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Use of carvacrol and thymol in shellac coating to control stem-end rot on 'Ruby Red' grapefruit and maintain fruit quality during simulated storage and marketing

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ABSTRACT

Diplodia stem-end rot (SER) caused by *Lasiodiplodia theobromae* is often the most important postharvest disease of fresh citrus fruit in warm and humid regions such as Florida. This disease is exacerbated by commercial degreening practices used to improve peel color of early season fruit. Essential oils are aromatic oily liquids obtained from plant organs that have been used to control plant diseases. This study screened nine compounds from essential oils against *L. theobromae* mycelial growth *in vitro* and only carvacrol and thymol exhibited strong inhibitory efficiency, with half maximal effective concentration to reduce mycelial growth of 0.045 and 0.037 mg mL^{-1}, respectively, in amended PDA medium. Carvacrol and thymol were then incorporated in a commercial shellac coating and applied on 'Ruby Red' grapefruit inoculated with *L. theobromae* to determine their activities against Diplodia SER *in vivo*. Fruit were artificially inoculated with *L. theobromae* 12 h before coating application or immediately after coating application and incubated at 29 °C with 90 % relative humidity (RH) for 48 h. When fruit were inoculated before treatment, shellac containing 10 mg mL^{-1} carvacrol or thymol inhibited lesion development by 59 % or 37 %, respectively, compared to shellac alone. When fruit were inoculated after treatment, coating fruit with shellac containing 1 mg mL^{-1} carvacrol or thymol inhibited lesion development by 43 % or 24 %, respectively, compared to shellac alone. This study also found that incorporating carvacrol or thymol into shellac coating inhibited fruit decay from natural infections and chilling injury compared to shellac alone, while not negatively impacting fruit weight loss, peel color, total soluble solids, or titratable acidity. The results suggest that shellac coatings containing carvacrol or thymol may provide a viable option for Diplodia SER control and quality maintenance on citrus fruit.

1. Introduction

In Florida, the grapefruit harvest season can stretch from late September to April or May. Early season grapefruit (September to December) usually reach internal maturity before the peel attains its characteristic external color. A commercial degreening treatment that exposes fruit to 5 ppm ethylene at 28–29 °C with 90–96 % relative humidity (RH) is often necessary in Florida because most consumers relate green color with immaturity (Ritenour et al., 2003a). However, the degreening environment significantly promotes the development of Diplodia stem-end rot (SER), a most common and important postharvest disease on grapefruit in Florida (Brown, 1986; Zhang, 2004). The outbreak of citrus greening (Huanglongbing) in Florida also dramatically increases postharvest Diplodia SER on citrus fruit, causing substantial economic losses (Zhao et al., 2015, 2016).

Diplodia SER is caused by *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. Spores of *L. theobromae* infect immature citrus fruit on the tree and then remain quiescent in the necrotic floral tissue (Brown, 1971). At or after harvest, hyphae grow through the developing abscission zone and into the peel, flesh, and central core of the fruit. Growth of *L. theobromae* is maximum at 30 °C, and RH at 95–100 % can promote conidia germination (Barmore and Brown, 1985). During degreening, high temperature and RH conditions promote fungal growth on fruit, while ethylene speeds the formation of the abscission zone, reduces natural resistance of fruit tissue to pathogen, and promotes hyphal penetration into the flesh (Barmore and Brown, 1985; Brown and Burns, 1998). Recently, outbreak of citrus greening in Florida increased *L. theobromae* population, largely because of increased deadwood and

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weakened twigs that harbor the fungus (Zhao et al., 2015, 2016).

In Florida, the traditional commercial fungicides, thiabendazole (TBZ) and imazalil (IMZ), plus newer fungicides Graduate (fludioxonil) and Graduate A + (fludioxonil + azoxystrobin) have been reported effective against Diplodia SER (Zhang, 2012; Ritenour et al., 2016). However, some of the fungicides have been used for more than three decades and their intense and repeated use can result in pathogen resistance to the fungicides in the future. *Penicillium* isolates resistant to TBZ and IM have been reported in many packinghouses in California (Kinay et al., 2007). While such resistance to these fungicides is rarely observed in Florida, it is unclear if resistance will eventually develop with continued repeated use. Besides domestic sales, Florida exports a significant amount of grapefruit to a number of foreign countries including Japan, Europe, Taiwan, Korea, and Canada. Fungicide residues may also limit grapefruit exports because of lower maximum residue limits (MRLs) in some countries compared to the United States. For example, the IMZ MRL for grapefruit is 10 ppm in the U.S., but only 5 ppm for the export markets mentioned above. TBZ MRL for grapefruit is 10 ppm in the U.S., but only 7 ppm for Europe (Ritenour, 2019). Therefore, effective and safe alternative methods to control postharvest decay of citrus are needed.

In recent years, more attention has focused on exploitation of natural plant products that have little impact on human health or the environment to control postharvest disease. Essential oils are aromatic, oily liquids extracted from plant organs that are recognized as safe for human health and the environment, and research into their use in postharvest disease control has expanded in the past decade. These are complex mixture of natural compounds containing more than 40 components at different concentrations. The most abundant compound (s) present in these mixtures often exhibit the major function. Because of their antifungal activity and/or induction of plant systemic acquired resistance, essential oils have been reported to control postharvest disease caused by *Penicillium* spp., *Alternaria* spp., *Botrytis cinerea*, and *Monilinia fructicola* on a variety of fresh fruits and vegetables (Antunes and Cavaco, 2010). Jhalegar et al. (2015) found that dipping mandarin fruit in lemon grass oil, clove oil, eucalyptus, and neem oil significantly inhibited green (*P. italicum*) and blue (*P. digitatum*) mold. du Plooy et al. (2009) also reported that *Mentha spicata* and *Lippia scaberrima* essential oils, as well as pure limonene and carvone, incorporated into a wax coating significantly reduced green (*P. italicum*) and blue (*P. digitatum*) mold on oranges.

