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Effect of pre-harvest calcium chloride applications on fruit calcium level and post-harvest anthracnose disease of papaya

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ABSTRACT

Anthracnose disease of papaya, caused by *Colletotrichum gloeosporioides* Penz, can cause extensive postharvest losses. The goal of this research was to use pre-harvest calcium applications to reduce anthracnose disease. Six pre-harvest foliar calcium sprays were applied biweekly to papaya trees in experimental orchards at Universiti Putra Malaysia. Additional in vitro and in vivo tests were carried out to test the effect of calcium on fruit calcium content, spore germination, mycelial growth and disease severity. Calcium chloride at 1%, 1.5% and 2.0% concentrations significantly decreased spore germination. Calcium content of papaya fruit was significantly increased by calcium sprays at a concentration of 2.0% in 2012 and 2013. In vivo studies showed that increasing calcium content in fruit by calcium sprays at 1.5 and 2.0% concentrations significantly reduced anthracnose incidence of fruits during five weeks storage at 12 ± 2 °C, and delayed initiation of disease symptoms by four weeks.

1. Introduction

Papaya, *Carica papaya* L., is a large perennial plant with rapid growth (Paull and Duarte, 2011). It is a significant fruit in the Malaysian economy, ranking third after durian and banana. The Eksotika II cultivar is a high yielding F_1 hybrid with good quality that was released by the Malaysian Agricultural Research and Development Institute (MARDI). The cultivar has gained popularity in the local and export market (Shukor and Shokri, 1997); however, postharvest pathogens inflict significant losses in some years (Paull et al., 1997). Anthracnose, caused by *Colletotrichum gloeosporioides* Penz, is one of the most devastating pathogens in storage for papaya. Inoculum originates from drying, infected leaf petioles, which under favourable conditions (moist for a few hours) produce appressoria and subsequent infections that remain quiescent until ripening (Chau and Alvarez, 1983). The disease typically is managed with fungicides or hot water exposure, but hot water can damage fruits and the pathogen has become resistant to some of the fungicides currently in use (Djioua et al., 2010). With increased interest in non-fungicidal management approaches, researchers are looking for new ways to maintain disease-free fruit in the postharvest milieu (Ali et al., 2010).

Calcium is a key plant nutrient that has a significant role in cell functions, including reducing softening and senescence of fruits (Barker and Pilbeam, 2007). Many disorders of crops, such as bitter pit in apple, cork spot in pear, and blossom end rot in tomato that are caused by calcium deficiency could be reduced by calcium spraying (Kader, 2002). However, there are few reports about effects of calcium on infection of tropical fruits by *C. gloeosporioides*. Ghani et al. (2011) found that calcium can increase anthracnose resistance in dragon fruit. In addition, calcium decreased susceptibility to anthracnose in banana (Chillet et al., 2000). In papaya, there are a few reports about the role of calcium in anthracnose disease, and they are mostly related to postharvest uses (Mahmud et al., 2008). There is some evidence that calcium is antagonistic to *C. gloeosporioides* and it may have potential use as an alternative method in integrated disease management (Biggs et al., 2000). The objective of this study was to examine the effect of pre-harvest applications of calcium chloride on anthracnose disease of papaya.

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2. Materials and methods

2.1. Trial description

Papaya trees (cultivar Eksotika II) in an orchard at Agro-tech Unit, University Agriculture Park (TPU), Universiti Putra Malaysia, Serdang, Selangor were selected for calcium application experiments. Trees were eight months old, approximately 2.2 m tall. The trees were spaced 3 m by 3 m with two plants in each 3 × 3 m plot. Commercial fertilization rates (12:12:17:2 N:P:K:Mg) were used uniformly each month around the canopy periphery for all treatments according to Malaysia Ministry of Agriculture recommendations (Basir, 2005). Irrigation was accomplished with overhead sprinklers at approximately 4-day intervals. Weeds were controlled by mowing as needed. The experiment was carried out in 2012 and repeated in 2013. In 2012, solutions of 0, 0.5, 1.0, 1.5 and 2.0% (w/v) calcium chloride ($CaCl_2 \cdot 2H_2O$ (99% CC, SYSTERM®, Malaysia) with 0.03% v/v Tween 20 as surfactant were sprayed 21 days after flower anthesis to the fruits and leaves of papaya trees until run-off (three positions from top to bottom and three positions from bottom to the fruits and leaves (approximately 1.5 L per tree) using a 16 L knapsack sprayer (Jun Chong®, Malaysia, with a solid cone nozzle, nozzle cap, 0.70–0.85 L/min). Spray applications began in June and were repeated every two weeks for six times, with the final spray applied four days before fruit harvesting (13 August). Fruits of uniform size and shape were harvested at index 2 (green with trace of yellow), washed with water and allowed to air dry. Then, they were assigned randomly to five different treatments. After that, fruits in each treatment were packed in commercial boxes and stored at 12 ± 2 °C and 85–90% relative humidity for five weeks. In 2013, the experiment was carried out with 0, 1.5 and 2% calcium chloride to the fruits and leaves of eight month old papaya in TPU. Sprays were applied on 15 January, 2013, with the final spray applied two days before fruit harvesting (28 March). In all other respects the 2013 trial was managed using the same procedures as in the previous season.

