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Correlation of *Diplodia* (*Lasiodiplodia theobromae*) infection, huanglongbing, ethylene production, fruit removal force and pre-harvest fruit drop☆

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ABSTRACT

Citrus greening or huanglonbing (HLB) is thought to be caused by *Candidatus Liberibacter* asiaticus (*C*Las) and is devastating the citrus industry worldwide. One symptom of HLB disease is excessive pre-harvest fruit drop. Recently, higher incidence of *Lasiodiplodia theobromae* (*Diplodia*) was found in HLB-symptomatic orange calyx abscission zones (AZ-C) than in non-symptomatic fruit, and the infection was positively correlated with the reduction in fruit detachment force (FDF), suggesting that *Diplodia* infection may be involved in the HLB-related pre-harvest fruit drop. To verify the hypothesis, we conducted two experiments. Experiment 1 was conducted by shaking HLB-affected 'Hamlin' and 'Valencia' orange trees during the harvest season (twice for 'Hamlin' and once for 'Valencia'). The fruit that dropped from trees upon shaking were collected (D), and the fruit retained on trees after shaking were harvested (R). Fruit ethylene production was measured, and the levels of *Diplodia* and *C*Las in AZ-C of D and R fruit were analyzed. The results revealed significantly higher levels of *Diplodia* in D compared with R fruit; and ethylene was produced from more than half of the D fruit but none of the R fruit. Ethylene production was positively correlated with *Diplodia* level in D fruit. In experiment 2, a preliminary trial on the effect of fungicide (Quadris Top) application on incidence of *Diplodia* infection and fruit drop was investigated. The experiment was conducted in a commercial grove with essentially 100% of the trees being HLB-symptomatic, and included five citrus cultivars ('Early Gold' orange, 'Midsweet' orange, 'Murcott' tangor, 'Navel' orange and 'Ray Ruby' grapefruit). *Diplodia* levels were lower and FDF significantly higher in fungicide-treated compared to non-treated 'Early Gold', 'Midsweet' and 'Murcott' fruit, and consequently, the fruit drop was reduced by 45%, 30% and 46% that of non-sprayed controls, respectively. For 'Navel' or 'Ray Ruby' fruit, there was no significant change between sprayed and non-sprayed controls in the level of *Diplodia*, FDF or fruit-drop. The results consistently showed a positive correlation between *Diplodia* infection and fruit drop in HLB-affected fruit, indicating the possible involvement of the fungus in HLB-related excessive fruit drop. This suggests that control of *Diplodia* fungal infection in the field may reduce HLB-associated pre-harvest fruit drop.

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1. Introduction

Huanglongbing (HLB, also known as citrus greening), a devastating disease of citrus, has spread throughout the major citrus producing regions in Asia, Africa and the Americas, resulting in severe losses for the citrus industry worldwide (Gottwald, 2010). HLB is associated with *Candidatus Liberibacter* spp., a Gram-negative, phloem-limited bacterium (Bastianel et al., 2005). The Asian form of HLB, *Candidatus Liberibacter* asiaticus (*C*Las), is currently present in the U.S. *C*Las was first confirmed in Southeast Florida in 2005 (Gottwald, 2010), and now is established in all Florida citrus production areas. Transmission of *C*Las is predominantly mediated by its insect vector, the Asian citrus psyllid (ACP) (*Diaphorina citri*), but also can be transmitted by grafting.

HLB causes substantial economic loss by reducing the productive capacity and shortening the life span of infected trees, as well

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as promoting fruit drop (Bové, 2006). Yield reduction can reach 30–100% depending on the proportion of the canopy affected and the age of trees during infection (Bassanezi et al., 2011; Gottwald, 2010).

Disease symptoms include leaf chlorosis (blotchy mottle and others), twig dieback, poor fruit coloration, reduced fruit size, misshapen fruit and reduced fruit quality (Baldwin et al., 2010; Dagulo et al., 2010). An orange-brown stain may be present at the calyx abscission zone (AZ-C) located at the pedicel–fruit interface (Bové, 2006). Many fruit abscise prematurely at the calyx abscission zone (AZ-C) (Graca, 1991). As the severity of HLB progresses, pre-harvest fruit drop increases and results in significant loss of yield (Bassanezi et al., 2011; USDA-NASS, 2014, 2015). During the 2012–13 Florida harvest season, the amount of pre-harvest fruit drop for a season not affected by freezes, hurricanes or other weather issues was greater than expected and the most in more than 40 years (Bouffard, 2013). The 2013–14 and 2014–15 seasons were progressively worse. Pre-harvest fruit drop averaged between 9% and 11% between 2009 and 2012, while fruit drop increased to 18% and 31% in the last three seasons for early-midseason and 'Valencia' varieties respectively, resulting in fruit losses worth more than $150 million annually (USDA-NASS, 2015).

Different approaches have been tried to alleviate the fruit drop problem in HLB-affected orchards, but to date no therapeutic treatments have proven significantly effective. In one case, acidification of the rhizosphere reportedly increased root density and reduced fruit drop by 6% (Graham et al., 2014). Use of plant growth regulators (PGRs) including gibberellic acid, 2,4-dichlorophenoxyacetic acid (2, 4-D), 2,4-D Isopropylester (2,4-D IPE) to reduce HLB-related pre-harvest fruit drop was found to be inconsistent (Albrigo, 2014), although they have been commercially used to retard mature citrus fruit abscission for decades (El-Otmani et al., 2000). The enhanced nutritional programs adopted by growers to reduce tree disease symptoms unfortunately did not affect fruit drop (Gottwald et al., 2012).

Understanding the mechanism for HLB-related fruit drop would help to develop an effective control strategy. For HLB-affected citrus, the factors responsible for the excessive fruit drop seem elusive, although the phloem impairment (Kim et al., 2009) and loss of roots (Johnson et al., 2012) have been linked to fruit starvation and water stress, which may contribute to the HLB-associated pre-harvest fruit drop. It was reported that HLB symptomatic and asymptomatic fruit from symptomatic trees produced less ethylene than "healthy fruit" (harvested from *C*Las negative trees) (Rosales and Burns, 2011). Ethylene is a gaseous phytohormone that is known to play a pivotal role in promotion of organ abscission (Tadeo et al., 2008). The observation that HLB fruit do not enhance ethylene production suggests that some causal agents other than HLB itself might be involved in the excessive pre-harvest fruit drop.