Unfortunately, high concentrations of essential oil or their compounds can cause injury to fresh fruits and vegetables. Amiri et al. (2008) reported that dipping fruit in an aqueous 1 mg mL^{-1} eugenol solution resulted in peel injury on apple. Our previous study also found that dipping fruit in an aqueous 2 mg mL^{-1} carvacrol or thymol solution resulted in phytotoxic peel injury on 'Valencia' oranges, but no injury developed when the same or even greater concentrations were incorporated into the wax coating. It was also reported that wax coating immediately after heat treatment greatly reduced the development of visible heat injury on 'Marsh' grapefruit. These coatings can slow fruit water loss and restrict oxygen diffusion to inhibit oxidative peel browning and symptom development (Ritenour et al., 2003b). Another advantage of incorporating essential oil or their compounds into wax coatings is that coating may cause slow release of essential oil components. In dipping treatment, the protective activity provided by essential oils decreases right after treatment because of their volatility. Therefore, coating with essential oils can achieve the same effectiveness with lower concentration than dipping treatments. Kouassi et al. (2012) found that *Cinnamomum zeylanicum* essential oil incorporated into shellac wax controlled citrus blue mold better than the same extract concentration administered in ethanol. In addition, coating citrus fruit with such a wax is an important process in Florida commercial packinghouse to reduce subsequent water loss and improve gloss, which means incorporating essential oil or their compounds into wax can be practical for commercial applications.

The objectives of this study were, 1) to screen compounds from essential oils for their efficiency against *Lasiodiplodia theobromae in vitro*; 2) to evaluate the ability of the most effective compounds (carvacrol and thymol), incorporated into a commercial shellac coating, to control Diplodia SER on artificially inoculated 'Ruby Red' grapefruit, and 3) to evaluate the effects of coating with carvacrol and thymol on fruit quality during simulated storage and marketing.

2. Materials and methods

2.1. Pathogen isolate and chemical materials

Lasiodiplodia theobromae isolate D-5 (wild-type) was isolated from lesions of infected 'Ruby Red' grapefruit and identified by its morphological, reproductive, and cultural characteristics. A pathogenicity test (Koch's postulate) was successfully performed by re-inoculating healthy grapefruit with D-5 resulting in identical decay lesions. Benzaldehyde, carvacrol, cinnamaldehyde, trans-cinnamaldehyde, citral, eucalyptol, eugenol, menthol, and thymol were purchased from Sigma-Aldrich Co. (St. Louis, Mo., U.S.A.) and kept at 4 °C. All the compounds were extracted and separated from natural herbal plants. Tween-20 and anhydrous ethanol, as emulsifiers and solvents, respectively, were purchased from Sigma-Aldrich Co. (St. Louis, Mo., U.S.A.) and kept at 25 °C. Shellac wax (HS 590, JBT FoodTech Inc., Lakeland, FL) was kept at 4 °C and allowed to warm to room temperature before use.

2.2. Antifungal screening

For benzaldehyde, carvacrol, citral, cinnamaldehyde, trans-cinnamaldehyde, eugenol, and eucalyptol, volatile test was performed as described by Narciso (2009) and Sun et al. (2014) with some modifications. Twenty mL of sterilized PDA was poured into each 90-mm-diameter Petri dish. One mycelial plug, 3 mm in diameter, from the margin of a 4-d-old fungal colony, was removed with a sterile cork borer and placed in the center of each plate. Three sterilized insect pins with a 6-mm-diameter filter paper disc on each top was inserted into the edge of each plate, around the mycelial plug. One μL of compound was then added into each filter paper disc to make the final volatile concentration of 50 μL L^{-1}. The volatile concentration was calculated by dividing the volume of the compound on the filter paper to the volume of space in the petri dish. Control plates had no compound on the filter paper disc. Plates were sealed using parafilm and incubated at 29 °C in dark for 4 d. Colony diameter was determined as the average of two perpendicular measurements. The diameter of the mycelial plug was subtracted from the average colony diameter. The percent inhibition was calculated relative to the growth of the control colony for each colony growing in the treated plates. There were four replicates for each compound, and the experiment was repeated.

For menthol and thymol, two solid compounds, amended PDA test was performed as described by Zhang (2012) with some modifications. Menthol and thymol were diluted in anhydrous ethanol to prepare stock solutions of active ingredient at 100 mg mL^{-1}. One hundred μL of stock solution was added to 100 mL of molten PDA after cooling down to 55 °C to obtain the final concentration of active ingredient at 0.1 mg mL^{-1}. Anhydrous ethanol was tested by itself at 1 mg mL^{-1} in PDA medium, and unamended medium was used as the control. Twenty mL of amended PDA was poured into each 90-mm-diameter petri dish. One 3-mm-diameter mycelial plug was placed in the center of each plate and incubated at 29 °C in dark for 4 d. The percent inhibition was calculated as above. There were four replicates for each compound, and the experiment was repeated.

2.3. EC_{50} value of carvacrol and thymol against Lasiodiplodia theobromae mycelial growth in vitro

Carvacrol and thymol were diluted in anhydrous ethanol to prepare

stock solutions of active ingredient at 100 mg mL^{-1}. Different amounts of stock solution were added to molten PDA after cooling to 55 °C to obtain final concentrations of active ingredient of 0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 mg mL^{-1}. Anhydrous ethanol was tested by itself at 0.1, 0.2, 0.5, 1, 2, and 5 mg mL^{-1} in PDA medium, and unamended medium was used as the control. Twenty mL of amended PDA was poured into each petri dish. One 3-mm-diameter mycelial plug was placed in the center of each plate and incubated at 29 °C in darkness for 2 d. The percent inhibition was calculated as in 2.2. There were four replicates for each concentration, and the experiment was repeated. Half maximal effective concentration (EC_{50} values) were calculated based on the logarithmic models (relations between percent inhibition and compound concentration). To determine if carvacrol and thymol were fungistatic or fungicidal, mycelial plugs that failed to grow on compound-amended medium were transferred onto new PDA medium without any treatment and mycelial growth was observed after 2 d incubation.

2.4. Fruit inoculation

To determine the optimal artificial inoculation method, various methods were tested. 'Ruby Red' grapefruit were washed with water on the packingline brush bed and stored at 10 °C with 90 % RH until use (storage length approximately 3 d for the first experiment and 7d for the repeated experiment). The fruit were randomly separated into three groups, surface sterilized with 75 % (v/v) ethanol (Yan et al., 2012), and wounded with a sterilized nail at the fruit blossom, equator, or stem area (3 mm deep by 3 mm wide), respectively. For stem areas, the buttons were removed from the fruit and the fruit were wounded in the stem cavity. One 3-mm-diameter mycelial plug from the margin of a 3 d-old fungal colony was placed mycelia side down on each wounded area, and the fruit were placed in a tray with the wound area side up. Inoculated fruit were incubated either at degreening temperature (29 °C) or room temperature (23 °C), both at 90 % RH and in darkness. For controls, wounded but un-inoculated fruit were incubated at both conditions. The percentage of fruit developing Diplodia SER lesions was evaluated 48 h after inoculation. The experiment was conducted as a completely randomized design with three replicates, with ten inoculated fruit per replicate. The experiment was repeated.

