



Comparative Effects of Postharvest Fungicides for *Diplodia* Stem-end Rot Control of Florida Citrus

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Diplodia stem-end rot caused by *Diplodia natalensis* is one of the most important citrus postharvest diseases in Florida. Fungicide application is an important measure to effectively control *Diplodia* stem-end rot. In this study the activities of new postharvest fungicides azoxystrobin (AZY), fludioxonil (FLU), and pyrimethanil (PYR) for *Diplodia* stem-end rot control were evaluated and compared both in vitro and in vivo to those of existing fungicides imazalil (IMZ) and thiabendazole (TBZ). All fungicides inhibited the mycelial growth of the fungus on potato dextrose agar (PDA), and varied in their activities. The EC₅₀ of FLU, TBZ, IMZ, PYR, and AZY against the fungal mycelial growth was estimated at 0.011, 0.148, 0.787, 3.052, and 26.371 ppm, respectively. The decay control efficacy tests of the fungicides were conducted on 'Fallglo' tangerine hybrids and 'Pineapple' oranges using dipping, drenching, and packing line spraying application methods. At three weeks of fruit storage at 21 °C after fruit treatments with aqueous fungicides at 1,000 ppm in three separate tests, TBZ, IMZ, and FLU reduced the decay by 73.5% to 96.7%, more effectively than PYR and AZY, which reduced the disease by 17.4% to 67.0%. TBZ performed better than or similarly to IMZ and FLU. PYR performed similarly to AZY. Overall studies indicate that TBZ, IMZ, and FLU, but not AZY and PYR, are effective for *Diplodia* stem-end control on Florida citrus.

Diplodia stem-end rot, caused by *Diplodia natalensis* Pole-Evens [Syns. *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. and *Botryodiplodia theobromae* Pat.], is a common and serious postharvest disease on citrus in Florida and other warm, humid regions (Brown and Eckert, 2000). All citrus cultivars are susceptible to this disease, and its severity can be significantly enhanced by ethylene degreening treatment, particularly prolonged degreening duration and high ethylene concentrations (Barmore and Brown, 1985; Brown, 1986; Zhang, 2004). *Diplodia* stem-end rot originates from the latent infections of *D. natalensis* established in necrotic tissue on the surface of the fruit calyx and disk (button) on trees in groves (Brown and Burns, 1998). The fungus grows and penetrates the natural openings that are formed during early stages of button separation from the fruit at abscission (Brown and Wilson, 1968). The effective control of *Diplodia* stem-end rot is achieved by an integrated disease management system with postharvest fungicide application as the core component (Brown and Miller, 1999; Ismail and Zhang, 2004). The good cultural practice in the groves could reduce the pathogen population size by maintaining vigorous trees and minimizing the amounts of dead wood (Brown and Eckert, 2000). After harvest, if fruit ethylene degreening treatment is needed, it should be minimized, avoiding unnecessarily prolonged degreening time and high ethylene concentration (Ismail and Zhang, 2004; Ritenour et al., 2011). Postharvest fungicide application (drenching and/or packing line spraying) is one of the important measures in *Diplodia* stem-end rot control system in Florida's commercial packinghouses (Brown and Eckert, 2000; Ismail and Zhang, 2004). Thiabendazole (TBZ), imazalil (IMZ), and sodium o-phenylphenate (SOPP) are the existing commercial fungicides and have been used for *Diplodia*

stem-end rot and other decay control for more than 25 years (Eckert and Brown, 1986). TBZ is the most effective fungicide against *Diplodia* stem-end rot, followed by IMZ, and SOPP has some activity (Brown and Eckert, 2000). In recent years, three new fungicides, azoxystrobin (AZY), fludioxonil (FLU), and pyrimethanil (PYR), have been registered for citrus postharvest disease control, particularly for *Penicillium* decay control (US EPA, 2004a; US EPA, 2004b; US EPA, 2009). Many studies have shown that the new fungicides are effective for *Penicillium* decay control (Adaskaveg et al., 2004; Kanetis et al., 2007; Smilanick et al., 2006; Zhang, 2007). Some information on the new fungicides FLU and PYR for *Diplodia* stem-end rot control of Florida citrus has been available (Zhang, 2007, 2009). However, more information on all new fungicides is still needed. The objectives of this study were to evaluate the activities and efficacies of the new fungicides AZY, FLU, and PYR for inhibiting fungal mycelial growth in vitro and for *Diplodia* stem-end rot control in vivo, in comparison to the existing fungicides TBZ and IMZ.