Recently, higher incidence of *Lasiodiplodia theobromae* (formerly known as *Diplodia natalensis*; hereafter termed *Diplodia*), an opportunistic fungal pathogen, was found in HLB-symptomatic orange AZ-C than in non-symptomatic fruit from non HLB-symptomatic trees (Zhao et al., 2015). *Diplodia* is the causal agent of stem end rot (SER) which is not typically a field problem, but is a common postharvest disease (Brown, 1986). Following infection of the calyx, *Diplodia* typically remains quiescent while the fruit is attached to the tree, and the fungus does not usually start to colonize the fruit tissue until after harvest (Brown and Wilson, 1967). However, the colonization of *Diplodia* was found in the HLB-affected orange fruit prior to harvest, and the infection was correlated with fruit ethylene production and reduction in fruit detachment force (FDF), suggesting the possible involvement of *Diplodia* in the excessive fruit drop (Zhao et al., 2015).

In this study, we further investigate the role of *Diplodia* in fruit drop in two different experiments. In the first experiment, HLB-affected 'Hamlin' and 'Valencia' orange trees were manually shaken during the harvest season, *Diplodia* and *C*Las levels in fruit AZ-C, as well as the fruit ethylene production were compared between the fruit that dropped from trees upon shaking (D) and the fruit that remained on trees after shaking (R), with an aim to determine the relationship between *Diploidia* infection and fruit drop. In the second experiment, a fungicide (Quadris Top) was applied in a HLB-affected grove on five citrus types/cultivars in an effort to control *Diplodia*. The citrus types/cultivars included 'Early Gold' orange, 'Midsweet' orange, 'Murcott' tangor, 'Navel' orange and 'Ray Ruby' grapefruit, and spray treatments were followed by evaluation and comparison of *Diplodia* and *C*Las levels, FDF, and fruit drop between the sprayed and non-sprayed controls, with an aim to establish a relationship between changes in *Diplodia* level and fruit drop in the presence of *C*Las.

2. Materials and methods

2.1. Trees used in the tree shaking experiment

Six-year old 'Hamlin' and 'Valencia' orange trees (*Citrus sinensis* (L.) Osbeck), about 2.5–3.0 m tall, on 'Swingle' citrumelo (C. paradisi Macf. × Poncirus trifoliata (L) Raf.) rootstock, in a commercial grove located in Southern Florida, were selected for the experiment. The selected trees were similar in size, had tested *C*Las positive by qPCR using the method of Li et al. (2006), were grown under similar agro-climatic conditions and received common cultural practices and the grower's standard pest and disease management (UF/IFAS, Florida Citrus Pest Management Guide, 2016). 'Hamlin' fruit were sampled on 1 Dec., 2014 and 5 Jan., 2015, and 'Valencia' fruit were sampled on 8 Apr. 2015. Each sampling included nine trees. Ground under the trees was cleaned of dropped fruit and leaves just before shaking the trees, and trees were shaken manually. The dropped fruit from the trees upon shaking were collected (D), and the retained fruit after shaking were harvested (R). Thirty fruit were randomly picked from each of the D and R groups for ethylene measurement and DNA isolation.

2.2. Measurement of ethylene production

Ethylene production was determined for D and R fruit by incubating individual fruit in 1 l glass jars which were sealed for 1 h. One ml of headspace gas was withdrawn from each jar using a gas tight syringe and analyzed for ethylene by gas chromatography (Hewlett-Packard 5890, Avondale, PA) equipped with a flame ionization detector and an activated alumina column.

2.3. Trees used in the fungicide spray trial

The experiment was carried out on five citrus types/cultivars: 'Murcott' tangor (*Citrus reticulata* × *C. sinensis*, 13 year old trees) on 'Volkameriana' lemon rootstock, 'Navel' (Glen Navel) orange (*C. sinensis*, 40 year old trees), on sour orange (*C. aurantium*) rootstock, 'Early Gold' orange (*C. sinensis*, 15 year old trees), 'Midsweet' orange (*C. sinensis*, 15 year old trees), and 'Ray Ruby' grapefruit (*C. paradise*, 8 year old trees) on 'Swingle' citrumelo (*C. paradisi* × *Poncirus trifoliata*) rootstock. The trees were located in different parts of a large commercial grove in Indian River Co., Florida. The trees were grown under similar agro-climatic conditions, received common cultural practices and the grower's standard pest and disease management treatments. Application of agrochemicals was consistent for each group of experimental trees and was typical of commercial product practices in the region and included foliar nutritional treatments, insecticides to control Asian citrus psyllids and copper for control of citrus canker (UF/IFAS, Florida Citrus Pest Management Guide, 2016). The experiments were performed with 10 replicate trees

Table 1
The schedules for Quadris Top application, sampling for qPCR testing, fruit detachment force (FDF) measurement, and fruit drop count.

	Dates applied fungicide (2014)				Dates sampled for qPCR (2014)			Dates measured FDF (2014)		Dates evaluated fruit drop (2014–2015)			
	#1	#2	#3	#4	#1	#2	#3	#1	#2	#1 (2014)	#2 (2014)	#3 (2014)	#4 (2014–2015)
Early Gold Orange	4/4	5/30	8/29	10/31	4/17	6/10	9/11	9/11	10/30	9/11–10/2	10/2–10/30	10/30–11/12	–
Navel Orange	4/4	5/30	8/29	10/31	4/17	6/10	9/11	9/11	10/30	9/11–10/2	10/2–10/30	10/30–11/12	–
Midsweet Orange	4/4	5/30	9/19	12/12	4/17	6/10	10/2	10/2	11/24	10/2–10/30	10/30–11/24	11/24–12/22	12/22/14–1/16/15
Murcott Tangor	4/4	5/30	9/19	12/12	4/17	6/10	10/2	10/2	11/24	10/2–10/30	10/30–11/24	11/24–12/22	12/22/14–1/16/15
Ray Ruby Grapefruit	4/4	5/30	9/19	12/12	4/17	6/10	10/2	10/2	11/24	10/2–10/30	10/30–11/24	11/24–12/22	12/22/14–1/16/15

and two treatments (fungicide sprayed and non-sprayed control) for each of the five citrus types/cultivars. There were 5–7 buffer trees between sprayed and unsprayed controls. All of the experimental trees were HLB-symptomatic, and the presence of *C*Las was verified later by qPCR analysis as described below.

2.4. Fungicide, dosage and timing of application

The fungicide Quadris Top (Syngenta Canada Inc.) was used within allowed application rates. Quadris Top contains both a strobilurin and a triazol fungicide in a pre-mix formulation. The concentration of Quadris Top was 3.3 g/l of water (within the label recommended rate), and was applied to whole tree canopies until runoff. The fungicide was applied 4 times over the season, starting in April and ending in October or the middle of December depending on the timing of commercial harvest for each citrus type/cultivar. The dates of application for each cultivar are listed in Table 1.