To determine the invasion time of *L. theobromae* into fruit fresh, 'Ruby Red' grapefruit were wounded as above at the fruit blossom area. One 3-mm-diameter mycelial plug from the margin of a 3 d-old fungal colony was placed on each wounded area, and the inoculated fruit were incubated at 29 °C with 90 % RH in darkness, based on results from the previous experiment. The mycelial plugs on wounded areas were removed 6, 12, or 24 h after inoculation. The percentage of fruit developing Diplodia SER lesions was evaluated 48 h after inoculation. The experiment was conducted as a completely randomized design with three replicates, each replicate consisting of ten inoculated fruit. The experiment was repeated.

2.5. Carvacrol and thymol incorporated into a shellac coating to control Diplodia SER on artificially inoculated 'Ruby Red' grapefruit

'Ruby Red' grapefruit were washed with water on the packingline brush bed and stored at 10 °C with 90 % RH until use (storage length approximately 10 d for the first experiment and 15 d for the repeated experiment). Carvacrol and thymol were diluted in anhydrous ethanol to prepare stock solution of the active ingredient at 500 mg mL^{-1}. Different amounts of the stock solution and Tween-20 (0.1 %, v/v) were mixed with shellac wax to obtain final concentrations of 1, 2, 5, and 10 mg mL^{-1} of active ingredient. The mixtures were stirred at 600 rpm for 5 min before administering the treatment. Inoculated fruit, coated with shellac wax alone, were used as a coating control, and inoculated but un-coated fruit were used as a control.

'Ruby Red' fruit were randomly separated into two groups, with each group containing 180 fruit. One group of fruit was inoculated 12 h before applying the coating treatment, while the other was inoculated immediately after applying the coating treatments. When fruit were inoculated prior to coating, fruit were inoculated with mycelial plugs at the blossom areas and incubated at 29 °C with 90 % RH for 12 h before the mycelial plugs were removed from the wound. Then, the fruit were coated by spraying fruits on a brush bed with shellac wax containing different concentrations of carvacrol or thymol and dried using a heated air dryer on the packingline, which were all similar to commercial citrus packingline. After coating, the fruit were incubated again at 29 °C with 90 % RH in darkness. When fruit were inoculated after coating, fruit were coated and dried on the packingline as above, and then immediately inoculated with mycelial plugs at the blossom areas and incubated at 29 °C with 90 % RH in darkness. For all fruit, the percentage of fruit developing lesions was evaluated 24 h after inoculation, and lesion area was evaluated 48 and 72 h after inoculation. The experiment was conducted as a completely randomized design with three replicates per treatment, each replicate consisting of ten fruit that were coated together in a batch on the packingline. The experiment was repeated.

2.6. Effects of carvacrol and thymol incorporated into a shellac coating on grapefruit quality during simulated storage and marketing conditions

'Ruby Red' grapefruit were used in quality experiment immediately after harvest. The fruit were washed with water alone and coated with shellac wax containing 10 mg mL^{-1} of carvacrol or thymol as in 2.5. Fruit coated with shellac alone were used as a coating control. Washed but un-coated fruit were used as a control. After coating, the fruit were stored at 10 °C with 90 % RH (simulated commercial storage condition) for 8 weeks followed by 1 week at 25 °C with 60 % RH (simulated commercial marketing condition). Fruit natural decay, weight loss, firmness, color change, total soluble solids (TSS), and titratable acid (TA) were evaluated after 4- and 8-weeks cold storage, and after an additional week storage at room temperature. For chilling injury evaluation, the fruit were stored at 5 °C with 90 % RH for 8 weeks followed by 1 week at 25 °C with 60 % RH. The experiment was conducted as a completely randomized design with four replicates, each replicate consisting of 30 fruit that were coated together in a batch on the packingline. The experiment was repeated.

Fruit quality was evaluated as described by Yan et al. (2016) with some modifications. Natural decay incidence was calculated as the percentage of the decay fruit to the total. Weight loss was evaluated by weighing each fruit after coating and reweighing after storage. Weight loss was calculated as the percent of the loss relative to the initial weight. Fruit firmness was evaluated by pressing each fruit with a 1-cm-diameter, flat-tipped, cylindrical probe at three evenly spaced locations around fruit equator using a texture analyzer (Stable Micro Systems, model TA-XT2, Godalming, England). After contacting the fruit surface, the probe was set to travel at a speed of 8 mm s^{-1} and the maximum force recorded. Peel color was measured at three evenly spaced locations around the fruit equator using a Minolta Chroma Meter (model CR-300; Minolta Camera Corp., Ramsey, NJ). Color was reported as a*/b*, and the Δa*/b* value increases with fruit peel color turning from green to yellow. Fruit juice TSS (°Brix) was measured using a temperature-compensating refractometer (Spectronic Instruments, Rochester, N.Y.). Juice TA (% citric acid) was measured by titrating juice to pH 8.3 with sodium hydroxide (NaOH) using an automatic titrimeter (model DL 12, Mettler, Highstown, NJ). Chilling injury index was estimated visually as the percentage of peel surface showing chilling injury, where: 0 = no chilling injury; 1 = less than 25 %; 2 = 26 %–50 %; 3 = 51 %–75 %; and 4 = more than 75 % of the peel surface showing chilling injury.

2.7. Statistical analysis

All data were analyzed with one-way analysis of variance (ANOVA)

using the statistical software SAS 9.4 for Windows (SAS Institute Inc., Cary. N.C.). Treatment means were separated using Tukey test at $\alpha = 0.05$. For *in vitro* experiments, the half maximal effective concentration to reduce mycelial growth (EC_{50} value) was determined by fitting a logarithmic function.