Materials and Methods

FUNGICIDES. Pyrimethanil (PYR) [Penbotec®, 40% active ingredient (AI)] was obtained from Janssen Pharmaceutica, Beerse, Belgium. Imazalil (IMZ) (Freshgard® 700, 44.6% AI) was obtained from JBT Foodtech (Lakeland, FL). Thiabendazole (TBZ) (Mer-tect® 340-F, 42.3% AI), azoxystrobin (AZY) (Abound, 25% AI) and fludioxonil (FLU) (A9859A, 20% AI) were obtained from Syngenta Crop Protection Inc. (Greensboro, NC). AZY (99.5% AI) was obtained from Chem Service (West Chester, PA).

CITRUS FRUIT. Tangerine (*Citrus reticulata* Blanco) hybrids ('Fallglo'), and oranges [*Citrus sinensis* (L.) Osbeck, cv. Pineapple] were used in tests. Fruit that did not receive any postharvest treatments were obtained from a local commercial citrus packinghouse (Hunt Brothers, Lake Wales, FL). All tests were conducted at the

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facility of the University of Florida Citrus Research and Education Center (UF/CREC) at Lake Alfred, FL.

EFFECTS OF FUNGICIDES ON THE MYCELIAL GROWTH OF *D. NATALENSIS*. Individual fungicides (AZY, FLU, PYR, IMZ, and TBZ) were incorporated into autoclaved Difco™ potato dextrose agar (PDA) at a range of concentrations from 0 up to 100 ppm (AI) before pouring the medium into plates. Different concentration ranges for different fungicides were chosen due to the different sensitivities of the fungicides to the fungus. These sensitivities were determined prior to these bioassays. For example, the concentration range for FLU was 0 to 0.1 ppm, and 0 to 100 ppm for AZY and PYR. For AZY bioassay, the chemical (99.5%, Chem Service) was used to make the different concentrations of AZY in the tests, and all treatments were amended with salicylhydroxamic acid at 100 µg/mL to inhibit the alternative oxidase pathway (Wise et al., 2008). *D. natalensis* isolate D-20 (wild-type) was grown on PDA for 2 d at 30 °C before it was transferred to testing plates. PDA plugs (0.5-cm diameter) were cut from the edge of *D. natalensis* colonies and transferred onto the centers of fungicide-amended PDA plates. One fungal plus was used for each plate. Five plates were used for each concentration of a fungicide. The PDA plates with the fungus were incubated at 30 °C for 24 h, and the diameters of radial mycelial growth of the fungus were recorded. The experiment was conducted twice. EC₅₀ values were calculated based on the logarithmic models (relationships between percent fungal mycelial growth inhibitions and fungicide concentrations) for each of the fungicides. The models were fitted by transferring fungicide concentrations to logarithm.

EFFECTS OF NEW FUNGICIDES ON DIPLODIA STEM-END ROT CONTROL ON CITRUS FRUIT. The efficacies of new fungicides for Diplodia stem-end rot control on citrus fruit were evaluated using three different application methods to simulate the commercial packinghouse operations. Early-season fruit 'Fallglo' tangerine hybrids and mid-season fruit 'Pineapple' oranges were used in the tests. Ethylene degreening treatments of the fruit were conducted to enhance the Diplodia stem-end rot development and simulate the commercial operations. Fruit were not inoculated with the pathogen, and Diplodia stem-end rot was caused by natural infections. The following are the three fungicide application methods used in the different tests:

DIPPING APPLICATION. 'Fallglo' tangerine hybrids that were obtained from a local packinghouse were washed and dried through a simulated commercial packing line system. Fruit were then subjected to ethylene degreening treatment (10 ppm) for 2 d in a degreening room. The new fungicides AZY, FLU, and PYR, and the existing fungicides TBZ and IMZ were tested. Aqueous fungicide solutions were made for each fungicide at 1,000 ppm level. Fruit were dipped into the fungicide solutions for 1 min, drained for 5 min, and then air-dried. Each treatment had three replicates, and each replicate contained 60 fruit. Treated fruit were placed in plastic crates (60 cm × 40 cm × 30 cm) and incubated at 21 °C and 90–95% relative humidity (RH). Diplodia stem-end rot was identified and recorded weekly for up to 4 weeks.