2.5. Fruit drop assessment for spray trial

When fruits were approaching maturity, the ground under the trees was cleared. Subsequently the number of fruits on the ground under each tree was recorded periodically (Table 1). Fruit remaining on the individual tree were counted just before harvest. No on-tree fruit counts were collected for 'Early Gold' oranges, however, due to an unscheduled commercial harvest. The percentage of dropped fruit from the individual tree relative to the estimated yield (fruit remaining on the tree plus dropped fruit) was calculated, except for 'Early Gold' where there was no estimated yield. Fruit drop rate over the maturation season was assessed via regression analysis.

2.6. Fruit detachment force (FDF) measurement

The dates of sampling for FDF measurements are listed in Table 1. Fruit with attached stems (3 fruit per tree × 10 trees) were randomly harvested from different positions around the tree. Fruit from the same tree were carefully put in a labeled plastic bag and the bags containing the fruit were put in a plastic bin, then transported to the laboratory in an air-conditioned van within 2 h after harvest. Immediately upon arriving, FDF was measured using a force gauge (Force Five, Wagner Instruments, Greenwich, CT). Stems were clipped to ~3 cm above the fruit, inserted into the gauge and the fruit was then twisted and pulled until it separated from the stem. FDF was measured in newtons (N).

2.7. DNA extraction *for samples from the tree shaking experiment*

The AZ-C plus central fruit core (about 5 mm of tissue) of D and R fruit was excised using a 10 mm dia. cork borer. DNA of individual fruit was extracted from 100 mg plant tissue using DNeasy Plant Mini Kit (Qiagen Inc., 'Valencia', CA) following manufacturer's instructions. DNA quality (260/280 and 260/230 ratio) and quantity were assessed by spectrophotometry (Nano Drop, Thermo Scientific, Waltham, MA).

2.8. DNA extraction *for samples from the fungicide spray trial*

The dates of sampling for DNA extraction (qPCR) are listed in Table 1. Ten fruitlets per experimental tree were collected in April and June while 3 fruit per experimental tree were collected in September and October for DNA isolation and pathogen detection by qPCR assay. For the fruitlets, the stem was cut and removed with a razor blade, and the fruitlet AZ-C was excised (~2–3 mm thickness) from the stem side to be used for DNA isolation. For the more mature fruit, after FDF measurement, the fruit side of AZ-C plus central fruit core (about 5 mm of tissue) was excised using a 10 mm dia. cork borer. Samples from the same tree were pooled together for DNA extraction. DNA extraction method was the same as described above for D and R fruit.

2.9. Quantitative PCR

For *C*Las detection, primers HLBasf and HLBr and probe HLBp were used targeting 16S rRNA genes of *C*Las (Li et al., 2006). For *Diplodia* detection, specific primers targeting a *Diplodia* β-tubulin gene (GenBank #DQ458858.1) were designed with software Primer Express 3.0.1 (Zhao et al., 2015). TB-F: ATGGCTCCGGTGTGTAAGTGT; TB-R: TGCTACAGGTCAGCGATTGC. PCR mixtures with a total volume of 15 μl contained 7.5 μl of TaqMan PCR master mix or SYBR Green PCR Master Mix (Applied Biosystems), 250 nM each primer, 150 nM probe (for *C*Las detection), and 100 ng of template DNA. PCR amplifications were performed in a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The PCR cycling parameters were as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 S, and 60 °C for 1 min, with fluorescence signal capture at each stage of 60 °C. For SYBR® Green PCR, the default melt curve (disassociation) stage is continued after the 40 cycles of PCR to check the specificity of the individual PCR. Cycle threshold (Ct) values were analyzed using ABI 7500 Software version 2.0.6 (Applied Biosystems) with a manually set threshold at 0.02 and automated baseline settings.

2.10. Statistical analysis

SAS Version 9.3 (SAS Institute, Cart, NC) was used for analysis of variance (ANOVA) to determine mean separation for fruit detachment force (FDF), ethylene production, fruit drop, *C*Las and *Diplodia* Ct values. Individual trees were treated as replicates in the statistical analysis. In the case of FDF, since 3 fruit from each tree were measured, the average value of the 3 readings was taken as the FDF value for that tree (replicate). Real Statistics Resource Pack software (Release 4.3), Copyright (2013–2015) (Zaiontz, 2015) was used for regression analysis of fruit drop over time to assess fruit drop rate and comparison of regression line slopes (SlopesTest) to compare the fruit drop rates between sprayed and unsprayed control. Statis-

tical significance of differences was determined at the 95% ($p = 0.05$) confidence interval, where $p < 0.05$ was considered to be significant.

3. Results

3.1. Comparison of *C*Las and Diplodia levels, and ethylene production in d and r fruit

The *C*Las and *Diplodia* titers in fruit AZ-C, fruit ethylene production were compared between D and R fruit. The results of qPCR analysis showed all the fruit were *C*Las positive, with Ct values ranging from 19.8 to 31.2 (Fig. 1A1–C1). When compared between D and R fruit, although the average *C*Las Ct values trended lower in D than in R fruit for all the three batches of fruit (Fig. 1A1–C1), the difference in *C*Las Ct values was statistically significant only for the batch of 'Hamlin' fruit sampled on 1 Dec., 2014 (Fig. 1A1, $p < 0.05$). *Diplodia* Ct values distributed in a wider range (18.5–40.0) than that of *C*Las, especially for the D fruit (Fig. 1A2–C2). *Diplodia* Ct values in D fruit were significantly lower than in R fruit for both 'Hamlin' and 'Valencia' varieties in all the three samplings (Fig. 1A2–C2, $p < 0.001$), indicating significantly higher *Diplodia* levels in D than in R fruit. No ethylene production was detected in any of the R fruit in the three samplings under the methods used; however, ethylene production was detected in more than half of the D fruit (63% and 60% of the 'Hamlin' D fruit sampled on 1 Dec., 2014 and 5 Jan., 2015, respectively; 53% of the 'Valencia' D fruit sampled on 8 Apr., 2015) (Fig. 1A3–C3). Statistical analysis indicates that ethylene production from D fruit was significantly higher than from R fruit for both 'Hamlin' and 'Valencia' varieties in all the three samplings (Fig. 1A3–C3, $p < 0.001$).