3. Results

3.1. Antifungal screening

The inhibitory effects of seven compounds on mycelial growth of *L. theobromae* were evaluated using a volatile-based method, while menthol and thymol were evaluated using a PDA-amended method. The results demonstrated that only carvacrol volatile (50 μL L^{-1}) and thymol (0.1 mg mL^{-1}) in amended PDA exhibited significant effective inhibition activities against *L. theobromae in vitro*, with the percent inhibition 88 % and 86 %, respectively, which was also proved by the repeated experiment. Benzaldehyde, cinnamaldehyde, trans-cinnamaldehyde, citral, eucalyptol, eugenol, and menthol did not suppress mycelial growth of *L. theobromae*. Anhydrous ethanol in PDA medium did not inhibit mycelial growth of *L. theobromae* (data not shown).

3.2. EC_{50} value of carvacrol and thymol against Lasiodiplodia theobromae mycelial growth in vitro

Mycelial growth of *L. theobromae* was effectively inhibited by carvacrol and thymol in a concentration dependent manner, with higher concentration having higher antifungal activity. The relationship of percent inhibition of fungal mycelial growth and compound concentration fitted the logarithmic model (Fig. 1 A and B). The effective concentrations of carvacrol and thymol to inhibit mycelial growth by 50 % (EC_{50} value) of *L. theobromae* were 0.045 and 0.037 mg mL^{-1}, respectively. Carvacrol and thymol at 0.5 mg mL^{-1} killed *L. theobromae*, as viable mycelium could not be re-isolated from treated PDA (data not shown), and repeated experiment showed the similar results. Anhydrous ethanol in PDA medium did not inhibit mycelial growth of *L. theobromae* (data not shown).

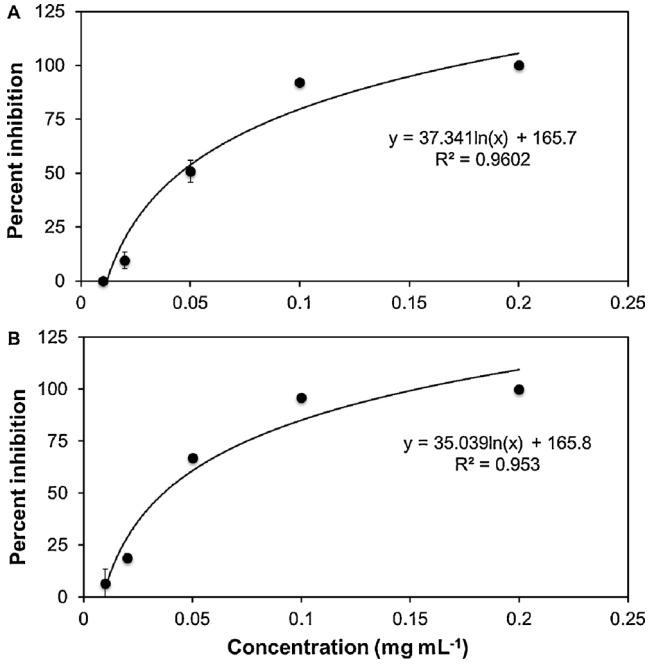

Fig. 1. Inhibition of mycelial growth of *Lasiodiplodia theobromae* by different concentrations of carvacrol (A) or thymol (B). Amended PDA plates containing various concentrations of these were inoculated with mycelial plugs of *L. theobromae* and incubated at 29 °C in darkness for 2 d. Each point represents the mean of four replicates and the vertical bar indicates the standard error.

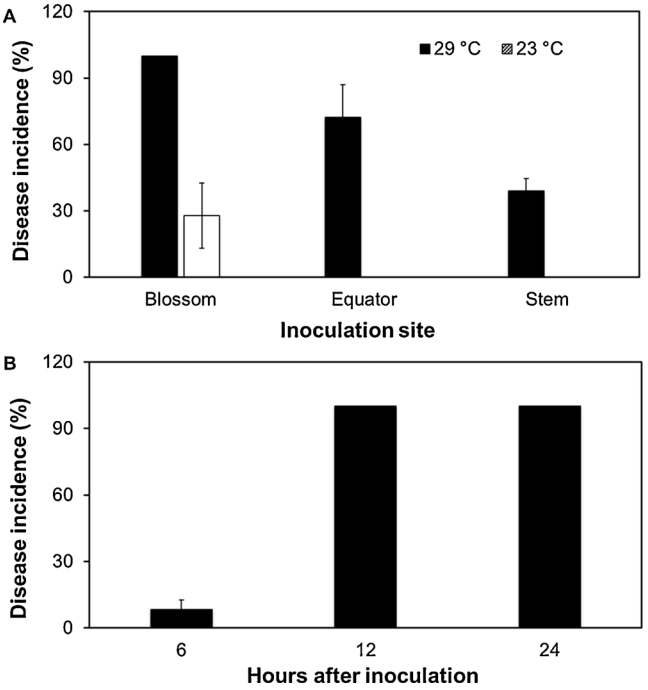

Fig. 2. Effects of inoculation sites and incubation conditions on Diplodia SER development on 'Ruby Red' grapefruit (A), and time required for *Lasiodiplodia theobromae* inoculations to establish in 'Ruby Red' grapefruit (B). 'Ruby Red' grapefruit were artificially inoculated with mycelial plugs on the blossom, equator, or stem area and incubated at 29 °C with 90 % relative humidity (RH) or at room temperature (23 °C) with 90 % RH (A). 'Ruby Red' grapefruit were artificially inoculated with mycelial plugs on the blossom area and incubated at 29 °C with 90 % RH. Mycelial plugs were removed 6, 12, or 24 h after inoculation (B). The percentage of these fruit developing lesions was evaluated 48 h after inoculation. Each value is the mean of three replicates of 10 fruit and the vertical bars indicates standard error. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Fruit inoculation

After 48 h incubation, no wounded but un-inoculated fruit developed Diplodia SER lesions, regardless of the storage temperature (data not shown). At 29 °C with 90 % RH, fruit inoculated with mycelial plugs of *L. theobromae* at the blossom, equator, or stem areas developed 100 %, 72 %, or 39 % infection after 48 h incubation, respectively (Fig. 2 A). At 23 °C with 90 % RH, only fruit inoculated with mycelial plugs at the blossom area developed infection (28 %). To determine the infection time, mycelial plugs on wounded fruit blossom area were removed 6, 12, or 24 h after inoculation, which resulted in 8.3 %, 100 %, or 100 % infection, respectively, after 48 h incubation at 29 °C with 90 % RH (Fig. 2 B). Similar results were got from the repeated experiment.

