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Postharvest ASM dipping and DPI pre-treatment regulated reactive oxygen species metabolism in muskmelon (*Cucumis melo* L.) fruit

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ABSTRACT

Pink rot caused by *Trichothecium roseum* is one of the most important postharvest diseases of muskmelon. The present study was to evaluate how disease resistance in muskmelon fruit (*Cucumis melo* L. cv. Yujingxiang) was affected by dipping with 100 mg/L acibenzolar-S-methyl (ASM) and 50 μM diphenylene iodonium (DPI), a NADPH oxidase specific inhibitor. Lesion diameters on the fruit inoculated with *T. roseum* were significantly decreased ($P < 0.05$) by dipping with 100 mg/L ASM. Decreased lesion development was associated with the accumulation of H_2O_2, release of superoxide anion (O_2^-), enhancement activities of NADPH oxidase (NOX), superoxide dismutase (SOD), ascorbate peroxidase (APX), and inhibition of catalase (CAT) activity. Antioxidant content including ascorbic acid (AsA) and reduced glutathione (GSH) was also induced by ASM treatment. Compared with ASM treated fruit, fruit treated with DPI prior to ASM treatment exhibited larger lesion diameter. Moreover, DPI treatment inhibited ASM-induced H_2O_2 and O_2^- accumulation, the increase of NOX, SOD, APX activities and content of ascorbic acid (AsA), and reduced glutathione (GSH). Cytochemical studies indicated that H_2O_2 and O_2^- were mainly deposited in the intercellular space and cell walls. These results suggest that pre-treatment with DPI prevented accumulation of ROS induced by ASM and resulted in serious disease symptoms, highlighting the important role of ROS in ASM-induced resistance in muskmelon fruit.

1. Introduction

Muskmelon (*Cucumis melo* L.) is easily infected by *Trichothecium roseum*, which causes pink rot in the fruit and leads to considerable postharvest losses (Bi et al., 2006). Generally, postharvest diseases of muskmelon can be controlled by synthetic fungicides such as imazalil (Aharoni et al., 1992), iprodione and azoxystrobin (Ma et al., 2004). However, the continuous use of fungicides has increased public concern regarding contamination of fruit with fungicidal residues, development of fungicide resistance by pathogens, and the rising environmental risk (Tripathi and Dubey, 2004; Bi et al., 2010).

The chemical plant activator, acibenzolar-S-methyl (ASM, commercially known as Bion® or Actigard), a functional analog of salicylic acid (SA), has been developed for systemic induction of disease resistance from fungi, bacteria and viruses (Friedrich et al., 1996; Lawton et al., 1996). It has been demonstrated that preharvest or postharvest ASM treatment induces disease resistance in a wide range of fruit and vegetables including apple (Sklodowska et al., 2010), strawberry (Cao et al., 2010, 2011), peach (Liu et al., 2005), pear (Cao et al., 2005), muskmelon (Ge et al., 2008; Ren et al., 2012), orange (Moscoso-Ramírez and Palou, 2013), banana (Tang et al., 2010), mango (Zhu et al., 2008) and tomato fruit (Iriti et al., 2007). The resistance is not based on direct inhibition of the pathogens, but on faster and stronger activation of defense responses including generation of reactive oxygen species (ROS) (Cao et al., 2011; Ren et al., 2012), activation of the phenylpropanoid pathway (Zhu et al., 2008; Liu et al., 2014), accumulation of pathogenesis-related proteins (PRs) and PR gene expression (Cao and Jiang, 2006; Zhu et al., 2008; Quaglia et al., 2011). The rapid and transient generation of large amounts of ROS, especially hydrogen peroxide (H_2O_2), by the consumption of molecular oxygen is one of the earliest responses of host cells under various abiotic and biotic stresses (Torres et al., 2006). A variety of enzymes have been implicated in ROS generation including NADPH oxidase (NOX), lipoxygenases and oxalate oxidases (Shetty et al., 2008). ROS

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generation changes the redox balance of host cells and affects a range of cell signaling and regulatory functions through interactions with enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR), and the non-enzymatic antioxidants ascorbic acid (AsA) and reduced glutathione (GSH) (Apel and Hirt, 2004; Foyer and Noctor, 2009). The ascorbate–glutathione (AsA–GSH) cycle has played an essential role in keeping the balance between ROS production and elimination for maintaining cellular redox homeostasis (Apel and Hirt, 2004; Gill and Tuteja, 2010). Previous studies in our laboratory showed that postharvest ASM treatment increased disease resistance to *T. roseum* by increasing H_2O_2 levels in muskmelon fruit (Ren et al., 2012). Similar results have been reported in pear (Cao and Jiang, 2006), peach (Liu et al., 2005), strawberry (Cao et al., 2011) and mango (Zhu et al., 2008) fruit. However, few investigations have been reported on the role of ROS in ASM-induced resistance using diphenylene iodonium (DPI), a NADPH oxidase specific inhibitor, to inhibit ROS generation in muskmelon fruit.

The objectives of this study were to investigate the effects of postharvest treatment with ASM and DPI on the inhibition of pink rot caused by *T. roseum* in muskmelon fruit during storage at 25 °C, to evaluate the effects of DPI and ASM on ROS metabolism (O_2^-, H_2O_2, NOX, SOD, CAT, APX, GR, AsA and GSH), to localize the accumulation of O_2^- and H_2O_2 in cells in order to determine the potential role of ROS in ASM-induced disease resistance in muskmelon fruit.