DRENCHING APPLICATION. A simulated commercial drenching system was established at the UF/CREC facility (Zhang, 2007) and used in this test. About 40 L of the fungicide suspension were applied in a drench cycle. The flow rate was approximately 20 L/min. 'Fallglo' tangerine hybrids were placed into plastic crates. Three plastic crates were stacked on top of each other as a treatment to simulate a commercial drench procedure. Each plastic crate contained about 70 fruit representing a replicate. Concentration of each tested fungicide in drench suspension was made at 1,000

ppm. The tested fungicides were AZY, FLU, PYR, TBZ, and IMZ. Fruit were drenched with the aqueous fungicide solution for 3 min, and drained for 4 min (commercial standard practice) prior to ethylene degreening treatment. After drenching, the fruit were subjected to ethylene degreening treatment (10 ppm ethylene, 28 °C and 90–95% RH) for 3 d. The treated fruit were then incubated at 21 °C and 90–95% RH for up to 3 weeks. Diplodia stem-end rot was identified and recorded weekly.

PACKING LINE APPLICATION. Pineapple oranges were subjected to ethylene degreening treatment (10 ppm) at 28 to 30 °C and 90–95% HR for 2 d before treating fruit with fungicides. Fruit were washed, dried, and randomized through a packing line system, and fruit were then treated with an aqueous fungicide suspension (1,000 ppm) by a non-recovery dripping system through the packing line system. The delivery rate of the fungicide suspension to the fruit was about 50 mL per 60 fruit. Treated fruit were dried at approximately 52 °C for 2 min, and then packed into 4/5 bushel cartons. The tested fungicides were AZY, FLU, PYR, TBZ, and IMZ. The fruit that were not treated with any fungicides served as controls. Each treatment consisted of 4 replicates, and each replicate contained 60 fruit. Treated fruit were then incubated at 21 °C and 90–95% RH. Diplodia stem-end rot was identified and recorded weekly for up to 4 weeks.

DATA ANALYSIS. Analysis of variance of data was performed using the JMP statistical package (SAS Institute Inc., Cary, NC). Percentage decay data were transformed to arcsine values before the data analysis. Treatment means were compared using the Tukey–Kramer HSD multiple range test ($P \leq 0.05$). Actual Diplodia stem-end rot incidences were presented in all figures.

Results

INHIBITION OF FUNGAL MYCELIAL GROWTH BY POSTHARVEST FUNGICIDES IN VITRO. When the fungicides were incorporated into PDA at concentrations up to 100 ppm depending on individual fungicides, all five fungicides AZY, FLU, PYR, IMZ, and TBZ inhibited the mycelial growth of *D. natalensis* in PDA, but their inhibiting activities varied largely (Table 1). The relationship of fungicide concentration and percent inhibition of fungal mycelial growth better fitted the logarithmic model than other models such as the linear model (data not shown). Based on the EC₅₀ values, FLU (EC₅₀ = 0.011 ppm) was the most effective, and AZY (EC₅₀ = 26.371 ppm) was the least active against the mycelial growth of *D. natalensis* under test conditions (Table 1). The order of their fungal inhibiting activity was: FLU > TBZ > IMZ > PYR > AZY based on their EC₅₀ values (Table 1).

DIPPING APPLICATIONS. 'Fallglo' tangerine hybrids were subjected to ethylene degreening treatment for 2 d before fungicide treatment to simulate the commercial degreening procedures as

Table 1. Effects of postharvest fungicides on mycelial growth of *Diplodia natalensis* on potato dextrose agar growing medium

Fungicide	EC ₅₀ (ppm) ²
Thiabendazole	0.148
Imazalil	0.787
Fludioxonil	0.011
Pyrimethanil	3.052
Azoxystrobin	26.371

²EC₅₀ means the concentration of a fungicide that shows 50% fungal mycelial growth inhibition of its maximum effect under the test conditions (30 °C incubation for 24 h).

well as to increase *D. natalensis* infections. After 'Fallglo' tangerine hybrids were degreened with ethylene, dipped into fungicide solutions (1,000 ppm) for 1 min and then incubated at 21 °C for 4 weeks, the control fruit showed a *Diplodia* stem-end rot incidence of 81.1% (Fig. 1). FLU, PYR, TBZ, and IMZ significantly ($P < 0.05$) reduced the disease incidence by 46.6% to 90.4%, and AZY did not significantly control the disease (Fig. 1). FLU, IMZ, and TBZ performed similarly and reduced decay incidence by 76 to 90.4%. PYR was much less effective than FLU, IMZ, and TBZ for *Diplodia* stem-end rot control under these conditions.