3.2. Correlation of Diplodia and *C*Las ct values, and correlation of ethylene production and ct values of Diplodia and *C*Las

The correlation analysis was conducted between Ct values of *Diplodia* and *C*Las for both D and R fruit, as well as between ethylene production and Ct values of *Diplodia* and *C*Las, respectively, for only D fruit, because no detectable ethylene was found for R fruit by the method used. The results indicated that there was a positive linear correlation between *Diplodia* and *C*Las Ct values for all the three batches of fruit analyzed (Fig. 2A1–C1), but with stronger correlations for D fruit ($R^2 = 0.71–0.73$) than for R fruit ($R^2 = 0.51–0.62$). And there were negative linear correlations between ethylene production and Ct values of *Diplodia* and *C*Las in D fruit (Fig. 2A2, 3; B2, 3 and C2, 3), indicating positive correlations with *Diplodia* and *C*Las. However, the correlations of ethylene production with *Diplodia* levels ($R^2 = 0.79–0.80$) were stronger than that with *C*Las ($R^2 = 0.53–0.69$) for all the three batches of fruit analyzed (Fig. 2A2, 3; B2, 3 and C2, 3)

3.3. Diplodia and *C*Las levels in the fungicide spray trial

Fruit samples collected at time points early in the fungicide spray program (April), during the beginning of the rainy season (June) and when the fruit were approaching maturity (September for 'Early Gold' and 'Navel'; October for 'Midsweet', 'Murcott' and 'Ray Ruby') were analyzed for *C*Las and *Diplodia* levels by qPCR (Table 1). The results indicated that all experimental trees were *C*Las positive, and the average *C*Las Ct values for sprayed and control groups were very close, showing no difference at the three different sampling time points. The average *C*Las Ct values for 'Early Gold', 'Midsweet' and 'Navel' were similar (around 25), while 'Murcott' and 'Ray Ruby' showed higher *C*Las Ct values (Ct values around 22 and 21, respectively) (Fig. 3) However, the differences in *C*Las Ct values were not statistically significant. In April, *Diplodia* was non-detectable or close to zero (Ct values from 37.8 to 40) for the five citrus cultivars for both sprayed and non-sprayed controls, but *Diplodia* levels increased (Ct values decreased) as the season progressed. Although an increasing trend of *Diplodia* level was observed in all the five citrus types/cultivars as the season progressed (Fig. 3), the extent of *Diplodia* increase was different between sprayed and unsprayed controls for some of the types/cultivars. 'Early Gold' and 'Murcott' showed significantly lower ($p < 0.05$) *Diplodia* levels (Ct values were 35.8 and 36.1, respectively in June; 33.9 and 35.2 in Sep/Oct) than that of their respective non-sprayed controls (31.7 and 32.1 in June; 30.2 and 29.8 in Sep/Oct) (Fig. 3A and C); sprayed 'Midsweet' also showed lower, but not statistically significant *Diplodia* levels (Ct values were 35.6 in June and 33.2 in Sep/Oct) compared to their non-sprayed controls (32.3 in June and 30.1 in Sep/Oct) (Fig. 3B). The results indicate that Quadris Top had some effect on reducing *Diplodia* growth in cultivars of 'Early Gold', 'Murcott' and 'Midsweet' (Fig. 3A–B). Meanwhile, for 'Navel' orange and 'Ray Ruby' grapefruit, the sprayed and non-sprayed control showed minimal or no differences in *Diplodia* titer (Fig. 3D and E).

3.4. Fruit detachment force

Comparison of FDF between sprayed and non-sprayed control groups are presented as average FDF values and the distribution of the FDF values (Fig. 4). 'Early Gold', 'Midsweet' and 'Murcott' showed the most significant differences in FDF between sprayed and non-sprayed controls, as reflected by both average FDF and the distribution of FDF (Fig. 4A–C). The average FDF was significantly higher for sprayed trees (34.4 N, 35.6 N and 24.7 N, for 'Early Gold', 'Midsweet' and 'Murcott', respectively) than for non-sprayed controls (28.8 N, 29.4 N and 17.2 N, respectively) ($p < 0.01$). Analysis of FDF frequency distributions revealed that fungicide-treated fruit were distributed in a higher range than for non-treated fruit, which were 30–40 N vs. 20–30 N for 'Early Gold', 30–40 N vs. 20–40 N for 'Midsweet', and 20–30 N vs.10–20 N for 'Murcott' (Fig. 4A–C). In contrast, although 'Navel' showed higher average FDF for the treated compared to the non-treated (Fig. 4D), the FDF for the majority of both groups was distributed in the same range (30–40 N). 'Ray Ruby' showed no difference in average FDF and distribution of FDF for sprayed and non-sprayed controls (Fig. 4E).

3.5. Fruit drop assessment

As fruit approached maturity, the numbers of dropped fruit for each replicate (tree) were recorded periodically until harvest. Overall, averaging fruit drop number in the maturation season for all fruit cultivar/types, the sprayed fruit dropped an average of 78 fruit per tree, while non-sprayed controls dropped an average of 109 fruit per tree; but the reduction in fruit drop was not statistically significant ($p = 0.08$). However, when assessed by each citrus cultivar/type, the reduction in fruit drop was significant in three of the five citrus cultivar/types (Figs. 5 and 6). The fruit drop rates were assessed by regression analysis of fruit drop number over time (Fig. 5). Fruit drop rates (dropped fruit/day, the slopes of regression lines in Fig. 5) for trees sprayed with Quadris Top were smaller ($p < 0.05$) compared with their non-sprayed controls during the maturation season for 'Early Gold', 'Midsweet' and 'Murcott' (Fig. 5A–C), which were 0.196 ± 0.015 vs. 0.358 ± 0.029 for 'Early Gold', 0.281 ± 0.022 vs. 0.410 ± 0.038 for 'Midsweet', and 1.541 ± 0.079 vs. 3.014 ± 0.167 for 'Murcott' (Fig. 5A–C). Effects of fungicide treatment on fruit drop rate for 'Navel' and 'Ray Ruby' were not significant (Fig. 5D and E). Cumulative fruit drop per tree during the maturation season is summarized in Fig. 6A. Fungicide treatment resulted in significant decreases in fruit drop for 'Early Gold' ($p < 0.05$), 'Midsweet' ($p < 0.05$) and 'Murcott' ($p < 0.001$), reducing fruit drop by 45%, 30% and 46% of