3.4. Carvacrol and thymol incorporated into a shellac coating to control Diplodia SER on artificially inoculated 'Ruby Red' grapefruit

When fruit were inoculated 12 h before applying coating treatments, most fruit developed *L. theobromae* infection after 24 h incubation regardless of treatment, which was similar to the rate of infection (disease incidence) observed on inoculated but un-coated fruit (data not shown). However, shellac coating with either carvacrol or thymol significantly inhibited Diplodia SER lesion development (disease severity) in a concentration dependent manner (Fig. 3 A and B). Carvacrol and thymol at concentrations in the coating higher than 2 mg mL^{-1}

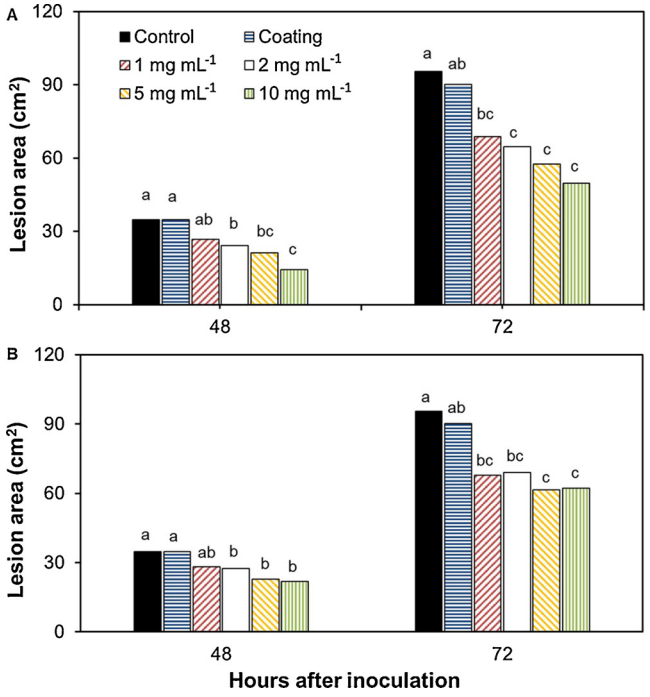

Fig. 3. Effects of coating fruit with shellac containing carvacrol (A) or thymol (B) on Diplodia stem-end rot lesion development on 'Ruby Red' grapefruit when fruit were inoculated 12 h before applying the coating treatment. 'Ruby Red' grapefruit were inoculated with mycelial plug of *Lasiodiplodia theobromae* and incubated at 29 °C with 90 % relative humidity (RH) for 12 h. Then the fruit were coated with shellac containing different concentrations of carvacrol or thymol and incubated at 29 °C with 90 % RH again. Coated fruit were washed and waxed with shellac alone. Control fruit were washed but not waxed. Each value is the mean of three replicates of 10 fruit. Means with different letters are significantly different based on Tukey test ($P \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly ($P \leq 0.05$) suppressed lesion development, with10 mg mL^{-1} carvacrol or thymol exhibited the greatest inhibition. After 48 h incubation, fruit coated with shellac containing 5 mg mL^{-1} or 10 mg mL^{-1} carvacrol inhibited Diplodia SER lesion development by 39 % or 59 %, respectively, while coating fruit with shellac containing 5 mg mL^{-1} or 10 mg mL^{-1} thymol inhibited Diplodia SER lesion development by 34 % or 37 %, respectively, compared to shellac alone ($P \leq 0.05$). Coating fruit with wax alone did not significantly inhibit Diplodia SER.

When 'Ruby Red' grapefruit were inoculated immediately after applying the coating treatment, those with carvacrol or thymol experienced significantly less Diplodia SER incidence and severity than those coated with shellac alone (Fig. 4 A and B). After 24 h incubation, when 92 % of the control fruit exhibited Diplodia SER lesions, significant reductions in disease incidence were observed started at 1 mg mL^{-1} oil and the inhibition increased in a concentration dependent manner. Fruit coated with shellac containing 1 mg mL^{-1} carvacrol or thymol exhibited only 5 % or 25 % disease incidence, respectively ($P \leq 0.05$). After 48 h incubation, all the fruit developed Diplodia SER lesions regardless of treatment (Fig. 4 C and D). However, carvacrol or thymol in the shellac coating significantly decreased lesion area on the fruit, even at low concentration. Carvacrol or thymol at 1 mg mL^{-1} suppressed lesion development by 43 % or 24 %, respectively, compared to shellac alone. Higher concentrations of carvacrol or thymol in the coating tended to exhibit greater effectiveness, but the differences were not significant. Repeated experiment showed the similar results.

3.5. Effects of carvacrol and thymol incorporated into a shellac coating on grapefruit quality during simulated storage and marketing conditions

After 8 weeks storage at 10 °C with 90 % RH, 23 % of control fruit developed natural decay, while fruit coated with shellac alone exhibited greater natural decay (31 %; Fig. 5 A). When the shellac coating contained either carvacrol or thymol, natural decay was significantly suppressed compared to shellac alone, only 17 % or 14 % of fruit showing natural decay, respectively ($P \leq 0.05$). After fruit were transferred to 25 °C with 60 % RH, natural decay developed quickly and treatment differences were no longer significant, although fruit coated with carvacrol or thymol still tended to develop less decay than the control or shellac alone.

Fruit weight loss increased with increased storage duration, but coating fruit with shellac significantly inhibited fruit weight loss during storage (Fig. 5 B; $P \leq 0.05$). Adding carvacrol or thymol to the coating retarded fruit weight loss slightly more than shellac alone. After 8 weeks storage, fruit coated with shellac wax containing carvacrol or thymol exhibited 19 % or 13 %, respectively, less weight loss compared to fruit coated with shellac alone ($P \leq 0.05$). After fruit were transferred to 25 °C with 60 % RH, fruit lost moisture quickly. Although relative treatment differences tended to continue, weight loss between those with shellac alone and those with carvacrol or thymol were no longer significantly different from each other, though still significantly less than the control ($P \leq 0.05$).