It can be also seen from Fig. 1 that the decay control efficacies of different fungicides changed significantly during the 4 weeks of fruit incubation at 21 °C. FLU, IMZ, and TBZ consistently and significantly controlled *Diplodia* stem-end rot, but AZY only delayed the disease progress. Although PYR performed better than AZY, it did not achieve a decay control at an acceptable level (Fig. 1).

DRENCHING APPLICATION TEST. To simulate commercial packing process, 'Fallglo' tangerine hybrids were drenched for 3 min with a fungicide solution (1,000 ppm) and then the fruit were subjected to ethylene degreening treatment. After fruit were stored at 21 °C for 3 weeks, *Diplodia* stem-end rot incidences were very high and the control fruit showed a disease incidence of 89.2% (Fig. 2). AZY, PYR, FLU, IMZ and TBZ reduced disease incidence by 17.4, 40.3, 73.7, 85.5 and 96.7%, respectively (Fig. 2). TBZ performed the best for the disease control, followed by IMZ and FLU. AZY and PYR only delayed disease progress and did not achieve an acceptable level of decay control (Fig. 2).

PACKING LINE APPLICATIONS. In this test 'Pineapple' oranges were first degreened with ethylene and then treated with a fungicide by spraying fungicide suspension to the fruit when they were run through a packing line system. During the 4 weeks of fruit incubation at 21 °C, *Diplodia* stem-end rot incidence on control fruit increased over time and reached to 31.9% at 4 weeks of the storage (Fig. 3). Fungicides significantly reduced *Diplodia* stem-end rot, but their efficacies for decay control largely varied. TBZ,

IMZ and FLU consistently showed good efficacies for *Diplodia* stem-end rot control over time, but AZY and PYR did not achieve an acceptable level of decay control efficacy (Fig. 3).

Discussion

In the current study, three new fungicides AZY, FLU, and PYR were evaluated for their antifungal activities in vitro and their efficacies in vivo for *Diplodia* stem-end rot control in comparison to the existing fungicides IMZ and TBZ. All three new fungicides reduced *Diplodia* stem-end rot incidences on citrus

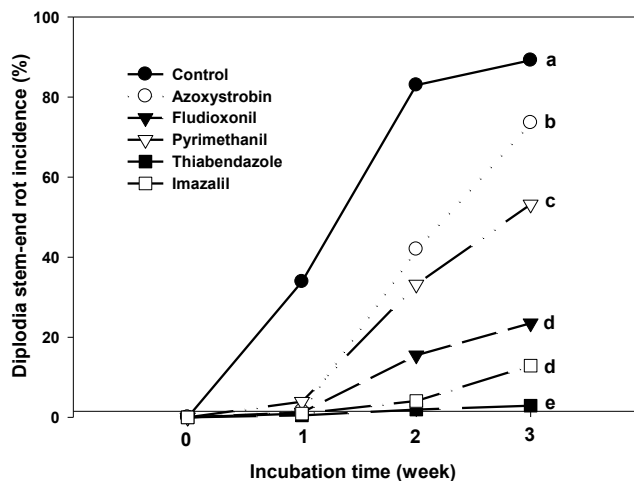


Fig. 2. Effects of postharvest fungicides on *Diplodia* stem-end rot control on 'Fallglo' tangerine hybrids using a simulated commercial drench application method. Fruit were degreened with ethylene (10 ppm) for 3 d after fruit drenching with fungicides. Disease incidences were cumulative data over 3 weeks of incubation at 21 °C and 90–95% relative humidity. Disease incidences at 3 weeks of fruit incubation were significantly different ($P \leq 0.05$) if the letters are different based on Tukey-Kramer HSD multiple range test.

