6 h. Fruit of uniform size and appearance without visible symptoms of disease and mechanical injury were selected for the experiments.

2.2. Pathogen

Isolation of *C. gloeosporioides* and preparation of spore suspensions (1×10^6 spores per milliliter) were performed as described in Zhang et al. (2013).

2.3. NO treatment and inoculation

Mango fruit were disinfected with 2% (v/v) sodium hypochlorite for 2 min, rinsed with tap water, air-dried and then divided randomly into 2 treatment groups, with 350 fruit for each group. The first group was immersed in 0.1 mM sodium nitroprusside (SNP) (Sigma–Aldrich, Shanghai, China) in water at 25 ± 1 °C for 5 min, and the other group (control) was immersed in distilled water for 5 min. Twenty-four hours after treatment with SNP or water, a uniform wound (3 mm deep × 6 mm wide) was made at the equator of each fruit using a sterile nail. The white latex was removed from the wound using a pipette, and then 20-μL of spore suspension of *C. gloeosporioides* (1×10^6 spores per milliliter) was inoculated into the wounded site using a pipette. Inoculated fruit were stored at 25 ± 1 °C and RH 85–90%. Lesion diameter was recorded at 2, 4, 6 and 8 d after inoculation. Three replicates were performed for each treatment, and each replicate contained 30 fruit. Uninoculated fruit were stored under the same conditions to evaluate natural decay and physio-biochemical parameters at regular intervals.

2.4. Natural disease incidence and index

Natural disease incidence was expressed as the proportion of fruit showing black lesions relative to the total number of fruit in each treatment. Data were expressed as percentage of affected fruit.

Natural disease index was evaluated by assessing the extent of total lesion area on each fruit surface using the following scale (Zheng et al., 2012) with minor modifications: 0, no visible decay; 1, <5% decay spots; 2, 5–25% decayed area; 3, 25–50% decayed area; and 4, >50% decayed area. The disease index was calculated according to the following formula: $\sum$(disease scale × number of fruit in each scale)/(number of total fruit × 4) × 100%. Each treatment contained three replicates, and 30 fruit were included in each replicate.

2.5. In vitro antifungal activity

The effects of NO (0.1 mM SNP) on the *in vitro* growth of *C. gloeosporioides* and spore germination were tested following the method described by Zhang et al. (2013). Briefly, SNP solution or sterile water (control) was mixed with potato dextrose agar (PDA) to give a total volume of 15 mL per Petri plate (90 mm diameter). SNP concentrations in the PDA were 0 and 0.1 mM. After the PDA had solidified, an 8-mm diameter disk of mycelial mat from a 1-week-old culture was placed in the center of each Petri plate containing PDA and SNP. Mycelial growth as expressed by diameter (mm) was recorded after 2, 4, 6 and 8 d of incubation at 25 ± 1 °C. Each treatment concentration was replicated three times, and the experiment was repeated twice.

For measurement of spore germination rate of *C. gloeosporioides*, aliquots of 100 μL of spore suspension at 1×10^9 L^{-1} were individually transferred to glass tubes with potato dextrose broth (PDB), which contained SNP concentrations at 0 and 0.1 mM. All the tubes were put on a rotary shaker at 1.7 s^{-1} at 25 °C and incubated for up to 12 h. Approximately 200 spores were measured at 3 h intervals for germination rate per replicate, with 3 replicates for each treatment. The experiment was repeated twice.

2.6. Sample collecting

For analysis of enzymes and metabolites, the fruit exocarp (peel) tissue around the equatorial region of each fruit was removed with a stainless vegetable peeler and cut into small pieces every 2 d during storage. Each treatment replicate at each sampling time contained 3 fruit, with 3 replicates for each treatment. The tissue samples were rapidly frozen in liquid nitrogen and stored at −80 °C until use.

2.7. Extraction and assay of defense-related enzymes

One gram of frozen exocarp tissue was ground with various pre-cooled buffers (4 °C) using a FSH-2A homogenizer (Jiangsu Jintan Corp., Nanjing, China) to prepare extracts for assay of the following enzymes: 5 mL of 100 mM boric acid buffer (pH 8.8) containing 4% (w/v) polyvinylpyrrolidone (PVP), 1 mM ethylene diamine tetraacetic acid (EDTA) and 50 mM β-mercaptoethanol for phenylalanine ammonia lyase (PAL, EC 4.3.1.5); 5 mL of 50 mM Tris–HCl buffer (pH 8.9) containing 15 mM β-mercaptoethanol, 4 mM $MgCl_2$, 5 mM ascorbate acid, 10 μM leupeptin, 1 mM phenylmethanesulfonyl fluoride, 0.15% (w/v) PVP and 10% glycerin for cinnamate-hydroxylase (C4H, EC 1.14.13.11); 5 mL of 0.2 M Tris–HCl buffer (pH 8.0) containing 0.1 M DTT for 4-coumarate: CoA ligase (4CL, EC 6.2.1.12); 5 mL of 50 mM sodium phosphate buffer (pH 7.0) for peroxidase (POD, EC 1.11.1.7); 5 mL of 50 mM