2. Materials and methods

2.1. Fruit and chemicals

Muskmelon (cv. Yujinxiang) fruit were harvested from Minqin county in Gansu Province, China, at the beginning of physiological maturity (firmness at 7.8×10^5 N/m^2, soluble solids content 9.08%) and then packaged in cartons and transported to the laboratory within 24 h, and subsequently stored at room temperature (25 °C, RH 55–60%). ASM was kindly provided by Syngenta Company (Switzerland) as a wettable granule formulation (ai. 50%, commercially as Bion®). DPI was purchased from Sigma–Aldrich (USA).

2.2. Treatments

Fruit were selected for uniformity of size, ripeness and absence of defects, and washed with tap water, air-dried, then disinfected with 2% (v/v) sodium hypochlorite for 2 min. The fruit were pretreated with DPI by dipping the fruit with 50 μM DPI for 5 min. After air-drying for 2 h, the fruit were again dipped with 100 mg/L ASM (containing 0.05% Tween-80) for 10 min. ASM at a concentration of 100 mg/L was chosen for this study because this concentration induced resistance against *T. roseum* in muskmelon fruit (Bi et al., 2006; Ge et al., 2008). Fruit treated with distilled water were the controls. All fruit were air-dried, kept in cartons and stored at room temperature for subsequent experiments. Three replicates per treatment were made, each replicate containing 12 fruit, and the whole experiment was performed twice.

2.3. Pathogen and inoculation

T. roseum was isolated from naturally decayed muskmelon fruit and maintained on potato dextrose agar (PDA) at 25–27 °C. Spore suspensions of *T. roseum* were prepared by flooding the 7-day-old culture with 10 mL sterile distilled water containing 0.01% Tween-80. The inocula were diluted to 1×10^6 spore/mL and confirmed using a haemocytometer.

Inoculation was carried out 24 h after the treatment (Ge et al., 2008). The fruit were surface-sterilized with 75% ethanol, then four wounds were made with a sterilized needle (3 mm deep × 2 mm wide) around the equator of each fruit. A volume of 20 μL of the spore suspensions were injected into each wound. After air-drying, the fruit were put in cartons and incubated at room temperature. The lesion diameter was recorded 2 days after inoculation.

2.4. Sample collecting

Three grams of tissues were detached from 4 to 8 mm below the skin around the equator of the fruit at 0, 6, 12, 24, 48, 72, 96, or 120 h after treatment. Each sample was packed in aluminum foil individually and frozen in liquid nitrogen immediately, and kept at −80 °C until biochemical analysis.

2.5. Production rate of O_2^- and H_2O_2 content assay

Production rate of O_2^- was determined according to the method of Ren et al. (2012) with minor modifications. Frozen tissue (3.0 g) was homogenized in 3 mL of 100 mM phosphate buffer (pH 7.8) amended with 0.1% polyvinylpolypyrrolidone (PVPP), then centrifuged at 12,000 × *g* for 15 min at 4 °C. For each sample, 2 mL of the supernatant was incubated with 1 mL of phosphate buffer (pH 7.8) and 0.5 mL of 10 mM hydroxylamine hydrochloride solution for 30 min at 25 °C. 4-Aminobenzene sulfonic acid (1 mL, 17 mM) and 1 mL, 7 mM α-naphthylamine was added for a further 40 min. Four mL of *n*-butanol was added into the reaction mixture, and then the *n*-butanol phase was used for the determination of O_2^-. The rate of O_2^- production was expressed as ΔOD_{530}/min/g FW.

H_2O_2 content was determined according to Prochazkova et al. (2001) with some modifications. 3.0 g of frozen tissues was homogenized in 3 mL of cold acetone and then centrifuged at 12,000 × *g* for 15 min at 4 °C. For each sample, 1 mL of the supernatant was re-centrifuged followed by the addition of 200 μL of 20% titanium tetrachloride, and 200 μL of concentrated ammonia solution to precipitate the titanium-hydro peroxide complex. Precipitate was washed repeatedly by cold acetone and dissolved in 3 mL of 1 M H_2SO_4 and then re-centrifuged. The H_2O_2 content was monitored by taking the absorbance at 410 nm and expressed as mg H_2O_2/g FW.

2.6. Cytochemical localization of O_2^- and H_2O_2

Accumulation of O_2^- at the sub-cellular level was determined using the DAB/$MnCl_2$-method (Steinbeck et al., 1993; Romero-Puertas et al., 2004). In the presence of O_2^-, Mn^{2+} is oxidized to Mn^{3+}, which then oxidizes 3,3′-diaminobenzidine-HCl (DAB) to an insoluble osmiophilic polymer. Tissues of 2 mm^3 were cut from fruit and incubated for 30 min in 0.1 M HEPES buffer (pH 7.2) containing 2.5 mM DAB, 0.5 mM $MnCl_2$ and 1 mM Na-azide. Control sections were incubated in the absence of either DAB or $MnCl_2$. Samples were then fixed in 1.25% (v/v) glutaraldehyde and 1.25% (v/v) paraformaldehyde in pH 7.2, 50 mM sodium cacodylate buffer (CAB) for 2 h at room temperature. Subsequently, tissues were washed twice for 10 min in CAB, post-fixed in 1% osmium tetroxide for 2 h. After washing in CAB for 10 min twice, tissue was dehydrated in a graded acetone, and embedded in Spurr's resin with flat embedding molds.

H_2O_2 production was assessed cytochemically by determination of cerium perhydroxide formation after reaction of $CeCl_3$ with endogenous H_2O_2 to form electron dense insoluble precipitates of cerium perhydroxides, $Ce[OH]_2OOH$ and $Ce[OH]_3OOH$ (Bestwick et al., 1997). Tissues of 2 mm^3 were excised from fruit and infiltrated with freshly prepared 5 mM $CeCl_3$ in 50 mM 3-(N-morpholino)propanesulphonicacid (MOPS) at pH 7.2 for 1 h.