Fruit firmness decreased continuously during storage, especially after transferring to 25 °C with 60 % RH (Fig. 5 C). Coating with shellac wax significantly retarded fruit softening. After 8 weeks storage and 8 + 1 weeks storage, fruit coated with shellac wax exhibited 13 % and 8 %, respectively, greater firmness than the control ($P \leq 0.05$). Incorporating carvacrol or thymol into the coating reduced the ability shellac alone to maintain fruit firmness. During storage, fruit peel color turned from green to yellow or orange, with the Δa*/b* value increasing (Fig. 5 D). This change was more dramatic after fruit were transferred to room temperature. By the conclusion of the experiment, shellac-coated fruit experienced significantly slower fruit peel color change than the control, while adding carvacrol or thymol to the coating enhanced color change further. After 8 weeks cold storage, fruit coated with shellac alone exhibited 71 % lower Δa*/b* than the control fruit ($P \leq 0.05$), while fruit coated with shellac containing carvacrol or thymol experienced 100 % or 94 %, respectively, lower Δa*/b* compared to shellac alone ($P \leq 0.05$). No treatments significantly affected fruit TSS, TA, or TSS:TA ratio (data not shown).

The grapefruit showed severe chilling injury when store at 4 °C for 8 weeks, and more chilling symptoms after transferring those fruit to 25 °C for 1 week (Fig. 6). Coating fruit with shellac tended to reduce chilling injury, but the difference was not significant, while the addition of carvacrol or thymol to the shellac coating significantly reduced chilling injury ($P \leq 0.05$). After 8 weeks storage at 4°C plus 1 week at room temperature, coated fruit containing carvacrol or thymol reduced chilling injury by 62 % or 59 %, respectively, compared to fruit with shellac alone ($P \leq 0.05$).

4. Discussion

L. theobromae grows fast *in vitro*. At its optimal growing conditions, a 90-mm-diameter petri dish will be covered by *L. theobromae* mycelium after 2–3 d. This makes *L. theobromae* more difficult to control than other pathogens. Liu et al. (2010) found that *L. theobromae* could not be inhibited by 0.1 mg mL^{-1} of tea polyphenol, a natural extract of tea. The present study also found that benzaldehyde, cinnamaldehyde, trans-cinnamaldehyde, citral, eucalyptol, and menthol did not suppress mycelial growth of *L. theobromae*, although they showed effective inhibition against growth of *Penicillium digitatum, Aspergillus ochraceus, Phyctema vagabunda, Penicillium expansum, Botrytis cinerea, and Monilinia fructigena* in other studies (Amiri et al., 2008; Hua et al., 2014; Sun

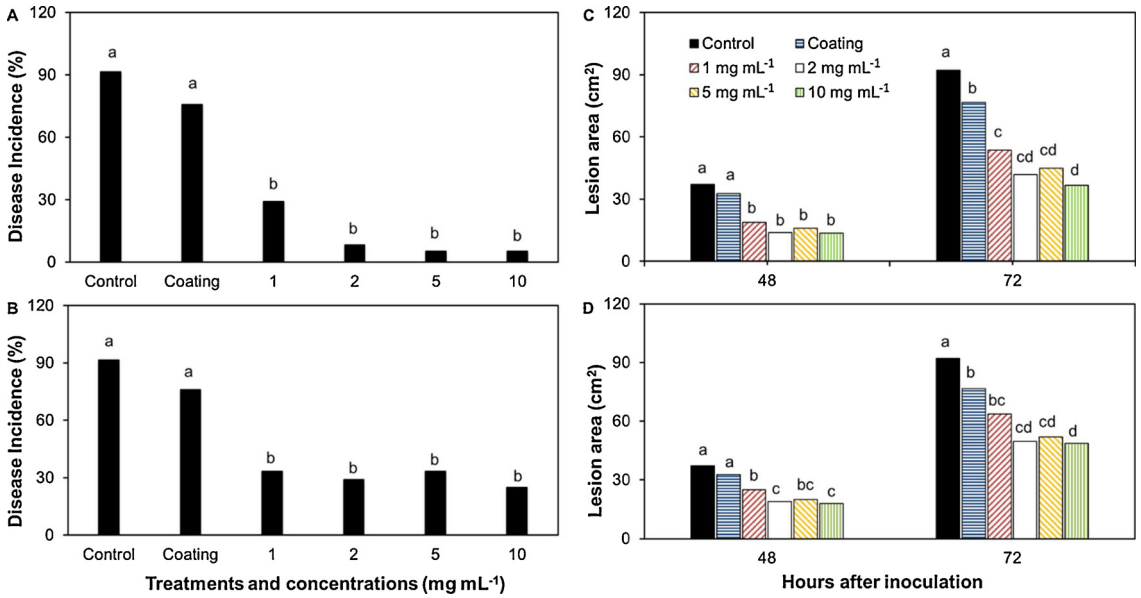

Fig. 4. Effects of coating fruit with shellac containing carvacrol (A and C) or thymol (B and D) on Diplodia SER incidence (A and B) and Diplodia SER lesion area (C and D) on 'Ruby Red' grapefruit inoculated with *Lasiodiplodia theobromae* immediately after applying the coating treatment. Afterwards, fruit were incubated at 29 °C with 90 % relative humidity (RH) and disease incidence evaluated after 24 h. Coated fruit were washed and waxed with shellac, while control fruit were washed but not waxed. Each value is the mean of three replicates of 10 fruit. Means with different letters are significantly different based on Tukey test ($P \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