2.2. Calcium measurement in fruit

In both years, samples of peel were taken with a metal blade scalpel from the middle part of fruits and dried at 60 °C in an air-circulating oven. Once dried, 0.25 g of the peel was digested in 5 ml 98% H_2SO_4 on a hot plate at 280 °C in a fume chamber for 7 min. Then, 10 ml H_2O_2 was added into the mixtures and the heating was continued for another 4 min. The solution mixtures were brought up to100 ml with distilled water. Calcium ion concentration was measured with an atomic absorption spectrophotometer (Perkin Elmer, Model AAS 3110, Palo Alto, California, USA), and results were expressed as mg calcium g^{-1} D.W. For calcium measurements in the peel, four replications with two fruits per replication arranged in randomized complete block design were used in both years.

2.3. In vitro evaluation of fungicidal activity of calcium

2.3.1. C. gloeosporioides isolate and culture conditions

C. gloeosporioides was isolated from infected papaya fruit. Eight small parts of the peel were sterilised with sodium hypochlorite and washed three times in distilled water, then dried and placed in Petri dishes that contained potato dextrose agar (PDA), and incubated at ambient temperature (28 ± 2 °C). When mycelial growth was observed, colonies were reisolated on to fresh PDA to obtain pure cultures (Ali et al., 2010). The isolates were identified by morphological and cultural characters based on a published description by Barnett and Hunter (1972) and confirmed at the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia. Identified *C. gloeosporioides* was maintained on PDA slants for usage.

2.3.2. In vitro mycelial growth

An agar disk (5 mm diameter) from a pure culture of *C. gloeosporioides* was placed in the center of PDA dishes containing different concentrations of calcium chloride (0, 0.5, 1.0, 1.5 and 2.0% w/v). The control dishes only contained PDA. Then, Petri dishes were placed at ambient temperature (28 ± 2 °C) for 7 days, at which time the fungus reached the edge of dishes.

2.3.3. In vitro conidial germination

To test the effect of calcium on spore germination, conidia were harvested by scraping them off the agar with a glass rod and sterile, distilled water. Then, the slurry was filtered through 4 layers of cheesecloth and adjusted to 20 ml. The number of conidia per ml was determined with a haemocytometer and adjusted to 5×10^5 conidia/ml (Obagwu and Korsten, 2003). One hundred microliters of this suspension were pipetted to PDA agar in dishes containing calcium chloride (0, 0.5, 1.0, 1.5 and 2.0%) and kept in the dark for 7 h at 28 ± 2 °C. The control dishes contained only PDA. Germination of 100 spores in 10 microscopic fields with magnification at 40× were used to calculate percent germination. A conidium was considered germinated when the germ tube was longer than conidium (El Ghaouth et al., 1992). Six replications of five petri dishes arranged in a completely randomised design were used for in vitro experiments, which were conducted twice.

2.4. In vivo assay of calcium against C. gloeosporioides

2.4.1. Anthracnose disease incidence and severity

In both years, to determine disease incidence and severity, fruits were put in commercial export boxes (EXOTIC STAR®, Kajang, Selangor, Malaysia) and stored at 12 ± 2 °C and 85–90% RH for five weeks. Disease incidence data were expressed as percentage of fruits showing anthracnose out of the total number of fruits in each treatment (Ali et al., 2010). Disease severity was measured as percentage of fruit surface with anthracnose disease. For disease incidence and severity, four replications with six fruits per replication arranged in a randomized complete block design with two factors (days in storage and calcium chloride concentrations), were used in both years.

2.4.2. Inoculation tests for lesion diameter of anthracnose in fruits

In 2012, after harvest, papaya fruit were washed with sodium hypochlorite (0.01%) for 3 min, rinsed with distilled water and air dried at ambient temperature (28 ± 2 °C). Fruits were wounded on three sides to a depth of three mm in diameter, immersed for 30 s in a conidial suspension of 10^5 conidia per millimeter and drained and placed at ambient temperature. Lesion diameter of fruits was measured after four and eight days. Lesion diameter per fruit was expressed as the average of the three values from each fruit. For lesion diameter, four replications with three fruits per replication arranged in randomized complete block design were used.

2.5. Microscopy observations

Small samples (1.5 mm^3) of the mid-region of papaya fruit peel, were cut and fixed in 4% glutaraldehyde at 4 °C for 24 h. Samples were washed in 0.1 mol/L sodium cacodylate buffer (pH = 7.6) for three times, and post fixed in 1% (w/v) osmium tetraoxide for 2 h. Then, they were rinsed again in 0.1 mol/L sodium cacodylate buffer (pH = 7.6). After that, tissues were dehydrated in graded series of acetone at 35–100%. Finally, samples were embedded in beam capsules and polymerized at 60 °C for 2 days. Ultra-thin sections