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Pre-harvest application of oxalic acid increases quality and resistance to *Penicillium expansum* in kiwifruit during postharvest storage

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ABSTRACT

Kiwifruit (*Actinidia deliciosa* cv. Bruno) fruits were sprayed with 5 mM oxalic acid (OA) at 130, 137, and 144 days after full blossom, and then harvested at commercial maturity [soluble solid content (SSC) around 10.0%] and stored at room temperature (20 ± 1 °C). Pre-harvest application of OA led to fruit with higher ascorbic acid content at harvest, slowed the decreases in fruit firmness and ascorbic acid content and increase in SSC during storage, and also decreased the natural disease incidence, lesion diameter, and patulin accumulation in fruit inoculated with *Penicillium expansum*, indicating that the OA treatment increased quality and induced disease resistance in kiwifruit. It was suggested that the increase in activities of defense-related enzymes and in levels of substances related to disease resistance might collectively contribute to resistance in kiwifruit against fungi such as *P. expansum* in storage.

1. Introduction

Kiwifruit (*Actinidia deliciosa*) is one of the fruits most appreciated by consumers due to its attractive sensory and properties and its nutritional properties, especially high ascorbic acid content, which provide health benefits (Beever & Hopkirik, 1990). However, kiwifruit is highly perishable and prone to storage diseases, particularly those caused by pathogenic fungi. Gray mold decay caused by *Botrytis cinerea* is the most common postharvest disease of kiwifruit. Blue mold decay caused by *Penicillium expansum* is also responsible for the significant postharvest loss in stored kiwifruit, as *P. expansum* is easily infects wounds produced during harvest and handling or through infection sites of other primary fruit pathogens during storage, and over-mature or long-stored kiwifruit are also susceptible to *P. expansum* infection (Hur et al., 2005). Compared to the other fungi, *P. expansum* is regarded as the main producer of patulin, a mycotoxin with mutagenic, carcinogenic, teratogenic and embryotoxic effects on humans (Puel, Galtier, & Oswald, 2010). Controls of blue mold and patulin contamination in usual hosts such as pears and apples have received much attention during storage and processing (Morales, Marín, Ramos, & Sanchis, 2010). However, besides pears and apples, some less common hosts including kiwifruits, apricots, peaches, and strawberries also support patulin production, indicating that a real threat of patulin contamination exists in less common hosts of *P. expansum* (Neri, Donati, Veronesi, Mazzoni, & Mar, 2010).

Oxalic acid (OA) is an abiotic elicitor that induces systemic resistance against disease caused by fungi, bacteria and viruses in plants (Mucharromah & Kuc, 1991). Postharvest application of OA is effective in decreasing disease incidence in fruits and vegetables such as mango (Zheng, Tian, Gidley, Yue, & Li, 2007), and pear (Tian, Wan, Qin, & Xu, 2006), and artichokes (Ruíz-Jiménez et al., 2014) during storage. This decreased disease incidence is attributed, alone or collectively, to maintenance natural disease resistance associated with delay of the ripening process, induced disease resistance, and inhibition of the development of post-harvest pathogens. Also, postharvest OA treatment enhances the fruit russet elimination and storability of kiwifruit (Zhang, Rao, Wang, & Zhang, 2006). Recently, Martínez-Espla et al. (2014) have reported that pre-harvest application of OA increases fruit size, bioactive compounds, and antioxidant capacity in sweet cherries at commercial harvest without affecting the on-tree ripening process. However, no information is available about effects of pre-harvest OA application on quality and disease resistance in kiwifruit, which is the main objective in this study, with special interest in its effect on the progress of blue mold decay and patulin contamination caused by *P. expansum*.

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2. Materials and methods

2.1. Material and treatment

Kiwifruit (*A. deliciosa*) cv. 'Bruno' was used for this study, and the experiment was carried out at a commercial orchard in Wenzhou city, China. Six, 9-year-old plants were selected in a completely randomized manner for water (control) or 5 mM OA (containing 0.5% Tween-20) treatment according to our preliminary investigations within a concentration range from 1 to 10 mM OA. With a hand-held mist sprayer, the selected plants were sprayed with water or 5 mM OA at 130 days after full blossom until the surfaces of leaves and fruits were fully wet, and then foliar sprayed at a 7-day interval an additional two times.

Fruits selected for uniformity of size and maturity and without physical injuries or apparent infection were harvested at 151 days after full bloom, when the soluble solid content (SSC) had reached about to 10.0%. The control and pre-harvest OA treated fruit were then divided into two groups respectively. One group was for observation of disease incidence and analytical determinations of quality parameters during storage at 20 ± 1 °C. Analysis of nine fruits each from nine plastic boxes, in triplicate, was undertaken at 3-day intervals for flesh firmness, SSC, titratable acid (TA), and ascorbic acid. Sixty fruits (three boxes) from each treatment were observed at 3-day intervals during storage in order to evaluate the disease index. Another group was for determination of rot's progress, patulin content and disease resistance of fruit challenge-inoculated with *P. expansum*.

The *P. expansum* strain that was obtained from the Institute of Botany, Chinese Academy of Sciences represented the most aggressive with regard to its ability to infect kiwifruit. Petri dishes containing potato dextrose agar were inoculated with this isolate and incubated at 28 °C for 2 weeks. Conidial suspension were prepared by adding 10 ml of sterile distilled water containing 0.85% NaCl and 0.01% (w /v) Tween 80 over the surface of 2-week old cultures and rubbing the surface of the agar with a sterile glass rod. The concentration of the *P. expansum* were adjusted to 1×10^6 - conidia ml^{-1} with the aid of a hemacytometer.