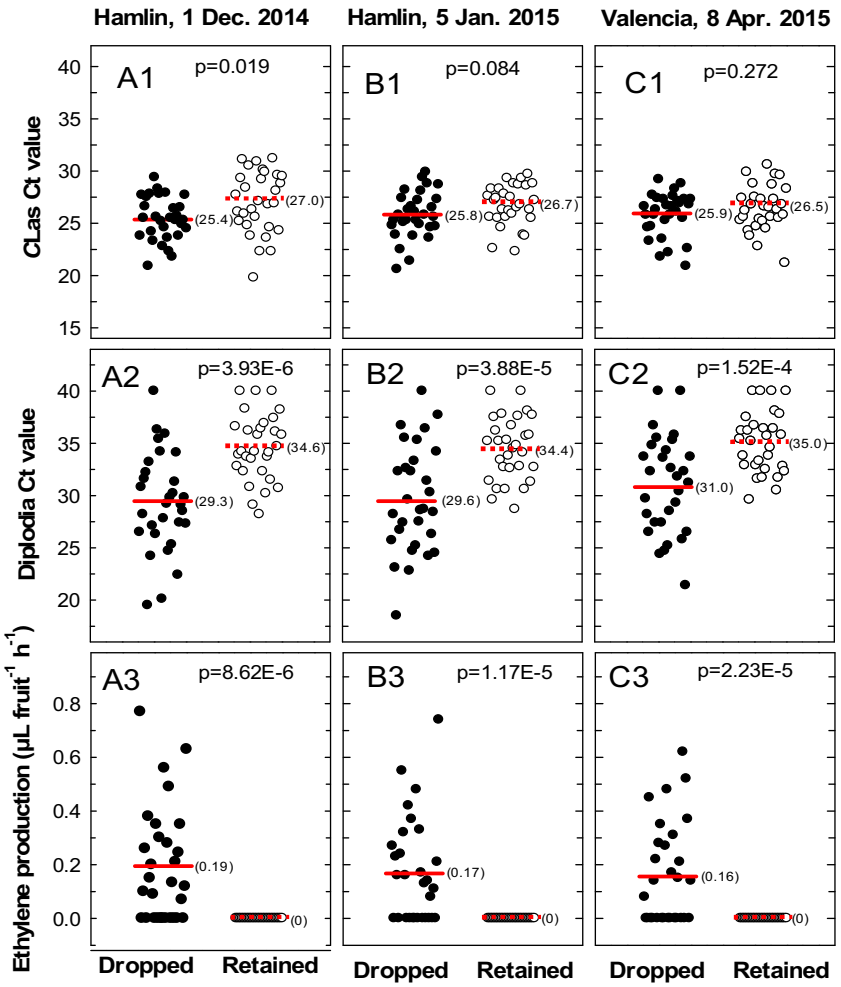

Fig. 1. *C*Las (A1, B1 and C1) and *Diplodia* (A2, B2 and C2) Ct values in the calyx abscission zone, and ethylene production (A3, B3 and C3) of the fruit dropped upon shaking the trees (Dropped) or fruit retained after shaking the trees (Retained) of 'Hamlin' sampled on 1 Dec., 2014 and 5 Jan., 2015, and the 'Valencia' on 8 Apr., 2015 (n = 30). The filled black circles and the solid lines represent the fruit dropped upon shaking the trees (Dropped), while the open circles and the dotted lines represent the fruit retained after shaking the trees (Retained).

non-sprayed controls, respectively (Fig. 6A). Percentage of fruit drop relative to estimated yield (retained fruit plus dropped fruit) is shown in Fig. 6B. Lower percent of fruit dropped during the maturation season from sprayed 'Midsweet' and 'Murcott' trees (could not be calculated for 'Early Gold') than non-sprayed controls ($p < 0.05$ and 0.001, respectively) (Fig. 6B). Meanwhile, no effect on fruit drop count or% of yield was observed for 'Navel' orange or 'Ray Ruby' grapefruit (Fig. 6A and B).

4. Discussion

Fungal infection is one of the major reasons for pathological fruit drop (Racskó et al., 2007). It is not surprising, because some phytohormones such as ethylene and jasmonates, that are known to promote fruit abscission, are induced during the plant defense response to fungal infection. For example, the infection of citrus petals with the fungus *Colletotrichum acutatum* results in post-bloom fruit drop (PFD) characterized by necrotic brown lesions in petals and drop of young fruit accompanied by increased ethylene production and accumulation of jasmonic acid (Lahey et al., 2004). *Diplodia* and other fungi have been associated with fruit drop of citrus (Chaudhary et al., 1994), including 'Shamouti' orange (Minz, 1946) and 'Kinnow' mandarin (Shaft et al., 2004).

Recently, higher incidence of *Diplodia* was found in (HLB)-symptomatic orange fruit AZ-C than in non-symptomatic fruit, suggesting that *Diplodia* infection could contribute to pre-harvest drop of HLB-affected citrus fruit (Zhao et al., 2015). However, since HLB disease is also correlated to pre-harvest fruit drop, it is difficult to separate the effects of the two diseases. More evidence is needed to establish the role that *Diplodia* plays in the pre-harvest drop of HLB-affected citrus fruit.

In this study, we examined the direct correlation of *Diplodia* infection, fruit ethylene production, FDF, and fruit drop in HLB-affected citrus fruit in different experiments. First, the relationship of Diploidia infection and fruit drop in HLB-affected 'Hamlin' and 'Valencia' fruit was demonstrated by comparing fruit ethylene production, *Diplodia* and *C*Las titers in AZ-C of fruit that dropped upon shaking the tree (D fruit, which have a looser AZ-C) with those that remained on the tree (R fruit). The results of this experiment indicated that the difference in *Diplodia* levels between D and R fruit was even more significant than that of *C*Las levels, being higher in D than in R fruit; and higher levels of Diploia in AZ-C was correlated with fruit ethylene production.

The role of *Diplodia* infection in fruit drop of HLB-affected fruit was further validated by application of a fungicide in the field on five citrus types/cultivars ('Early Gold' orange, 'Midsweet' orange, 'Murcott' tangor, 'Navel' orange and 'Ray Ruby' grapefruit).