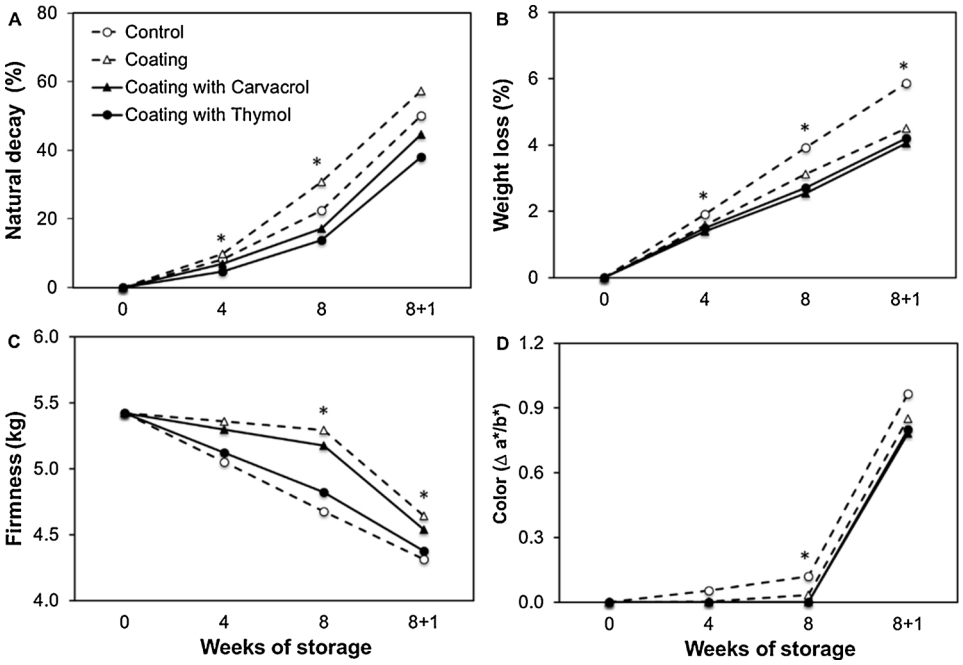

Fig. 5. Effect of coating fruit with shellac containing carvacrol or thymol on natural decay (A), weight loss (B), firmness (C), and color change (D) of 'Ruby Red' grapefruit. Grapefruit were coated with shellac with 10 mg mL^{-1} carvacrol or thymol, stored at 10 °C with 90 % relative humidity (RH) for 8 weeks, and then transferred to 25 °C with 60 % RH for 1 week. Coated fruit were washed and waxed with shellac, while control fruit were washed but not waxed. Each value is the mean of four replicates of 30 fruit. Values within each day marked by * are significantly different based on Tukey test ($P \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2014). Of natural compounds tested to date, carvacrol and thymol demonstrated the most effective inhibition activity against *L. theobromae in vitro*. Both carvacrol and thymol are phenolic compounds, and the phenolic ring plays an important role in its antimicrobial activity (Serrano et al., 2008). Carvacrol and thymol have been reported to also have antifungal activity against *Botrytis cinerea*, *Monilinia fructicola, Rhizopus stolonifer, Alternaria arborescens, Geotrichum candidum, Colletotrichum acutatum, Botryodiplodia theobromae, Phytophthora cactorum,* and *Cryponectria parasitica* (Kim et al., 2008; Numpaque et al., 2011; Plotto et al., 2002; Tsao and Zhou, 2000). They are also reported to kill gram-negative bacteria by disintegrating the outer membrane and releasing outer membrane-associated materials to the external medium (Helander et al., 1998). In the present study, the EC_{50} value of compounds *in vitro* against *L. theobromae* mycelial growth indicated that thymol showed stronger activity than carvacrol, but both compounds exhibited lower antifungal activity compared to traditional commercial fungicides. The previous study indicated that the EC_{50} value of fludioxonil (FLU), thiabendazole (TBZ), imazalil (IMZ), pyrimethanil (PYR), and azoxystrobin (AZY) against mycelial growth of *L. theobromae* were from 0.011 mg L^{-1} to 26.371 mg L^{-1} (Zhang, 2012).

To find out the optimal artificial inoculation method on grapefruit after harvest, the current study evaluated the effects of incubation temperature, inoculation site, and pathogen infection time on lesion development. The result demonstrated that high temperature is critical for Diplodia SER development on artificially inoculated fruit. This agrees with previous studies showing the optimal temperature for *L.*

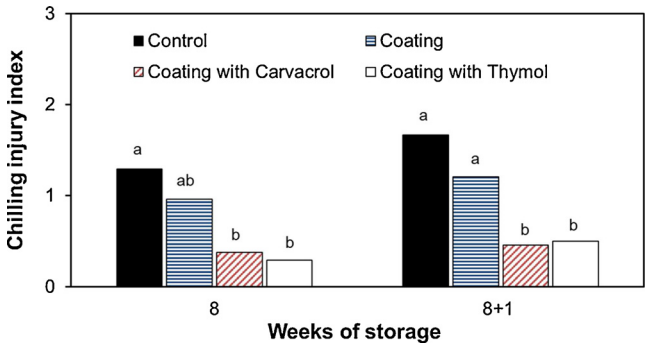

Fig. 6. Effect of shellac coatings with or without carvacrol or thymol on chilling injury of 'Ruby Red' grapefruit. Grapefruit were coated with shellac containing 10 mg mL^{-1} carvacrol or thymol, stored at 5 °C with 90 % relative humidity (RH) for 8 weeks, and then transferred to 25 °C with 60 % RH for 1 week. Each value is the mean of four replicates of 30 fruit each. Means with different letters are significantly different based on Tukey test ($P \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

theobromae growth was 30 °C and that high temperature (28 – 29 °C with 90 % RH) during degreening practices promotes Diplodia SER development on naturally infected fruit (Barmore and Brown, 1985; Brown, 1986). Although naturally infected fruit usually develop *L. theobromae* infection from the stem area, only 39 % of the fruit developed infection when the fruit were artificially inoculated at stem cavity at 29 °C with 90 % RH (Fig. 3 A). Diplodia SER originates from latent infections of *L. theobromae* in necrotic tissue on the surface of floral calyx and disc (button). In the current study, the latently infected buttons were removed from fruit at harvest to prevent natural latent Diplodia from developing (Barmore and Brown, 1985). After abscission, phenolics and lignin-like materials are synthesized and accumulated in cells of the separation layer, forming a protective layer that can limit penetration of *L. theobromae* into the fruit fresh (Barmore and Brown, 1985; Brown and Lee, 1993). Thus, inoculation at the stem area developed less infection during incubation. Inoculation using a mycelial suspension was also tested in a preliminary experiment, as described by Barmore and Brown (1985), but fruit infection was not consistent as the concentration of mycelial suspension was difficult to maintain and varied between experiments (data not shown). The current study provides an effective inoculation method for postharvest Diplodia SER study on citrus fruit, which is inoculating fruit with a mycelial plug at the fruit blossom wounded area and incubating the fruit at 29 °C with 90 % RH. The current tests indicate that *L. theobromae* can invade fruit fresh within 12 h under the current inoculation conditions, although lesions were not visible until 48 h after inoculation. This is the first report examining *L. theobromae* invasion time on artificially inoculated fruit and will provide information for further postharvest Diplodia SER studies on citrus fruit.