A uniform wound (3 mm wide and 5 mm deep) was made at the equator of each harvested kiwifruit with a sterile borer, and a 15 μl of the *P. expansum* suspension was introduced into each wound 4 h after wounding, and then each twenty fruits of the control or OA treatment were placed in a plastic box without fruit touched, and each box was wrapped in an unsealed 0.05 mm polyethylene bag to maintain a relative humidity and stored at 20 ± 1 °C. Analysis of nine fruits each from nine plastic boxes, in triplicate, was undertaken for measurements of lesion diameter, patulin content, activities of defense-related enzymes, and levels of resistance-related substances at 2-day intervals.

2.2. Measurements of flesh firmness, SSC, TA and ascorbic acid content

Flesh firmness was measured on two opposite sides of each fruit with a durometer (FHM-1, Japan). SSC was assessed in juice using a refractometer (Master-a, ATAGO ATC, Japan) and TA by titration of 10 ml of juice with 0.1 N NaOH up to pH 8.2 and expressed as gram of malic acid per liter of juice. The analysis of ascorbic acid was refer to Tanaka, Suda, Konda, and Sugahara (1985). Two gram of sample was homogenized in 6 ml of 5% (w/v) metaphosphoric acid, and then centrifuged at 12,000×*g* for 20 min at 4 °C. Ascorbic acid was measured by monitoring the decrease in absorbance at 265 nm in mixture containing 1 ml supernatant and 2 ml potassium phosphate buffer (100 mM, pH 6.8), which was initiated by addition of 1 unit of ascorbic oxidase.

2.3. Measurement of disease index and lesion diameter of fruit inoculated with P. expansum

The disease index for fruit was observed by assessing the extent of total disease symptoms on each fruit surface using the following scale: 0 = no visible disease symptoms; 1 ⩽ 10% disease spots; 2 = 10–20% disease spots; 3 = 20–50% disease spots; 4 ⩾ 50% disease spots. The disease index was calculated using the formula: ∑(disease scale × number of fruit in each class)/(number of total fruit × highest disease scale) × 100. Lesion diameter on fruit inoculated with *P. expansum* of fruit was measured by a vernier gauges using the cross method, and the unit was centimeter.

2.4. Determination of patulin

Patulin was measured according to the method of Neri et al. (2010). The visibly infected tissues were removed from the surrounding sound area of fruit and blended with a mixer. A sample of 5 g was homogenized with 5 ml water before adding 150 μl pectinase solution (1400 U g^{-1}), then held at 40 °C for 2 h and centrifuged (10,000×*g*, 20 min). The supernatant was extracted three times (20 ml each time) with ethyl acetate, cleaned with Na_2CO_3 and dried with Na_2SO_4. The sample was evaporated to dryness, re-dissolved in 2 ml of acetic acid (pH 4) and filtered through a 0.45 μm filter, then analyzed for the patulin concentration of the solution by an HP-1100 high-performance liquid chromatograph (Agilent, Waldbronn, Germany) equipped with a diode array detector (DAD) (Agilent) at 276 nm. Separations were done on a 250 × 4.6 mm i.d., 5 μm Hypersil BDS-C18 cartridge column (Agilent). The mobile phase, eluting at a flow rate of 1 ml min^{-1}, consisted of a mixture of water and methyl alcohol (v/v = 9:1). The temperature of the column was 30 °C, and the injection volume was 20 μl. To construct the calibration curve, standard solutions of patulin (Sigma) with concentrations of 2, 4, 6, 8, 10 and 20 μg ml^{-1} were injected in triplicate. The patulin content in the tissue was expressed as mg kg^{-1} FW.

2.5. Assay for activities of defense-related enzymes

Each two-gram flesh, from healthy tissues surrounding the rot lesion, were ground with 5 ml of different buffers containing 1% (v/v) polyvinyl polypyrrolidone (PVP) to assay different enzymes: sodium acetate buffer (50 mM, pH 5.0) for Chitinase (CHI) and β-1,3-glucanase (GLU); sodium borate buffer (200 mM, pH 8.8) for phenylalanine ammonia-lyase (PAL), Tris–HCl buffer (25% glycerin, 0.1 mM DTT, 100 mM, pH 8.0) for 4-coumarate CoA ligase (4CL); sodium acetate buffer (100 mM, pH 5.5) for polyphenoloxidase (PPO); and sodium phosphate buffer (50 mM, pH 7.4) for peroxidase (POD). The samples were homogenized with a Kinematica tissue grinder (Crl-6010, Kriens-LU, Switzerland) and centrifuged (10,000×*g*, 4 °C, 30 min). The supernatants were used to assay enzymatic activities. All steps in the preparation of extracts were carried out at 4 °C.

CHI activity was measured according to the method of Boller (1983), with some modification. The reaction mixture, containing 1 ml crude enzyme solution, 1 ml sodium acetate buffer (50 mM, pH5.0) and 1 ml 10 mg ml^{-1} colloidal chitin (diluted in 95% HCl and 50% ethanol) was incubated at 37 °C for 1 h, and then reacted in boiling water for 3 min. The reaction was stopped by centrifugation (12,000×*g*, 4 °C, 10 min). Then 0.5 ml of the supernatant was incubated at 37 °C with 10 μl 20% (w/v) desalted snail gut enzyme (Sigma) for 30 min then added 0.2 ml 0.6 M sodium borate buffer and placed in boiling water for 3 min. CHI activity was measured at 585 nm after mixing 2 ml dimethylaminobenzaldehyde (DMAB) and allowed to react for 20 min at 37 °C. One unit of CHI