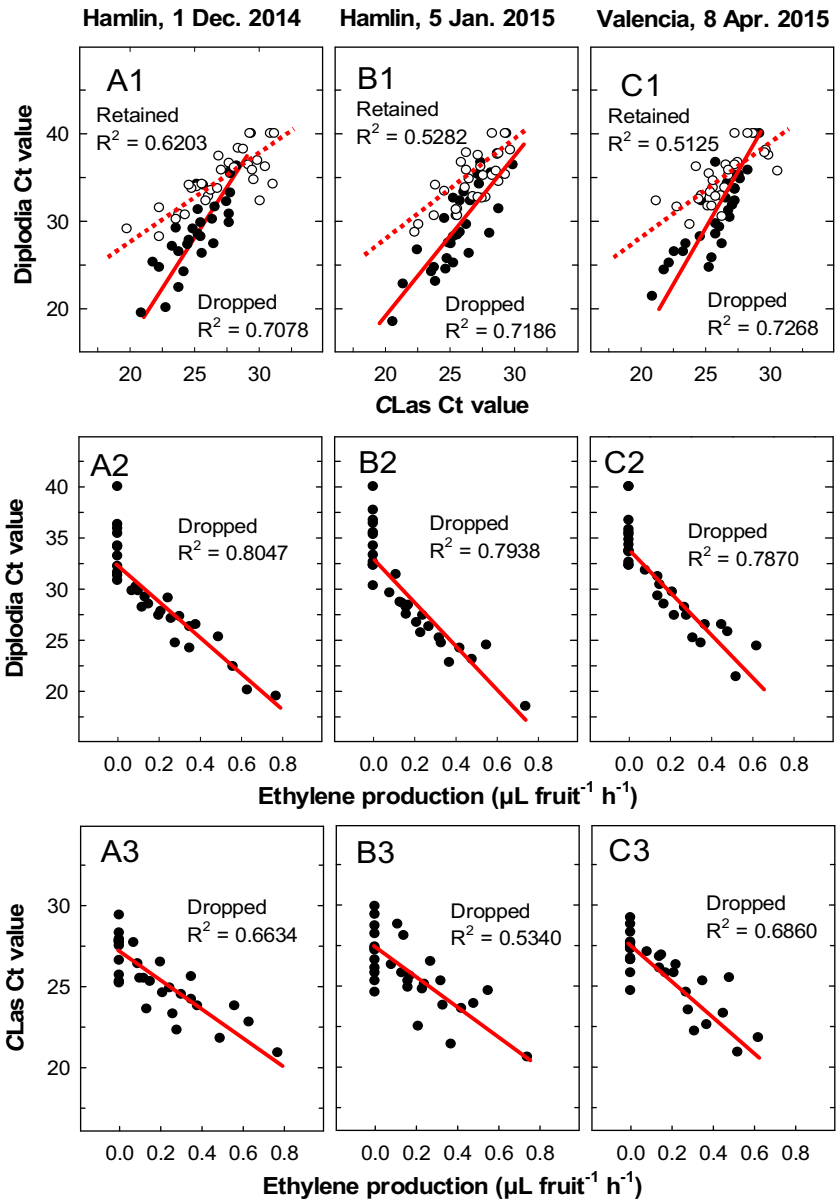

Fig. 2. The correlation between *Diplodia* and *C*Las Ct values (A1, B1 and C1) of the fruit dropped upon shaking the trees (Dropped) or fruit retained after shaking the trees (Retained); the correlation between *Diplodia* Ct value and fruit ethylene production (A2, B2 and C2), *C*Las Ct value and fruit ethylene production (A3, B3 and C3) of the fruit dropped upon shaking the trees (Dropped) of 'Hamlin' sampled on 1 Dec., 2014 and 5 Jan., 2015, and the 'Valencia' on 8 Apr., 2015 (n = 30). The filled black circles and the solid lines represent the fruit dropped upon shaking the trees (Dropped), while the open circles and the dotted lines represent the fruit retained after shaking the trees (Retained).

A pre-mix fungicide Quadris Top™ was selected because it contains two active ingredients: azoxystrobin (a Group 11 strobilurin fungicide) and difenoconazole (a Group 3 triazole fungicide) with translaminar and xylem-systemic properties. Having a mix of active compounds that have different modes of action may reduce the risk of fungicide resistance development (Brent and Hollomon, 1995), and the combined active ingredients likely increased efficacy against *Diplodia*.

The results indicate that Quadris Top reduced Diplodia infection in the AZ-C of 'Early Gold', 'Murcott' and 'Midsweet' fruit, and consequently, the fruit drop for these three cultivars was significantly reduced, as was evaluated by fruit drop rates and cumulative fruit drop per tree. The most significant effect was on 'Murcott' tangor, the cultivar that had the most severe pre-harvest fruit drop among the five cultivars evaluated, and also showed highest average *Diplodia* titer in non-sprayed controls when approaching fruit maturity. The reduction in fruit drop after fungicide application for these three cultivars is in line with the reduced *Diplodia* titer (increased *Diplodia* Ct values) and increased FDF. 'Navel' orange or 'Ray Ruby' grapefruit, however, did not show a change in *Diplodia* titer, FDF nor subsequent fruit drop compared to non-sprayed controls. Not surprisingly, *C*Las titer did not change in all the five cultivars after fungicide application. The results indicated that after fungicide application, the reduction in fruit drop of HLB-affected fruit was consistently associated with the reduction in *Diplodia* levels, where *C*Las remained unchanged. The results provided evidence that *Diplodia* infection plays a role in the drop of HLB-affected fruit.

Some growers in Florida reported that use of the fungicide Headline (also a strobilurin) on 'Valencia' orange resulted in lower fruit drop rates (Bouffard, 2014). Although it was used by the growers in order to prevent spread of black spot (*Guignardia citricarpa*), the