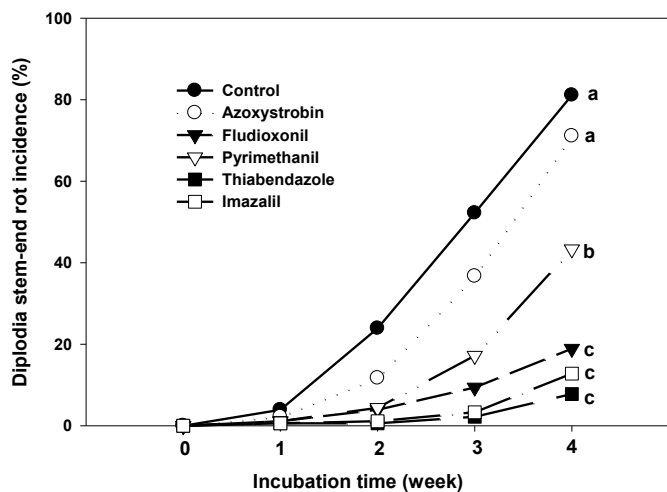


Fig. 1. Effects of postharvest fungicides on *Diplodia* stem-end rot control on 'Fallglo' tangerine hybrids using a dip application method. Fruit were degreened with ethylene (10 ppm) for 2 d before fungicide treatments. Disease incidences were cumulative data over 4 weeks of incubation at 21 °C and 90–95% relative humidity. Disease incidences at 4 weeks of fruit incubation were significantly different ($P \leq 0.05$) if the letters are different based on Tukey-Kramer HSD multiple range test.

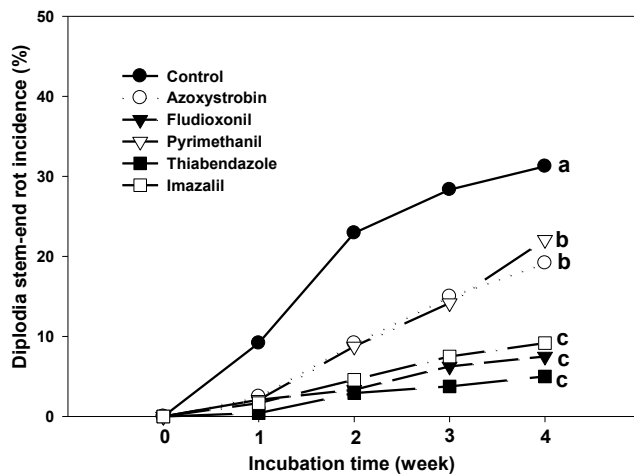


Fig. 3. Effects of postharvest fungicides on *Diplodia* stem-end rot control on 'Pineapple' oranges using a simulated commercial packingline application method. Fruit were degreened with ethylene (10 ppm) for 2 d before fungicide application. Disease incidences were cumulative data over 4 weeks of incubation at 21 °C and 90–95% relative humidity. Disease incidences at 4 weeks of fruit incubation are significantly different ($P \leq 0.05$) if the letters are different based on Tukey-Kramer HSD multiple range test.

fruit at certain levels depending on the duration of fruit storage after fungicide treatments, but only FLU showed a strong activity and acceptable efficacy for this decay control. Both TBZ and IMZ effectively and consistently reduced *Diplodia* stem-end rot incidences in all tests. FLU performed similarly to IMZ, and TBZ performed similarly to or better than FLU and IMZ for *Diplodia* stem-end rot control. AZY and PYR significantly reduced the disease incidence for about a week, and then lost their efficacies quickly after a week during fruit storage. This indicates that AZY and PYR may only possess some fungistatic activity and delay the disease progression.

In the present study, the EC₅₀ values of the fungicides in vitro against *D. natalensis* mycelial growth indicated that FLU showed the strongest activity followed by TBZ and IMZ. PYR showed much lower activity, and AZY had the least activity against the fungal growth. These in vitro fungal growth inhibition activities of the fungicides were consistent with their efficacies for *Diplodia* stem-end rot control observed on fruit in vivo. Although FLU has the strongest activity against fungal mycelial growth in vitro, it was not the most effective one in vivo for *Diplodia* stem-end rot control on fruit. This might be partially due to its less systemic activity in fruit tissue compared to IMZ and TBZ.

In this study, citrus fruit were subjected to ethylene degreening treatment before or after fungicide treatment to enhance pathogen infection and disease development. This ethylene treatment resulted in high or very high cumulative *Diplodia* stem-end rot incidences on tested fruit. The possible reasons that ethylene treatment promotes the *Diplodia* stem-end rot development have been reported as: 1) ethylene promotes the formation of abscission zone between fruit and button, allowing the pathogen to infect fruit tissues (Brown and Wilson, 1968); 2) ethylene may reduce natural fruit resistance to the fungus by triggering certain biochemical changes in the fruit rind (Zhang, 2004); and 3) ethylene stimulates the production of cell wall-degrading enzymes by *D. natalensis* (Brown and Burns, 1998). Since ethylene enhances *Diplodia* stem-end rot severity, fungicide applications before fruit degreening should be more effective than those after fruit degreening treatments (Brown and Miller, 1999; Zhang, 2004). In fact, TBZ and IMZ drenching of fruit before the degreening treatment is a common practice in Florida packing-houses (Brown and Miller, 1999). Since the new fungicide FLU, but not AZY and PYR, is effective for *Diplodia* stem-end rot control, FLU should to be a good alternative to TBZ and IMZ for *Diplodia* stem-end rot control (Zhang, 2007).

