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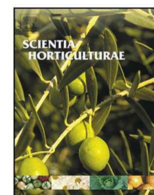

Effect of ethanol treatment on disease resistance against anthracnose rot in postharvest loquat fruit

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ABSTRACT

The effect of ethanol treatment on disease resistance against anthracnose rot in postharvest loquat fruit and its possible mechanism were investigated. Our data implied that treatment of loquat fruit with ethanol at 300 μl/l significantly inhibited the anthracnose rot caused by *Colletotrichum acutatum* and maintained the overall quality. Ethanol treatment suppressed activities of catalase and ascorbate peroxidase while increased superoxide dismutase activity in *C. acutatum*-inoculated loquat fruit, thus resulting in a higher level of H_2O_2, which might serve as a crucial role to activate disease resistance. Meanwhile, the activities of defense-related enzymes including phenylalanine ammonia-lyase, peroxidase, polyphenol oxidase, chitinase and β-1,3-glucanase were significantly enhanced by the ethanol treatment. In addition, 300 μl/l ethanol exhibited an effective antifungal activity against spore germination and mycelial growth of *C. acutatum in vitro*. Therefore, these results suggest that ethanol can inhibit anthracnose rot in postharvest loquat fruit, possibly *via* inhibiting pathogen growth directly and inducing disease resistance indirectly.

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1. Introduction

Loquat fruit, a special fruit originating from south China, is well known for its good taste, juiciness and rich nutrition. Normally, this fruit ripens during the rainy and warm season in China and is sensitive to mechanical injury or microbial infection, which causes a significant economic loss for the farmers. Anthracnose rot caused by fungi of *Colletotrichum acutatum* is the major disease of loquat fruit after harvest (Cao et al., 2008a). Cold storage is a widely used preservation technology to reduce decay and maintain the special flavor; however, cold storage will induce a series of chilling symptoms such as flesh leatheriness and lignification, either of which finally seriously deteriorates the storage quality of loquat fruit (Cao et al., 2010). Traditionally, control of postharvest disease of loquat fruit is mainly dependent on fungicides. However, because of the negative impact of fungicides residues on environment and human health, alternative measures for controlling postharvest diseases in loquat fruit have been strongly demanded (Schirra et al., 2011).

Ethanol is a 'Generally Recognized As Safe' (GRAS) compound and is, therefore, considered safe for use in conjunction with food (Anonymous, 1993). Ethanol dips and vapors have been reported to control postharvest diseases of some harvested commodities. For example, ethanol has a potential for inhibiting development of gray mold decay and improving shelf-life of table grapes, whether it is applied by means of a dipping treatment (Lichter et al., 2002) or a pad generating vapors (Lurie et al., 2006). Postharvest ethanol vapor treatments also have beneficial effect on reducing decay of Chinese bayberries (Wang et al., 2011). Additionally, ethanol dips also have been reported to control postharvest decay of cherries (Karabulut et al., 2004), peaches and nectarines (Margosan et al., 1997). However, little information is available regarding the effect of ethanol on induction of disease resistance in postharvest fruits. Thus, the objectives of this study were (1) to examine the effect of exogenous ethanol vapor treatment on disease resistance against anthracnose rot in loquat fruit, (2) to investigate the antifungal activity of ethanol against *C. acutatum in vitro*, (3) to assess the quality change of loquat fruit by the ethanol treatment.

2. Materials and methods

2.1. Fruit and pathogen

Loquat (*Eriobotrya japonica* Lindl. cv. Jiefangzhong) fruits were hand-harvested at ripe stage from a commercial orchard in Fujian, China and transported within 12 h to our laboratory. The fruits were

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selected for uniform size and color and without visual defects. The selected fruits were then gently surface-rinsed with sterile distilled water to remove dust and any water soluble residue, and air-dried prior to ethanol treatment and pathogen inoculation.

C. acutatum was isolated from infected loquat fruit and maintained on potato-dextrose agar medium (PDA: extract of boiled potatoes, 200 ml; dextrose, 20 g; agar, 20 g and deionized water, 800 ml). Spores of *C. acutatum* were obtained from 2-week-old cultures incubated at 26 °C by flooding the cultures with sterile water. The concentration of spores was adjusted to 1.0×10^5 spores/ml with a hemocytometer.

2.2. Ethanol treatment

Preliminary experiment was conducted to determine the optimum concentration of ethanol vapor treatment for inhibiting anthracnose rot in loquat fruit. The loquat fruits were treated with ethanol vapor at different concentration (100, 300, 600, 900 and 1200 μl/l), and then stored at 20 °C for 8 days. The results indicated that 300 μl/l ethanol was able to markedly control the development of anthracnose rot in loquat fruit without impairing the fruit quality (data not shown). Therefore, the concentration was used for further experiments.