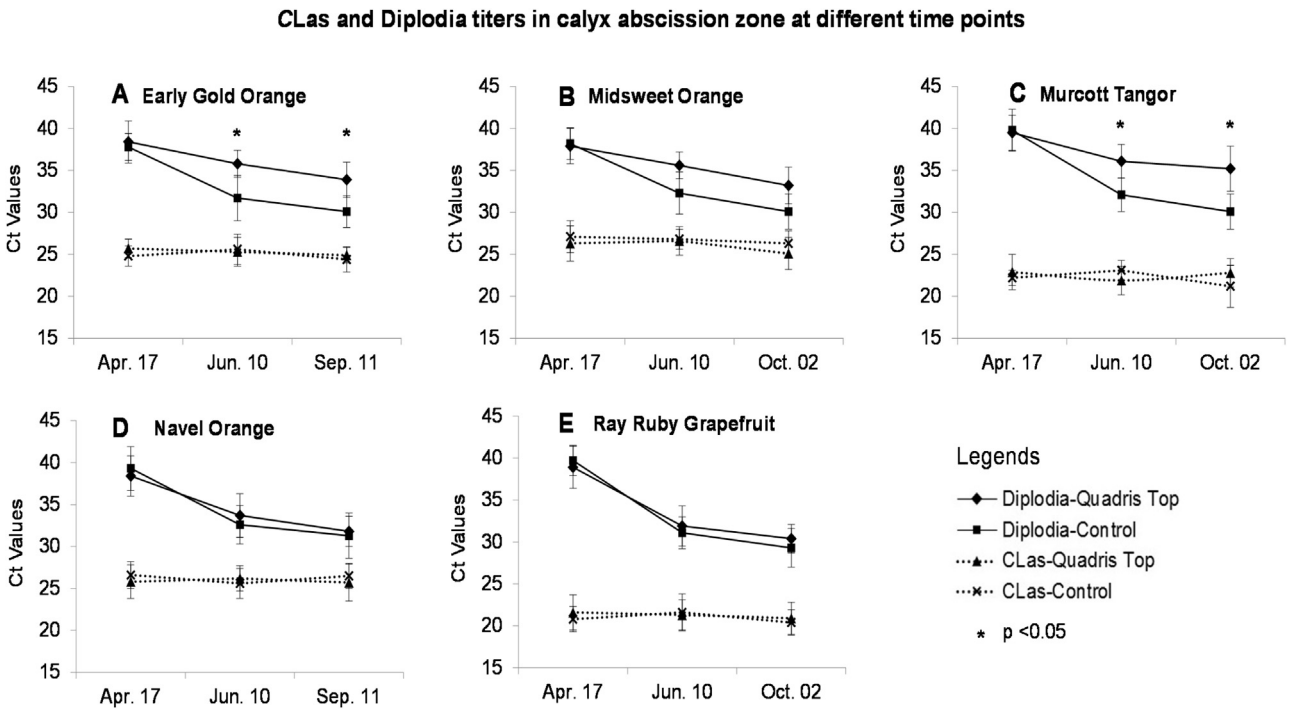

Fig. 3. CLas and *Diplodia* Ct values in the AZ-C at different time points during the developmental season for 'Early Gold' orange (A), 'Midsweet' orange (B), 'Murcott' tangor (C), 'Navel' orange (D) and 'Ray Ruby' grapefruit (E).

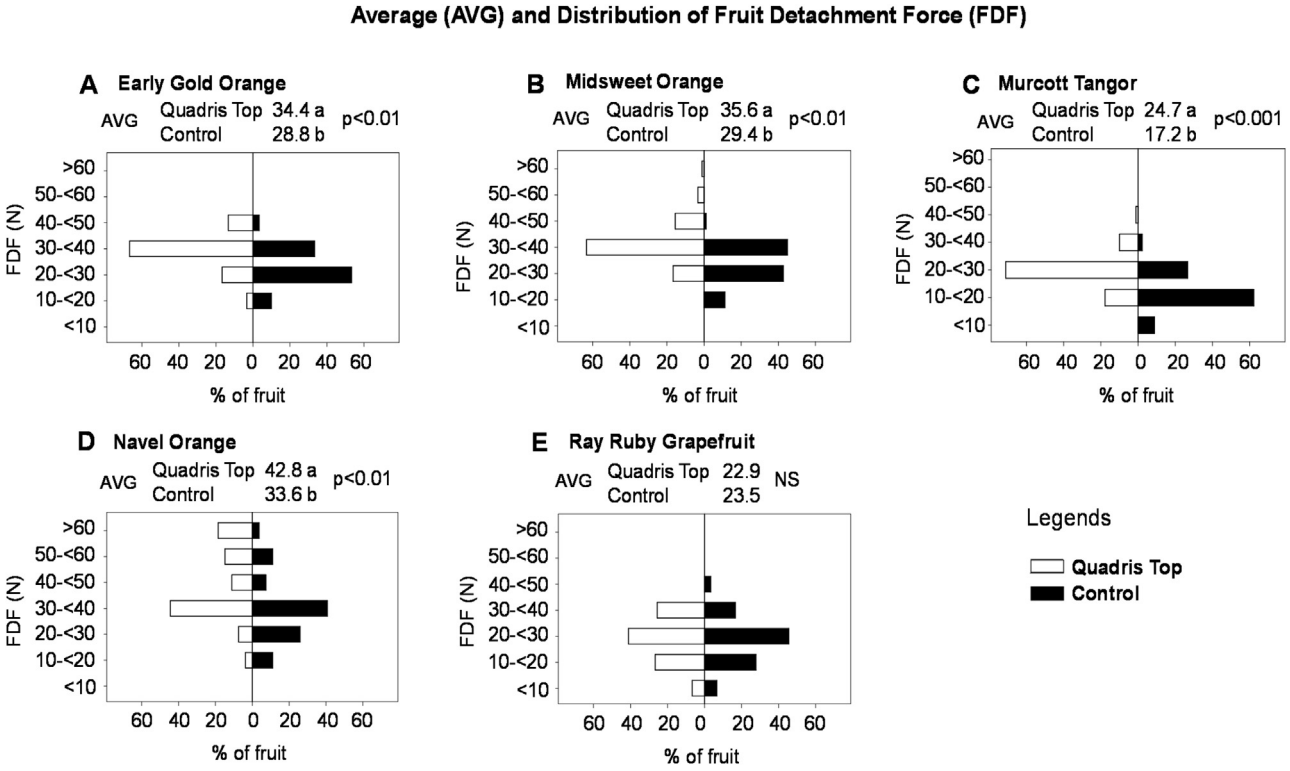

Fig. 4. Effects of fungicide treatment on fruit detachment force (FDF) in newtons (N) and distribution for 'Early Gold' orange (A), 'Midsweet' orange (B), 'Murcott' tangor (C), 'Navel' orange (D) and 'Ray Ruby' grapefruit (E).

effect of Headline on fruit drop was in agreement with our results of fungicide Quadris Top on 'Early Gold', 'Murcott' and 'Midsweet'.

The reason why fungicide Quadris Top worked better on some of the cultivars than others may be attributed to several factors. The first may be related to the developmental stage of the fruit/fruitlet when environmental temperature, rainfall and humidity became suitable for *Diplodia* growth. In the case of citrus postbloom fruit drop (PFD), the developmental stage of the flower buds directly influence the infection by *C. acutatum* and efficiency of PFD control (De Goes et al., 2008; Fagan, 1979). The second factor may be