It has been reported that the new fungicides FLU, AZY, and PYR are effective for the control of green mold caused by *Penicillium digitatum* on citrus fruit (Adaskaveg et al., 2004; Kanetis et al., 2007; Smilanick et al., 2006; Zhang, 2007). Developing fungicide resistance by pathogens is a common problem for disease control, especially for *Penicillium* species (Brown and Miller, 1999; Ismail and Zhang, 2004). *Penicillium* resistance to TBZ and IMZ has been reported in California citrus packing-houses (Holmes and Eckert, 1999; Kinay et al., 2007) and other regions (Perez et al., 2011; Wild, 1994). The existing and new postharvest fungicides TBZ, IMZ, AZY, FLU, and PYR have different modes of action compared to each other, and are valuable chemical tools for *Penicillium* fungicide resistance management. However, stem-end rots such as *Diplodia* stem-end rot in Florida are also important citrus postharvest problems. The appropriate use of currently available postharvest fungicides in an integrated postharvest disease control system is important to effectively control both *Penicillium* decays and other diseases such as *Diplodia*

stem-end rot in Florida packing-houses. Another common citrus postharvest fungal problem in Florida is *Phomopsis* stem-end rot caused by *Phomopsis citri* (Ismail and Zhang, 2004). TBZ and IMZ are effective for *Phomopsis* stem-end rot control (Ritenour et al., 2011). However, it is still not clear if the new fungicides AZY, FLU, and PYR are effective for *Phomopsis* stem-end rot control. This needs to be studied in the future.

AZY showed much less antifungal activity in vitro and a much lower efficacy for *Diplodia* stem-end rot control in vivo compared to the other fungicides tested in the current study. This could be related to its specific mode of action. AZY belongs to the strobilurin fungicide family, which has a quinone outside inhibitor (QoI) mode of action against many fungal pathogens (Kanetis et al., 2007). They are effective against fungal sporulation, spore germination, and mycelial growth of some other fungal pathogens (Kanetis et al., 2007; Mondal et al., 2005; Wise, 2008). Bushong and Timmer (2000) studied AZY for controlling citrus scab caused by *Elsinoe fawcettii* and melanose caused by *Diporthe citri* and found that AZY was more effective for disease control when it was applied at pre-infection stage of fungal pathogens, and had a poor disease control efficacy when it was applied at post-infection stage. In the current study, on harvested citrus fruit *D. natalensis* infection has been at a latent infection stage on the calyx tissue, and it reactivates and quickly grows into fruit tissue via the abscission zone formed as a result of ethylene degreening treatment. AZY was also much less effective in vitro against *D. natalensis* mycelial growth compared to other tested fungicides (Table 1). This body of information might explain why AZY did not perform well for the *Diplodia* stem-end rot control on citrus fruit degreened with ethylene. For postharvest citrus disease control however, AZY is not marketed as a single active ingredient in the product. Its manufacturer, Syngenta Inc., has registered it as a formulation called Graduate A+®, which contains two active ingredients, AZY (20.6%) and FLU (20.6%) (US EPA, 2009). Graduate A+® should have a positive effect for *Diplodia* stem-end rot control since it contains FLU. The synergetic effect of AZY and FLU for decay control is not known. The product Graduate A+® was not tested in the current study, and more studies on it are needed.

In conclusion, three newly registered citrus postharvest fungicides AZY, FLU, and PYR were evaluated both in vitro and in vivo in comparison to the existing postharvest fungicides TBZ and IMZ for *Diplodia* stem-end rot control using different application methods. The results demonstrated that FLU, but not PYR and AZY, was effective for *Diplodia* stem-end rot control. FLU performed similarly or slightly less compared to TBZ and IMZ. TBZ remains the most effective fungicide for this decay control. FLU appears to be a new alternative fungicide for citrus postharvest *Diplodia* stem-end rot control on Florida fresh citrus.

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