In this study, loquat fruits were surfaced-disinfected with 70% ethanol for 1 min and air-dried. Two wounds (uniform 4 mm deep × 2 mm wide) were made on the two sides of each fruit using the tip of a sterile dissecting needle. Aliquot (15 μl) of 1.0×10^5 spores/ml was inoculated into each wound site. After drying, fruits were randomly distributed in 2 lots of 100 fruits. Then each lot of the inoculated fruits was placed in a 100-l airtight container for 0 (control) or 300 μl/l ethanol treatment. An appropriate amount of ethanol liquid (purity ≥99.7) was soaked in some filter papers inside the container and incubated at 20 °C for 6 h, allowing the ethanol to evaporate into the container headspace. Following treatment, the containers were opened, and both lots of fruit were sealed in polyethylene-lined plastic boxes to retain high relative humidity (approximately 90%) and then incubated at 20 °C for 8 days. Disease incidence and lesion diameter on the each fruit were recorded every two days. When the visible rot zone beyond the wound area on each fruit was more than 2 mm wide, it was counted as an infected fruit. Tissue samples of healthy pulp in each replicate were collected before ethanol treatment (time 0) and at 2 day intervals during the incubation. The samples were mixed and immediately frozen in liquid nitrogen, then stored at −80 °C until used. Each treatment was replicated three times, and the whole experiment was conducted three times with similar results.

2.3. Measurement of enzymes activities

All extract procedures were conducted at 4 °C. Ten gram of the frozen samples was homogenized in 50 ml ice-cold 50 mmol/l sodium acetate buffer (pH 7.0) containing 1.33 mmol/l EDTA and 1% (w/v) polyvinyl pyrrolidone. The homogenates were then centrifuged at 10,000 × *g* for 15 min and supernatants were used for enzyme analysis. To measure PAL activity, 50 mmol/l sodium borate buffer (pH 8.7) containing 5 mmol/l β-mercaptoethanol was used.

SOD was extracted and assayed by the method of Rao et al. (1996). One unit of SOD activity was defined as the amount of enzyme that caused a 50% inhibition of nitro blue tetrazolium. CAT activity was extracted and assayed according to the method of Chance and Maehly (1955). One unit of CAT was defined as the amount of enzyme that decomposed 1 μmol of H_2O_2/min at 30 °C. APX activity was extracted and assayed as described by Nakano and Asada (1989). One unit of APX was defined as the amount of enzyme that oxidized 1 μmol ascorbate/min at room temperature. Phenylalanine ammonia-lyase (PAL) was extracted and assayed by the method described by Zucker (1968). One unit of PAL activity was defined as the amount of enzyme that caused the increase in absorbance at 290 nm of 0.01 in 1 h under the specified conditions. Peroxidase (POD) and polyphenoloxidase (PPO) were extracted and assayed using the method of Cao et al. (2008b). One unit of PPO activity was defined as the amount of enzyme that caused the increase in absorbance at 410 nm of 0.01 in 1 min under the specified conditions. One unit of POD activity was defined as the amount of enzyme that caused the increase in absorbance at 460 nm of 0.01 in 1 min. Chitinase and β-1,3-glucanase activities were measured according to the method of Abeles et al. (1971). One unit of chitinase activity was defined as the amount of enzyme required to catalyze the formation of 1 nmol product/h. One unit of β-1,3-glucanase was defined as the amount of enzyme catalyzing the formation of 1 μmol glucose equivalents/h.

Protein content in the enzyme extracts was estimated using the Bradford (1976) method, using bovine serum albumin as a standard. Specific activities of all the enzymes were expressed as units per milligram protein.

2.4. Measurement of total phenolic and H_2O_2 contents

Total phenolic content was determined according to the Folin-Ciocalteu procedure (Slinkard and Singleton, 1977). Five gram of the frozen samples was homogenized in 25 ml ice-cold 80% acetone, filtered and centrifuged at 10,000 × *g* for 20 min at 4 °C. Result was expressed as milligrams of gallic acid equivalent (GAE) per kilogram of fresh weight. To measure H_2O_2 level, 2 g of fresh tissue was homogenized with 10 ml ice-cold 100% acetone and then centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant was collected immediately for H_2O_2 analysis. The level of H_2O_2 was determined according to the method of Patterson et al. (1984) by monitoring the absorbance of the titanium-peroxide complex at 410 nm and expressed as μmol per gram of fresh weight.

2.5. Measurement of quality parameters of loquat fruit

Fruit firmness was measured on two paired sides of 10 fruits from each replicate (skin removed) with a TA-XT2i texture analyzer (Stable Micro System Ltd., UK) with a 5 mm diameter probe at a speed of 1 mm/s. Extractable juice rate was estimated from the weight loss from placental tissue plugs in response to low-speed centrifugation. Four plugs (7 mm wide and 10 mm thick) were placed over sterile cotton in a 50 ml centrifuge tube and centrifuged for 10 min at 1700 × *g* at 20 °C. The result was expressed as fresh weight loss of the tissue plugs after centrifugation. Total soluble solids (TSS) was determined using a refractometer at 20 °C. Vitamin C (Vc) was quantitatively determined by using 2, 6-dichlorophenolindophenol dye method as described by Jones and Hughes (1983) and the result was expressed as milligrams per gram of fresh weight.

2.6. Effect of ethanol on the growth of C. acutatum in vitro

2.6.1. Spore germination and germ tube growth

The effect of ethanol on spore germination and germ tube elongation of *C. acutatum* was assessed in potato dextrose broth (PDB) using the method of Tian et al. (2002) with some modifications. The suspension of 1×10^5 spores/ml was prepared as described above. Aliquots of 100 μl of the pathogen suspension were transferred to glass tubes containing 5 ml PDB with or without 300 μl/l ethanol. All tubes were put on a rotary shaker at 100 rpm at 26 °C. After 12, 24 or 36 h, the percentage of spore germination and germ tube length was calculated in three different microscopic fields. Approximately 100 spores of the pathogen were measured for germination rate and germ tube length. Spores were considered germinated when germ

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