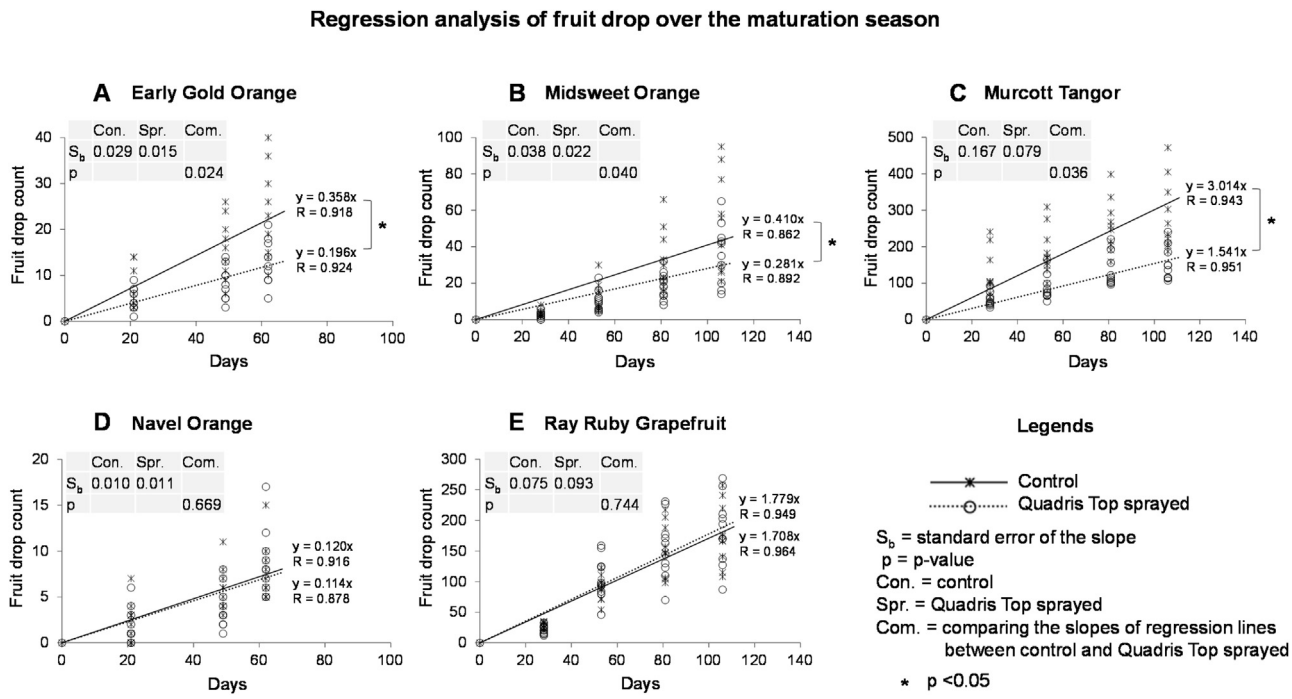

Fig. 5. Effect of fungicide application on fruit drop rate during the maturation season as assessed by regression analysis of fruit drop count over time (days). Day "0" for fruit drop count was when the ground under the trees was cleared 2–3 months before harvest and prior to fruit drop count assessments. A: 'Early Gold' orange, B: 'Midsweet' orange, C: 'Murcott' tangor, D: 'Navel' orange, E: 'Ray Ruby' grapefruit.

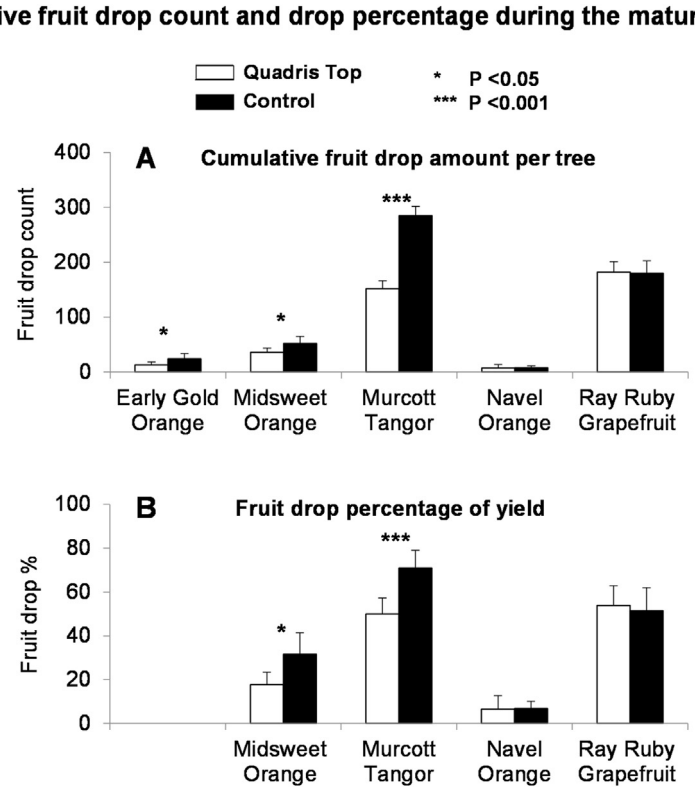

Fig. 6. Effects of fungicide treatment on total fruit drop (A) and percentage of fruit drop relative to retained fruit plus dropped fruit (B) during the maturation season.

related to the timing of fungicide application. The scheduling of Quadris Top sprays might work better for some cultivars than for others. Since *Diplodia* infects the fruit under the calyx (Brown and Wilson, 1967), where fungicides have minimal direct contact, it is important that fungicides be applied before *Diplodia* gets established in that area. Another important factor is the density of the tree canopy when the fungicide is applied. It is more difficult for fungicide sprays to penetrate and reach the target tissues inside a dense and thick canopy. It was noted that the 'Murcott' canopy was both smaller and thinner than the other 4 citrus types/cultivars

in the trial, due to 'Murcott' innate characteristics. Among the citrus cultivars in Florida, 'Murcott' has smaller size and tends to grow upright, thus has a "thinner" canopy than sweet orange and grapefruit (Wheaton et al., 1991). Finally, it is possible that the differences in the size of the abscission zones for the different citrus cultivar/types may have influenced the different responses to the fungicide spray treatment.

In this study, *Diplodia* was not detected in April but in June and later as the season progressed. This was, not surprisingly, correlated with the typical amount of rainfall and increase in temperature in Florida for that time of year. Rainfall is normally most abundant in Florida during the months of May, June, July, August, and September (Butson and Prine, 1968). A majority of the infections of immature fruit occurred during these months, as water is required for spore dissemination and infection by *Diplodia* (Brown and McCornack, 1969).

5. Conclusions

The results of these two experiments indicate that *Diplodia* infection, as an added biotic stress, exacerbates fruit drop by causing fruit to produce the abscission hormone, ethylene. These results suggest that fungicide application may facilitate the control of preharvest fruit drop of HLB-affected citrus. However, more work needs to be done to optimize the application conditions such as type of fungicide, dosage, frequency and timing of application, etc. Nevertheless, fungicide resistance and cost/benefit ratios of application and effect on fruit quality also need to be considered.

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