Diplodia SER is a serious postharvest disease in Florida. It infects immature citrus fruit on the tree, remains quiescent in necrotic tissue, and invades mature fruit through natural openings after harvest. In this study, 'Ruby Red' grapefruit were artificially wounded, inoculated with mycelial, and incubated at 29 °C with 90 % RH for 12 h before applying different coating treatments. During 12 h, hyphae of the fungus invaded fruit tissue without showing visible lesions. This process simulated natural *L. theobromae* infection and the results indicated a significant inhibition of carvacrol and thymol on lesion development. Zhang (2012) investigated five traditional fungicides in aqueous solutions at 1 mg mL^{-1} and found TBZ, IMA, FLU, PYR, and AZY reduced Diplodia SER incidence by 96.7 %, 85.5 %, 73.7 %, 40.3 %, and 17.4 %, showing higher inhibitory efficiency than the compounds in this study. However, all the fungicides were evaluated on naturally infected fruit rather than the inoculated fruit, and the amount of inoculum varied. The current study was the first report about incorporating these natural plant compounds in wax coating to control Diplodia SER on artificially inoculated fruit. Recently, many essential oils have been reported to control postharvest disease because of their direct antifungal activity (Antunes and Cavaco, 2010). Both du Plooy et al. (2009) and Fan et al. (2014) reported that wax coatings with essential oils showed significant effects against blue and green mold on artificially inoculated citrus fruit. Kouassi et al. (2012) investigated the efficiency of seven *C. zeylanicum* essential oil-wax formulations against blue and green mold on artificially inoculated orange fruit and found the efficacy not only depended on essential oil concentration in wax, but also related to the wax volume that remained on fruit and the retention of essential oil components on the fruit surface.

In the current study, the coating treatments were also applied on grapefruit before fruit were wounded and inoculated with *L. theobromae*. Both carvacrol and thymol exhibited high inhibitory efficiency on Diplodia SER incidence and severity. This may be due to the induction of systemic disease resistant responses within grapefruit, because the effects were significant at low concentration (1 mg mL^{-1}) and the efficacy did not increase with increasing concentrations. Bill et al. (2014) found that thyme oil reduced anthracnose on avocado fruit by stimulating plant defense related and antioxidant enzymes. One fungicide based on thyme oil, PROUD 3, is also labeled and used in commercial fresh fruit and vegetable packinghouse due to its local systemic effect. Both carvacrol and thymol are the main compounds in thyme oil. Besides thyme oil, the other essential oils were also reported to induce plant disease resistance. da Silva et al. (2014) found that essential oils from *Aloysia gratissima* exhibited both local and systemic effects to control powdery mildew in eucalyptus. Fan et al. (2014) also found that citral in wax coating increased antioxidant enzyme activity and inhibited green mold on citrus fruit. Therefore, the mode of action of carvacrol and thymol for Diplodia SER control may be a combination of both direct effects on the pathogen *L. theobromae* and host defense induction, but further tests are needed to confirm this.

Wax coating is widely used on citrus fruit in commercial packinghouses in Florida to reduce water loss and improve fruit appearance. In the current study, grapefruit were coated with shellac wax containing carvacrol or thymol and placed in simulated storage condition for 8 weeks followed by 1 week in simulated marketing condition to evaluate the effects of coating with carvacrol or thymol on fruit quality.

On Florida citrus fruit, most natural decay was caused by Diplodia SER and green/blue mold. The current study found that fruit coated with shellac alone exhibited significantly greater natural decay than uncoated fruit. This increase might be related to maintaining more moisture in the fruit that promoted pathogen growth and decay. When the shellac coating contained carvacrol or thymol, natural decay was significantly suppressed. du Plooy et al. (2009), Kouassi et al. (2012), and Fan et al. (2014) also found that essential oils incorporated into wax coating significantly reduced blue and green mold on citrus fruit. Therefore, carvacrol or thymol likely has value in reducing both Diplodia SER and green/blue mold on citrus fruit in Florida, but further investigation is needed.

The current study found that shellac coating inhibited fruit weight loss, fruit softening, and peel color changes, and incorporation of carvacrol or thymol in coating further inhibited fruit weight loss and color changes compared to coated fruit alone. Shellac coatings form a protective barrier on the fruit peel, inhibiting oxygen, carbon dioxide, and water diffusion, reducing fruit respiration and senescence, and inhibiting fruit weight loss, softening, and peel color changes (Mannheim and Soffer, 1996; Castillo et al., 2014). The addition of carvacrol and thymol into the wax might improve the film formation because of its oily property and enhance the protective efficiency. Both du Plooy et al. (2009) and Cháfer et al. (2012) also found that wax or chitosan coatings on orange fruit significantly reduced fruit weight loss and the addition of essential oils into the coating reduced this further. In agreement with Castillo et al. (2014) and du Plooy et al. (2009) who found no

significant difference in TSS and TA between citrus fruit coated with wax or essential oil amended wax and control fruit, none of the treatment significantly affected fruit TSS, TA, and TSS:TA ratio in this study.

Grapefruit is very sensitive to chilling injury. When stored at temperatures below 10 °C, the fruit become pitted and discolored, which influences fresh grapefruit marketability. The application of wax can form a thin film over the fruit and limit fruit gas exchange and water diffusion, which influences fruit chilling injury susceptibility (Dou, 2004; Albert, 1984). Wild (1993) and Kellerman et al. (2014) reported that wax treatment reduced chilling injury on citrus fruit, but it depended on the wax formulation. Dou (2004) also found that shellac wax provided better protection of fruit from chilling injury compared with carnauba wax and explained that was because shellac wax formed a continuous sheet, while carnauba wax formed large particles with gaps over the fruit surface. In the current study, the addition of carvacrol or thymol created thicker sheets over the fruit surface, inhibiting fruit weight loss and suppressed chilling injury.

5. Conclusion

These results indicated that incorporating carvacrol or thymol into the shellac coating, either before or after inoculation, did not prevent Diplodia SER development on artificially inoculated grapefruit (disease incidence), but did significantly reduce lesion development (disease severity). In addition, these treatments also significantly inhibited fruit decay from natural infection and the development of chilling injury during storage. Additional research is needed to evaluate incorporation of these compounds into different coatings (*e.g.* carnauba wax) and in combination with commercially used fungicides such as TBZ and IMZ. Sensory evaluations should also be conducted to determine if these compounds impact fruit aroma and taste after simulated storage, transport and marketing.

CRediT authorship contribution statement

Jiaqi Yan: Funding acquisition, Methodology, Visualization, Writing - original draft. **Jiuxu Zhang:** Investigation, Methodology, Writing - review & editing. **Cuifeng Hu:** Data curation, Formal analysis, Resources, Software. **Lili Deng:** Methodology, Resources, Validation. **Mark A. Ritenour:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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